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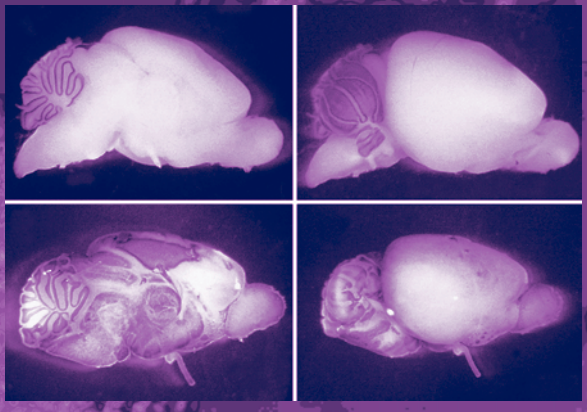

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Activity of the Germline-Specific *Oct4*-GFP Transgene in Normal and Clone Mouse Embryos

Michele Boiani, James Kehler, and Hans R. Schöler

1. Introduction

The first step in elucidating the function of a particular gene of interest involves defining its expression pattern. One way to do this is by using a reporter gene to tag the gene either in a construct (transgenesis) or in the genome (homologous recombination). Transgenes have been generated using different regions of the *Oct4* gene promoter to drive expression of either lacZ or GFP, which allows the activity of the *Oct4* promoter to be followed in living cells and tissues. The bacterial *LacZ* gene encodes the enzyme β-galactosidase, which cleaves the substrate X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) to produce a blue color reaction. The green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* gives a natural green fluorescence that can be viewed under blue light excitation without processing the biological substrate for any chemical/enzymatic reaction. GFP is a convenient reporter for several reasons:

- It is detected *in situ* under noninvasive viable conditions (*see see* **Note 1**).
- GFP allows the separation of the tissue testing positive down to the single cell. It is sensitive enough for biological assays; variants with different half-life and stability extend its use in a variety of applications and follow-up observations.
- Fluorescence persists after fixation with methanol or paraformaldehyde/glutaraldehyde.

This chapter shows how a transgene consisting of GFP under control of the *Oct4* promoter, has been used to further the study of embryonic and germ cells in mammals with respect to pluripotency. In particular, are methods described of how mouse germ cells are formed, how they can be isolated to purity, and

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what happens as the female germ cell (oocyte, egg) has its genetic material replaced with that from another cell that carries *Oct4*-GFP. All that is required is an inverted fluorescence microscope, a micromanipulator, a cell sorter, and standard cell culture abilities.

1.1. Germline and Soma

Germ cells, the source of sperm and eggs, have the ability to make a genetic contribution to the next generation. In fact, once they are mature, these are the only cells in the body needed for fertilization to form an entire organism. This was true until a few years ago, as germ cells are no longer unique in such ability. Genetic contribution to the next generation can also be made by somatic cell nuclei in a procedure known as cloning by nuclear transfer. However, this procedure, is not an efficient and safe means of reproduction, and, moreover, an oocyte cytoplasm is still required as a recipient for the nucleus transplant. The search for substitutes of in vivo produced oocytes is in progress ***(1)***.

In mammals, the germ cell pathway is complex and not well understood. In the mouse, germ cell precursors originate from the proximal region of the epiblast (primitive ectoderm) as early as 6.5 d postcoitum (dpc) ***(2)***. These cells, called primordial germ cells (PGCs), do not seem to be predetermined in the embryo, because they can also arise from other portions of the epiblast after being transplanted to the proximal region ***(3)***. Expression of Bmp4 in the extraembryonic ectoderm close to the proximal region is an essential requirement for the establishment of the germ cell lineage ***(4)***. Mouse PGCs are detected in the extraembryonic mesoderm at 7.2 dpc by alkaline phosphatase (AP) staining ***(5)***. It is unclear why PGCs temporarily leave the embryo proper, but it may be a way for the precursors of germ cells to establish their identity (cell type-specific DNA methylation of the genome that controls the expression of genes, also referred to as *epigenome* ***6***) when somatic cells are being determined. On the other hand, transient segregation in extraembryonic location may be a way to preserve pluripotency when, during gastrulation, companion embryonic cells become committed to a somatic fate. From 7.5 dpc to 10.5 dpc, PGCs are proliferating and moving to the gonadal ridge. Size estimates of the founding population of PGCs are difficult to make with any degree of accuracy. PGCs in the mouse embryo range from Ginsburg's 8 at 7 dpc ***(5)*** to Yoshimizu's 9-–34 after in vitro culture from 6.75 dpc ***(7)*** to Lawson's 45 after in vitro culture from 6.5 dpc ***(2)***. The ambiguity in early PGC counts reflects the difficulty of precisely timing development and reliably identifying these cells before 8 dpc. Overcoming this limitation has been the key to success in deriving germ cells from mouse embryonic stem (ES) cells ***(1)***. In theory, methods to identify PGCs should be based on features that are found exclusively in these cells, but in practice, such features are often characteristic

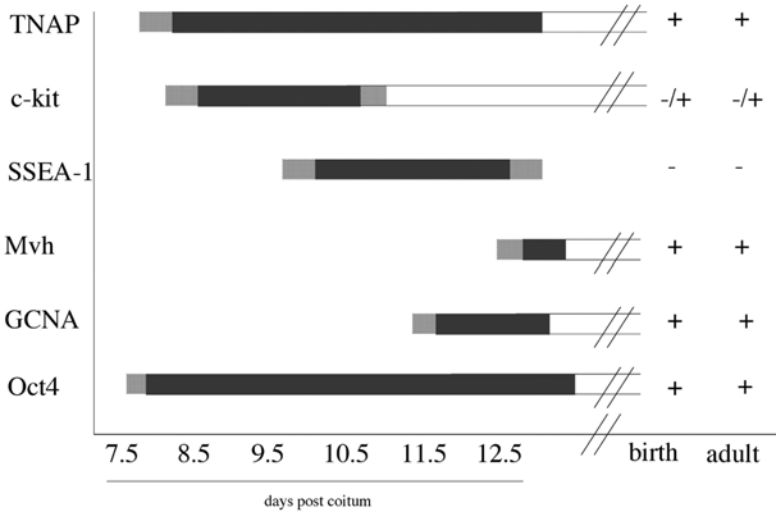


Fig. 1. Most common PGC markers and their expression profiles during mouse fetal development. For all markers but SSEA-1, expression continues (+) after birth; however, the level is lower.

but not exclusive to PGCs. Many marker genes have been used to identify germ cells in the mouse and other species, but not without limitations. A partial list of markers for mouse germ cells includes the following (summarized also in **Fig. 1**):

1.1.1. TNAP

Germ cells express high levels of the tissue nonspecific alkaline phosphatase (TNAP; ***8***), the product of the *Akp2* gene locus. TNAP is the PGC marker with the widest window of expression during germline development in the mouse. Enzymatic staining for TNAP was the first method to identify and isolate germ cells ***(9)***. However, prior to 8 dpc, the somatic cells of the epiblast produce AP as well, therefore, this staining does not effectively distinguish between the soma and germline so early in development. Transgenic mice have since been generated that express a lacZ reporter under control of the *TNAP* gene promoter ***(10)***.

1.1.2. c-kit

The c-kit tyrosine kinase receptor encoded at the *W* locus is expressed in some (but not all) stages of the germ cell pathway, i.e., mostly along the migratory route ***(11,12)***. Disruption of the W^e allele affects the proliferation of PGCs with very few reaching the gonadal ridge. The c-kit antigen is not exclusive to

germ cells, as it is found in other cell types (e.g., Sertoli cells, granulosa cells, melanocytes and hematopoietic stem cells).

1.1.3. SSEA-1 and Mvh

Other well-established PGC markers are the stage-specific embryonic antigen 1 (SSEA-1; ***13***,***14***) and the product of *mouse Vasa homolog* (*Mvh*) gene ***(15)***. Expression of SSEA-1 is first evident at 9.5 dpc and is downregulated at 12.5 dpc when Mvh appears. An interesting aspect of *Mvh* is the differing role of this gene in the male and female PGC lineage, such that *Mvh* –/– male mice are sterile, whereas homozygous homozygous null females are apparently normal.

1.1.4. GCNA

A germ cell nuclear antigen 1 (GCNA1) is initially detected in both male and female mouse germ cells as they reach the gonad at 11.5 dpc. GCNA1 is continually expressed in germ cells throughout all stages of gametogenesis until the diplotene/dictyate stage of meiosis I ***(16)***.

1.1.5. Oct4

Oct4, also named Oct3 and Pou5f1, is a member of the class V of POU (Pit-1, Oct-1, -2, Unc-86) transcription factors and is expressed in early mouse embryo cells and exclusively in germ cells after gastrulation ***(17)***. *Oct4* gene is highly expressed in PGCs at 7.2 dpc, but it is also present in the epiblast and surrounding cells. By 8.25 dpc, germ cells become the only *Oct4*-expressing (Oct4-positive) cells in the conceptus. Downregulation of *Oct4* expression from 7.2 dpc correlates precisely with loss of potential to form germ cells and the undertaking of a somatic cell fate. The relationship between *Oct4* and the germline is akin to the relationship between *Oct4* and pluripotency.

1.1.6. Oct4 Transgenes

Yeom and colleagues ***(18)*** characterized, cloned, and modified the *Oct4* gene regulatory sequences and generated an *Oct4*/*lacZ* fusion transgene. Yoshimizu and colleagues ***(19)*** then substituted *GFP* for *LacZ*. The modified *Oct4* promoter, *Oct4*-Δ*PE*, also known as *GOF-18*-Δ*PE*, was produced by deletion of the proximal enhancer (ΔPE) region. This significantly reduced the reporter signal in the embryonic epiblast and made the transgene a slightly earlier germ cellmarker than the *Oct4* gene. Coexpression with other germ cell markers ***(20***,***21)*** proves that *Oct4*-ΔPE/GFP identifies germ cells.

1.2. The Octamer-Motif Paradigma in Mammals

All known members of the octamer-motif gene family play a role in various developmental processes:

- *Oct1* and *Oct2* in B-cell maturation ***(22)***;
- *Oct3/4* in the maintenance of pluripotency (ES cells) and survival of germ cells ***(17)***; and
- *Oct6* in Schwann cell differentiation and nerve myelination ***(23)***.

The *Oct4* protein, in particular, was first identified in mice as a DNA-binding activity in extracts from mouse blastocysts and PGCs, as well as in embryonal cell lines, such as ES and embryonal carcinoma (EC) cells ***(17)***, and, subsequently has been shown to be expressed at various timepoints of the germline (*see* **ref. *24*** for review). The *Oct4* protein contains a bipartite DNA-binding domain (comprised of the POU-specific and POU-homeodomain) that binds the octamer motif (ATGCAAAT). POU proteins can also form homo- or heterodimers on a palindromic DNA motif (ATTTG CAAAT) that bears resemblance to the octamer (Palindromic Oct factor Recognition Element; [PORE]; ***25***). This recently identified structural property of POU factors may bring about the formation of novel protein–protein–DNA configurations, which may account for the functional versatility of these proteins in development. *Oct4* orthologs have been identified in mammalian species other than the mouse, such as human and bovine ***(26)***, and share a high degree of structural homology, suggesting that they may have an evolutionary conserved role in different species and even across vertebrate taxa (*see Oct4* ortholog in zebrafish; ***(27)***).

1.3. Oct4 at Work

Oct4 is essential for the pluripotential founder cells and the germline in the mouse embryo. The Oct4 protein activates certain key genes while repressing others.

Target genes activated by Oct4 include: *fibroblast growth factor 4* (*Fgf-4*), *Osteopontin* adhesion molecule (Opn), and transcriptional coactivator *Utf1*.

Target genes repressed by Oct4 include: *human chorionic gonadotropin* (*hCG*) α and β subunits in primates and *interferon* τ (*IFN*τ) in ruminants.

Other putative downstream genes of *Oct4* include *Rex-1*, *Sox-2*, *Creatine kinase B*, *Markorin 1*, *Importin beta*, *Histone H2A.Z*, and *Ribosomal protein S7* ***(28)***. Through its target genes, the domain of *Oct4* comprises the cells of the inner cell mass (ICM; *Fgf-4*), of the ICM and primitive endoderm (*Opn*), of the ICM, epiblast, and ES cells (*Utf1*), and the trophoblast cells (*hCG*, *IFN*τ). It is not clear how opposite processes of activation and repression can be brought about by the same transcription factor. Some cofactors (E1A-like activity, Sox2 activity OBF1-like activity; ***(22)***) may act on *Oct4* protein in a way that leads to activation or repression. After embryo gastrulation, *Oct4* is dispensable from somatic cells, and germ cells are the only cells of the organism known to maintain *Oct4* expression. In the adult, *Oct4* expression is restricted at low levels to oocytes and spermatogonia ***(29)***. In vitro, each of the three types of established mammalian pluripotent stem cells—ES, EC, and embryonic germ (EG) cells—

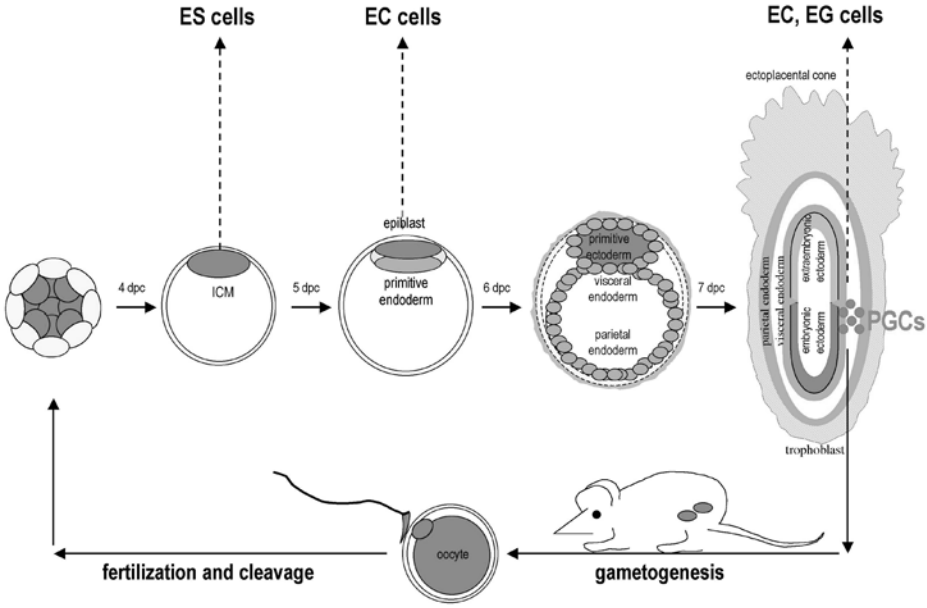


Fig. 2. Scheme of developmental pluripotency and how it can be tracked in the mouse embryo using *Oct4* gene expression as a marker. Oct4/GFP (here shown in dark gray) reflects the expression of the endogenous gene and allows viable observations. (Reproduced with permission from Academic Press.)

expresses *Oct4*, which becomes downregulated as the cells differentiate (**Fig. 2**). However, these cells constitute artificial model systems that do not exist in vivo. Although it has been proved that Oct4-positive mouse ES, EC and EG cells from 8.5 dpc PGCs can contribute to chimeras, it is not clear whether primordial germ cells or fetal germ cells have a potency to produce chimeras without previous exposure to in vitro culture ***(30)***. This raises the provoking question of whether Oct4 should be considered as being specific to the germline or rather as simply participating in defining the pluripotent state of various types of cells, including germ cells. Consistent with these contrasting views is the observation that spontaneous testicular germ cell tumors (seminomas) express *Oct4*, but teratomas that give rise to all three germ layers do not express *Oct4*. On the other hand, *Oct4* expression has been reported in somatic tumors ***(31)***.

In vitro, change in the Oct4 levels of mouse ES cells drives these cells into opposing cell fates. An increase/decrease in Oct4 levels leads to primitive endoderm/trophectoderm ***(32)***. What stays upstream and regulates *Oct4* is a matter of investigation. The germ cell nuclear factor (GCNF) has been shown

to bind the promoter region of *Oct4* and bring about its repression following gastrulation ***(33)***. The factor(s) involved in *Oct4* regulation prior to gastrulation are unknown. Until recently, the early lethality of *Oct4* null mutants ***(34)*** and the inability to derive germ cells directly from ES cell, in vitro, precluded the testing of whether *Oct4* is required in germline determination. The finding that we could induce *Oct4* deletion from the genome in germ cells while they are forming (Tomilin, A., unpublished results) has opened the door to understanding *Oct4*'s role in this process.

1.4. The Oct4/Gfp *Transgene as a Tool to Visualize Germ Cells*

Analysis of *Oct4*/LacZ transgenes ***(18)*** showed that a 18-kb genomic *Oct4* fragment (GOF-18) encompassing the *Oct4* coding and its 5' and 3' flanking sequences was large enough to drive gene expression comparable to that of the endogenous *Oct4* gene. Subsequent *in situ*/in vivo analyses were reinforced by transgenesis involving the substitution of GFP for LacZ. The *GOF-18*/*GFP* transgene, like the endogenous *Oct4* gene, was not an exclusive marker for mouse germ cells at 7.2 dpc, but was at 8.25 dpc. Deletions in the GOF-18 5'sequence revealed the existence of various *cis*-acting elements clustered in two regions upstream of the transcription initiation site. These two clusters, known as the PE and the distal enhancer (DE), are responsible for the restriction and maintenance of *Oct4* expression in specific cell lineages. *Oct4* expression in pre-implantation embryos and in PGCs is driven by the DE, whereas expression in the epiblast is enhanced by the PE (**Fig. 3**).

A deleted version of the *GOF-18* transgene lacking the conserved region II of the PE (GOF-18-ΔPE) is more weakly expressed in the epiblast than the full *GOF-18* transgene and becomes more readily restricted to PGCs shortly after they form (*see* **Note 2**; **Figs. 4–6**). The question arises as to when *GOF-18* expression begins to diverge from that of *GOF-18-ΔPE*. Both the endogenous *Oct4* gene and the transgene are silent in the early stages of mouse embryogenesis. The low levels of *Oct4* found in mature and fertilized oocytes are a carryover from oogenesis and do not seem to hold developmental relevance; in fact, a ZP3-Cre-induced deletion of *Oct4* produced no disruption in ovulation and preimplantation embryo development (Smith, A., personal communication). The onset of *GOF-18*/*GFP* expression is the same as that of *Oct4* and occurs at the eight-cell stage. By 4 dpc, expression of both transgenes becomes concentrated in the ICM of the blastocyst embryo, although this may not be readily apparent from GFP due to the half-life of the protein and its persisting fluorescence in the trophectoderm (TE). Differential transgene expression can be observed at 6.75 dpc when GOF-18/GFP is brightly and uniformly expressed all throughout the embryo proper, whereas GOF-18-ΔPE/GFP is weaker over-

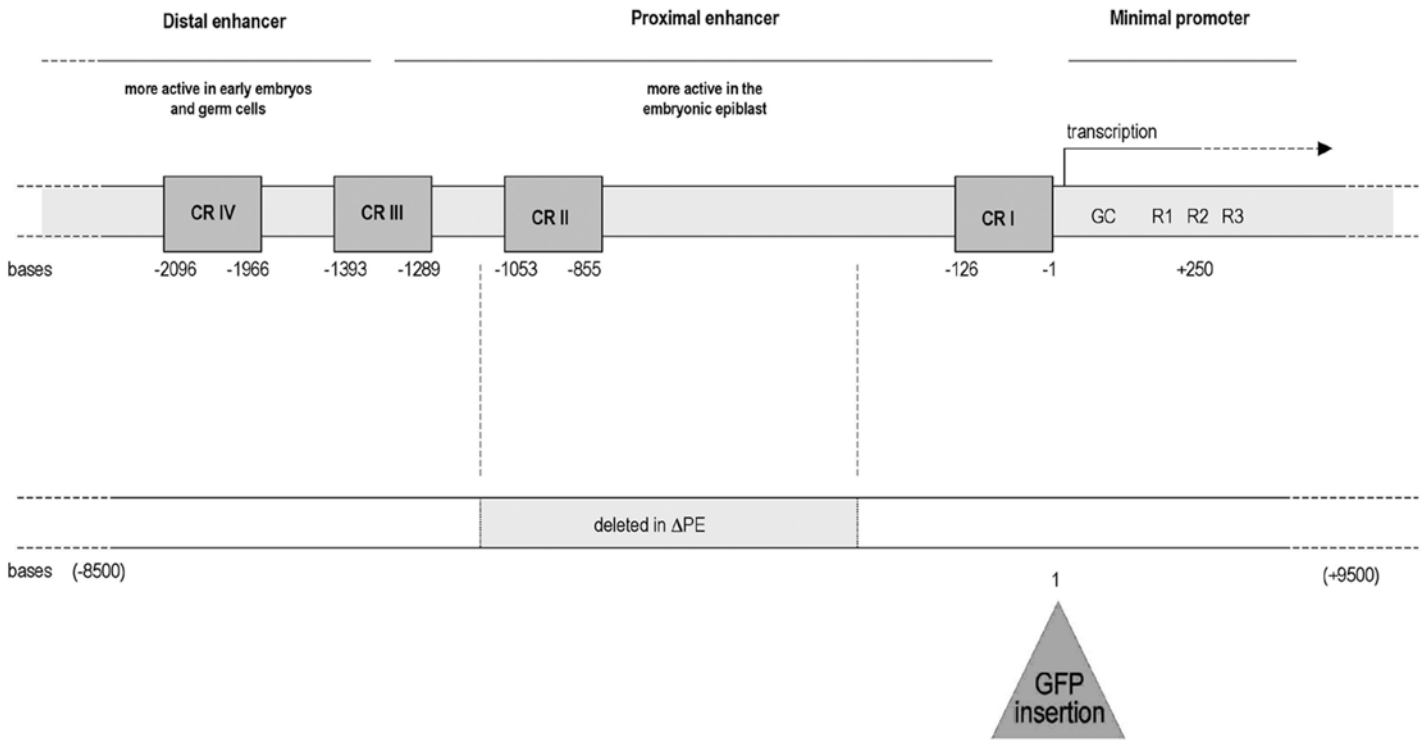


Fig. 3. Scheme of the Oct4/GFP construct incorporated into the OG2 mouse strain (courtesy of Dr. Jeffrey Mann): genomic Oct-4 fragment 18 kb (GOF-18) upstream control region. CR, conserved regions I, II, III, and IV***(25)***. (Reproduced with permission from Academic Press.)

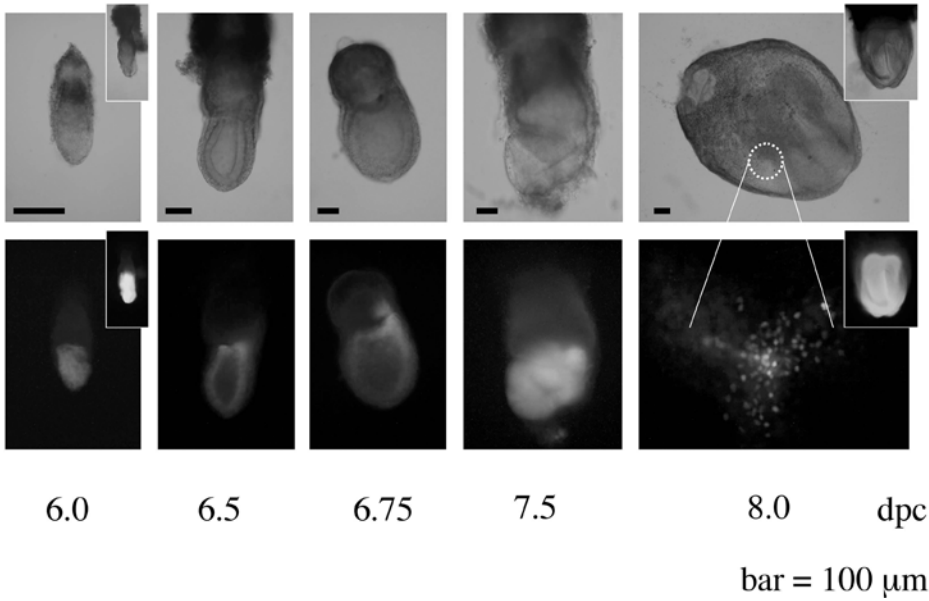


Fig. 4. Oct4-ΔPE/GFP is observed in the epiblast at 6.0 dpc. It becomes less intense overall at 6.5 and 6.75 dpc while persisting high in the posterior part of the embryo (allantoic bud). The increase observed at 7.5 dpc is transient and unexplained. At 8.0 dpc, Oct4-ΔPE/GFP is restricted to primordial germ cells. The small inserts at 6.0 and 8.0 dpc show the expression of Oct4/GFP for comparison with Oct4-ΔPE/GFP under the same light conditions.

all than GOF-18/GFP ***(19)***. The proximal region of the epiblast has more intense GOF-18-ΔPE/GFP fluorescence than the rest of the epiblast, which is consistent with gastrulation proceeding from the posterior to the anterior end of the embryo. By 7.25 dpc, the posterior end of the embryo emits stronger fluorescence than does the rest of the epiblast. Because PGCs arise in the posterior region of the embryo, it was speculated that GOF-18-ΔPE/GFP might be an early germ cell-specific marker. This notion was further supported by observations that only a cluster of 30–40 highly GFP-positive cells were seen at 8.0 dpc at the base of allantoic bud, and this number increased up to 60–80 cells by 8.75 dpc at the time when the GFP-positive cells were seen in the hindgut epithelium ***(19)***. Afterward, PGCs migrate along the hindgut (9.5 dpc), through the dorsal mesentery (10.5 dpc), and finally enter the forming gonadal ridges (11 dpc) (*see* **Note 3**). At 13.5 dpc (**Fig. 6**), both male and female germ cells cease dividing, anticipating the onset of meiosis in females (oogonia-to-oocytes) and the mitotic arrest in G0 in males (prospermatogonia). Based on

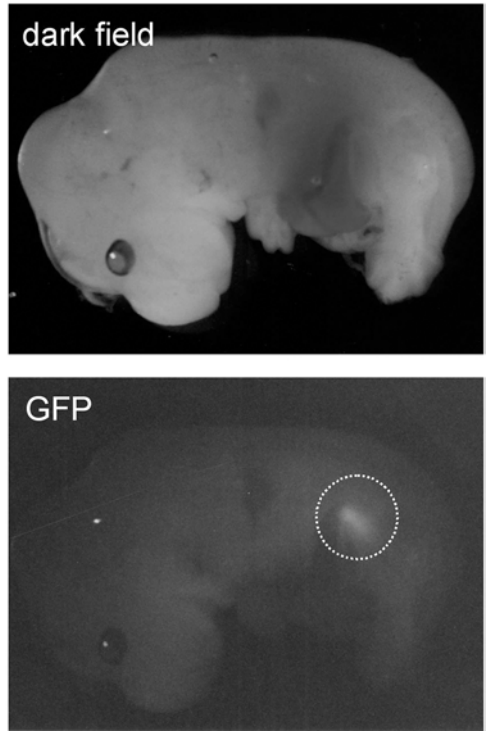
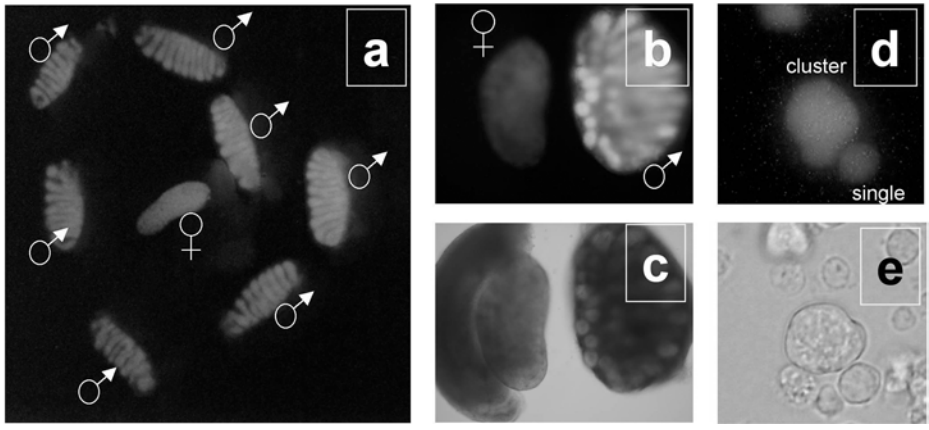


Fig. 5. Germ cells are the only cells where Oct4 and Oct4/GFP are expressed in a 13.5-dpc mouse fetus. They glow green through the body wall under epi-illumination with a blue light source and allow localization of the gonad (bottom, circled).



colocalization of Oct4-GFP and c-kit, Oct4-GFP indeed appears to be a germ cell-specific marker (*see* **Note 4**; **Fig. 7**). In sum, throughout mouse fetal germline development, expression of the GFP transgenes reflects that of the endogenous *Oct4* gene. After birth, both transgenes become downregulated in cells that remain positive for the native Oct4 protein by immunohistochemistry (e.g., spermatogonia). This suggests the presence of other regulatory elements excluded in the original GOF-18 genomic fragment. Fine resolution molecular analysis of the GOF-18 5' regulatory elements (conserved region [CR] I–IV; **Fig. 3**) is currently underway (Hübner, K., personal communication).

1.5. Expression of Oct4 After Gametogenesis

Oct4 is downregulated in germ cells upon entry in meiosis, and its coding mRNA is virtually absent in mature oocytes and sperm. Upon merging of the gametes at fertilization and cleavage of the resulting zygote, *Oct4* gene expression is turned on. The onset of zygotic *Oct4* expression occurs at the eight-cell stage. *Oct4* is expressed in all embryo blastomeres at the morula stage to be gradually restricted to the ICM of the mouse blastocyst at 3.5 dpc (*see* **Note 5**; **Fig. 8**). The ICM, which is the pluripotent founder cell population in the blastocyst, gives rise to the primitive ectoderm and endoderm. At the time of embryo implantation (4.5 dpc), Oct4 protein levels transiently increase in the primitive endoderm; from that point onward, its expression is limited to the primitive ectoderm. Although the primitive ectoderm is pluripotent, as it is the precursor of the embryonic germ layers (ectoderm, mesoderm, and endoderm), *Oct4* expression is not retained by derivatives of the primitive ectoderm after gastrulation, but it is ultimately confined to germ cells until they enter meiosis. This alternation of expansion (from germ cell to morula) and restriction (from blastocyst to germline) of the *Oct4* domains of expression led to the concept of an *Oct4* "totipotent cycle" (***24***; **Fig. 2**). According to this conceptual model, all the early embryonic cells have the potential to become germline unless their *Oct4* expression is silenced. If this happens, the cells exit the cycle and undergo somatic differentiation. It would be interesting to find what happens as *Oct4* is selectively and conditionally silenced in germ cells, i.e., if germ cells switch to

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Fig. 6. (*opposite page*) Gonads of either sex can be easily distinguished by the pattern of Oct4/GFP (top), i.e., the striped appearance being characteristic of the developing testis where germ cells are organized in cords **(A)**. Detail of forming ovary (**B**,**C**—left) and testis (**B**,**C**—right). Only a proportion of the gonadal cells glow green (**D**,**E**) and those are *bona fide* germ cells. Germ cells are found in clusters as the one in the center of **D**,**E**.

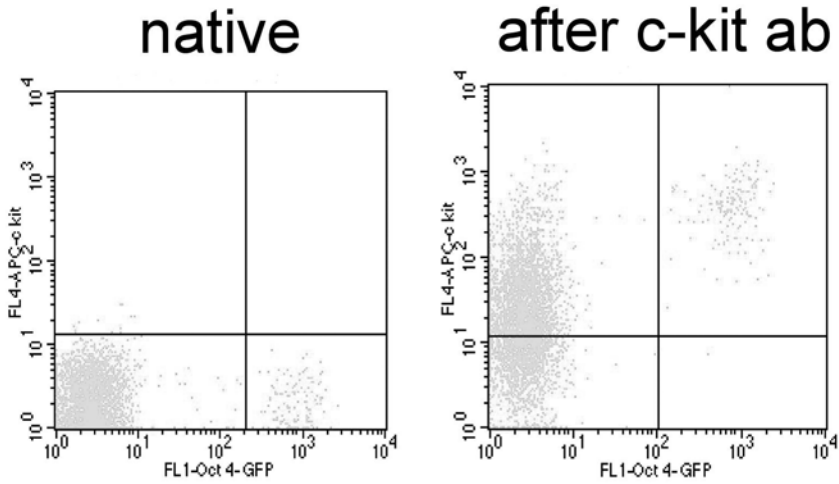

Fig. 7. FACS of 13.5-dpc OG2 gonadal cells before (left) and after (right) immunostaining with c-kit antibody. Cells expressing high GFP are also highly c-kit-positive.

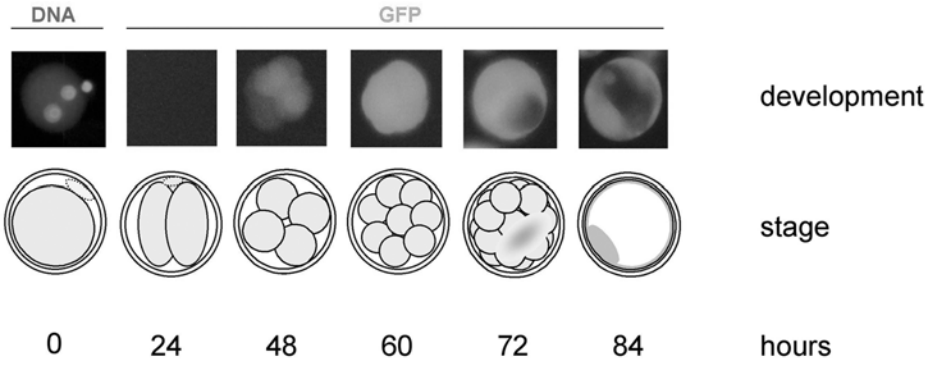

Fig. 8. After fertilization, Oct4-ΔPE/GFP is expressed during embryo cleavage, and it is clearly detected at the eight-cell stage.

a different cell fate or instead they die (Tomilin, A., personal communication). In summary, the developmental restriction of *Oct4* expression from the preimplantation embryo (all Oct4-positive cells) to the adult (germ cells as the only Oct4-positive cells) and the fact that in vitro, Oct4 is confined to undifferentiated EC, ES, and EG cells, further confirm the notion that *Oct4* plays a key role in developmental pluripotency.

1.6. Insight on Oct4/GFP Regulation from Nuclear Transfer Experiments

From this discussion it appears that *Oct4* regulatory elements alone are insufficient to drive its expression, but require the binding of some factor(s). Therefore, the next question becomes whether the silent state of *Oct4* in the cells that have "abandoned" the germline ***(24)*** is reversible and, if so, whether it is mediated by a physical modification of the gene locus (methylation, histone deacetylation, euchromatinization, and so on) or by the presence of transactivators. This topic has tremendous implications and widespread applications. In fact, fertilization is not the only way to initiate development and reproduction, as nuclear transfer from differentiated somatic cells into oocytes has shown that the oocyte cellular machinery is amazingly capable of supporting a somatic cell nucleus (*see* **Note 6**). But what is necessary to transform a nucleus that is restricted in potency into a nucleus that is totipotent and can recapitulate development, i.e., what are the differences between a somatic (differentiated) nucleus and a germline (totipotent) nucleus? Oct4 as a hallmark of totipotency in mammalian cells prompted the assessment of its status in clone embryos generated by nuclear transfer of somatic and germ cell nuclei into mouse oocytes.

The *Oct4* promoter is silent in somatic cells, but it is active in the germline and during preimplantation development. Thus, reexpression of the *Oct4*/GFP transgene in somatic cell-derived clones can be used as an indicator of reprogramming of the *Oct4* promoter in the transplanted nucleus ***(35)***. The existing *Oct4*/GFP transgene because of its high-product stability (enhanced GFP—24 h half-life), may be questioned as a tool for the fine resolution analysis of spatial and temporal changes in gene expression during and after reprogramming, for which a short-lived destabilized GFP (dGFP, 4 h half-life) would be more promptly responsive. On the other hand, persistence of eGFP provides a "memory" of reprogramming, whereby information gained from GFP is not circumstantial.

2. Materials

2.1. Reagents

1. phosphate buffered saline (PBS).
2. Ca^{2+}/Mg^{2+}-free Hank's buffered saline with 1 m*M* EDTA.
3. Trypsin 0.25% w/v solution with 1 m*M* EDTA (optional: add 100 ng/mL DNAse in the solution).
4. Fetal calf serum (FCS).
5. Bovine serum albumin (BSA).
6. HEPES-buffered CZB medium

7. Iscove's modified Dulbecco's medium (IMDM).
8. Alpha-minimum essential medium (α-MEM).
9. 40 kDa Polyvinylpyrrolidone (PVP) stock solution (16% w/v in H_2O).
10. Cytochalasin B stock solution (5 mg/mL in dimethylsulfoxide [DMSO]).

2.2 Oct4/Gfp *Transgenic Mice*

A transgenic mouse line expressing GFP only in germline after gastrulation (**Fig. 4**) has been generated at the Beckman Research Institute (City of Hope, Duarte, CA) by Dr. Jeffrey Mann. The construct used for transgenesis was generated at the European Molecular Biology Laboratory (EMBL, Heidelberg, Germany) by the research group of Dr. Hans Schöler. The original construct, comprising an 18-kb *Oct4* genomic fragment with the promoter region, was named GOF-18 ***(18)***. It was modified by deleting the conserved region II within the ΔPE and replacing the *LacZ* reporter with *eGFP* (**Fig. 3**). A number of transgenic mouse lines were obtained after pronuclear microinjection of the construct into (CBA/CaJ × C57BL/6J)F2 zygotes. One transgenic line, denominated OG2, was bred to homozygosity for the transgene and used as a parental strain to derive the experimental mice for the studies described here and elsewhere ***(35, 36)***. The hemizygous genotype was generated by mating OG2 males with C57BL/6J females and is referred to as (B6OG2)F1 throughout this work; it was used as a source of transgenic oocytes and embryos. Nontransgenic oocytes were obtained from C57BL/6J or (C57BL/6J × C3H/HeN)F1, also known as (B6C3)F1, mice (*see* **Notes 7** and **8**).

3. Methods

3.1. Recovery of Mouse Fetuses and Their Gonads

After mating of (B6C3)F1 females with OG2 stud males, pregnant females are sacrificed at 13.5–16.5 dpc (*see* **Note 8**). The uterine horns are dissected in PBS in a 10-cm plastic dish to release the fetuses. Fine dissection is carried out in HEPES-buffered CZB (HCZB in short) medium in a 6-cm dish on the stage of a stereomicroscope with epi-illumination (fiber optics). Once removed, the gonadal ridges are to be processed immediately. If not possible, they should be kept in HCZB medium on ice. The composition of HCZB and other media used in this study is described in **Table 1**.

3.2. Isolation of Fetal Gonadal Cells and Low-Scale Purification of Germ Cells Based on Oct4/GFP

The mildest way to isolate germ cells from fetal gonads is to incubate the tissue in Ca^{2+}/Mg^{2+} -free Hank's buffered saline with 1 m*M* EDTA at room temperature, pin the surface with a fine glass capillary, and collect the germ

cells as they naturally ooze out using a mouth-operated micropipet (50 µm ID). Another way is to incubate the gonad in 0.25% w/v trypsin with 1 m*M* EDTA for 10 min at room temperature, mince the tissue in IMDM containing 20% v/v FCS and 100 U each of penicillin and streptomycin until a cell suspension is obtained. The serum in the medium stops the action of the trypsin. In this study, mechanical isolation without chemical or enzymatic aid is preferred in order to preserve the microarchitecture of the tissue, i.e., the *in situ* relationship between germ cells (*see* **Note 9**). The gonad is torn into pieces using 30-gauge needles in a drop of HCZB medium, and the fragments are flushed in and out the tip of a mouth-operated micropipet (200 µm ID). Clouds of cells are displaced during the flushing, most of them being germ cells as indicated by GFP. More than 90% of the GFP-positive cells from 13.5-dpc OG2 gonads have been shown to stain positive for AP ***(36)***. Hence, the GFP-positive cells are *bona fide* germ cells. This mixed population of cells is transferred to HCZB medium on the stage of the inverted microscope, where germ cells are identified by GFP and picked up individually by a glass capillary (20 µm ID) operated by a micromanipulator.

3.3. High-Scale Purification of Fetal Germ Cells Based on Oct4/GFP

There are up to 2×10^4 total germ cells in one gonad of a 13.5-dpc mouse fetus; hence, manual recovery of so many pure germ cells is impractical. The endogenous GFP activity that identifies germ cells can be effectively combined to an automated procedure known as fluorescence activated cell sorting (FACS; **Note 10**). As noted in a few reports ***(20, 36)***, this procedure is useful. Gonadal ridges are dissected from 13.5-dpc male or female (B6OG2)F1 embryos. A single-cell suspension is prepared by digesting 10–20 gonadal ridges in 500 µL of 0.25% w/v trypsin with 1 m*M* EDTA for 10 min at 37°C. Tissues are disrupted by repeatedly pipetting with 100 ng/mL DNAse in the solution. Trypsin is inactivated by adding 4.5 mL of IMDM containing 20% v/v FCS and 100 U/mL each of penicillin and streptomycin. The cell suspension is run through a 40-µm nylon cell strainer (Falcon 352340) to remove any undigested fragments that could clog the flow cytometer. The cell suspension is centrifuged at 400*g*, the medium decanted, and the cell pellet is resuspended in 1 mL of PBS with 0.5% w/v BSA. To measure c-kit expression on PGCs, 0.1 µg of a monoclonal, rat anti-mouse c-kit/CD117 antibody conjugated with allophycocyanin (APC; BD Pharmingen Clone-2B8) is added to 1×10^6 cells in 100 µL of PBS-BSA in 5 mL polypropylene round-bottom, FACS tubes (Falcon 352054). The same concentration of a rat IgG_{2b}-APC antibody is used in parallel as an isotype control. For FACS analysis using a Becton Dickinson *FACScalibur* flow cytometer equipped with dual lasers, 1×10^6 cells are

re-suspended in 1 mL of PBS-BSA. For cell sorting using a *FACstar plus* flow cytometer, cell suspensions with a concentration between 3 and 4.5×10^6 cells/mL are used to facilitate high-speed sorting while minimizing the occurrence of clogs. Germ cell (GFP^+) and stromal (GFP^-) fractions are gated and deflected into separate 5-mL polystyrene tubes containing 2 mL of IMDM with 20% v/v FCS.

3.4. Recovery of Adult Germ Cells, Fertilization, and Development

Mature oocytes make up the primary biological reagent required to study how the expression of *Oct4*/GFP is regulated during embryo development. In order to ensure the observation of *de novo* transgene expression, oocytes are obtained from wild-type females and fertilized with OG2 sperm. Mature (B6C3)F1 oocytes are isolated from the cumulus masses released from the ovary, by exposure to hyaluronidase enzyme (*see* **Note 11**). Zygotes are then produced in vitro by fertilization (IVF) with OG2 sperm (*see* **Note 12**); or flushed from the uterus at 3.5 dpc after natural mating of (B6C3)F1 females with OG2 stud males. Oocyte storage and embryo culture takes place in microdrops of α-MEM medium (*see* **Note 13** and **Table 1**) under oil (*see* **Note 14**) on 35-mm plastic dishes. The medium is not supplemented with antibiotics, but sterile-filtered on 0.22 μm pore size cellulose membrane prior to use. In vitro culture lasts for 4 d (one-cell to blastocyst) and takes place in a 5% CO_2 incubator at 37°C.

3.5. Oct4/Gfp Expression in Oocytes Reconstructed with Somatic Cell and PGC Nuclei

Embryos can also be generated by a nuclear transfer procedure from a donor cell other than sperm into an oocyte, whereby fertilization and amphimixis are bypassed. When somatic cells are used as donors, this requires reactivation of the silent *Oct4* during development of the reconstructed oocyte. A convenient source of donor cells are the cumulus cells that surround the oocyte. Cumulus cells are recovered from the cumuli oophori of (B6OG2)F1 oocytes after hyaluronidase treatment, and stored at 4°C (*see* **Note 15**). As a control for Oct4 being already expressed in the nucleus donor cells, either ES, EC, or germ cells could be used. In this study, germ cells are used as they match cumulus cells for direct recovery from the body and absence of in vitro culture. Gonadal cells, including donor germ cells, are mechanically isolated from (B6OG2)F1 fetuses at 13.5–16.5 dpc and used fresh within 3 h. Germ cells are selected among gonadal cells by GFP visualization (*see* **Figs. 5** and **9**) (*see* **Subheading 3.8.**). Because female germ cells enter meiosis shortly after colonization of the gonadal ridge, whereas the male counterparts do not, male gonads are a more feasible source of nucleus donor cells.

Table 1
Manipulation and Culture Media Compositions

Component[a]	CZB[b] mg/L	M16[c] (activation) mg/L	Whittingham[d] mg/L	α-MEM[e] mg/L
Na_2HPO_4 $12H_2O$	–	–	56	–
NaH_2PO_4	–	–	–	122
KH_2PO_4	162	162	–	–
$CaCl_2$ $2H_2O$	250	–	264	265
$MgCl_2$ $6H_2O$	–	–	102	–
$MgSO_4$ $7H_2O$	290	293	–	200
Sodium pyruvate	36	36	55	110
Sodium lactate (60% syrup)	5.3 mL	3.3 mL	3.5 mL	–
NaCl	4760	5680	5803	6800
KCl	360	356	201	400
Glucose	1000	1000	1000	1000
HEPES, Na salt (free acid)	5200 (4760)	–	–	–
EDTA (disodium salt)	37	–	–	–
$NaHCO_3$	420	2101	2106	2200
BSA	–	4000	30000	4000
Other				
Amino acids (essential + NEAA)	–	–	–	1X
Vitamins	–	–	–	1X
gentamycin sulphate	20	20	20	–
Lipoic acid	–	–	–	+ (0.2)
PVP	1000	–	–	–
Phenol red	5	5	5	10
H_2O (milli-Q)	up to 1 L	up to 1 L	up to 1 L	up to 1 L

[a]All components from Calbiochem, except sodium lactate, sodium pyruvate, and phenol red (Sigma); gentamycin and BSA (ICN); water (Millipore).

[b]From **ref. *46***. Chatot, C. L., Ziomek, C. A., Bavister, B. D., Lewis, J. L., and Torres, I. (1989) An improved culture medium supports development of random-bred 1-cell mouse embryos in vitro. *J. Reprod. Fertil.* **86,** 679–688.

[c]From **ref. *47***. Whittingham, D. G. (1971) Culture of mouse ova. *J. Reprod. Fertil. Suppl.* **14,** 7–21.

[d]From **ref. *48***. Fraser, L. R. and Drury, L. M. (1975) The relationship between sperm concentration and fertilization in vitro of mouse eggs. *Biol. Reprod.* **13,** 513–518.

[e]α-MEM is purchased from Sigma (cat. no. M 4526).

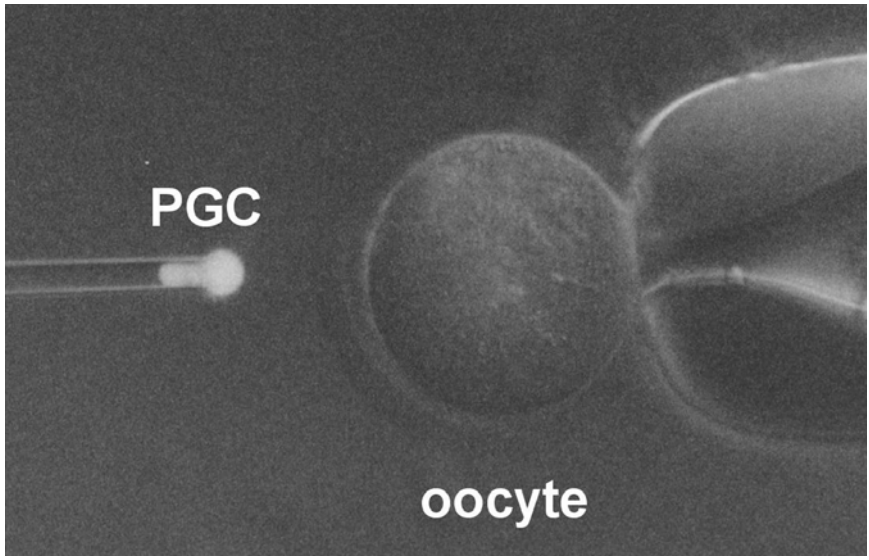

Fig. 9. Germ cells can be picked individually and used for specific purposes, such as injection of their nucleus into an oocyte (cloning by nuclear transfer), single-cell PCR, or other.

Bypassing fertilization and amphimixis with nuclear transfer during the cloning procedure requires extensive micromanipulation and technical skills. Micromanipulations are performed in HCZB medium (enucleation with PVP 0.1% w/v and 1 µg/mL cytochalasin B; nuclear transfer with 1% w/v PVP and no cytochalasin B; **Notes 16** and **17**) at 28°C using piezo-assisted capillary needles. The enucleation (15 µm ID), injection (8 µm ID), and holding (80 µm OD) needles are operated in a large drop (250 µL) of HCZB medium under silicon oil (*see* **Note 14**) on a 6-cm glass-bottomed dish. The enucleation and injection needles are loaded with 4–5 µL of mercury from the back down to the tip, which increases the momentum, thereby enhancing piezo pulse effectiveness. Batches of 20 oocytes are prepared in a drop of enucleation medium on the stage of the inverted microscope, equilibrated for 2 min, and deprived of their own chromosomes (metaphase II plate) in approx 10 min by gentle capillary aspiration (*see see* **Note 17**). After recovery in α-MEM for 2 h, the enucleated oocytes are processed together with the nucleus donor cells (retrieved from their storage at 4°C) in another drop of nuclear transfer medium (**Note 17**). Twenty to 30 donor cells are aspirated in the capillary at once, to be rapidly injected one-by-one into oocytes. After nucleus injection, the oocytes are left in the drop on the stage of the microscope for 10 min, then transferred to a drop of a 1:1 part mixture of HCZB and α-MEM medium out of the incubator for 1 h, and finally transferred to α-MEM for incubation at 37°C in 5% CO_2 atmo-

sphere. Two to 3 h later, the reconstructed oocytes are activated in Ca-free M16 medium (**Note 17**) supplemented with 10 m*M* $SrCl_2$, 5 µg/mL cytochalasin B, 0.5% v/v DMSO, and 1X RPMI vitamins. Six hours later, the clones are washed off the activation chemicals by several passages in Whittingham medium and transferred to α-MEM, where they are allowed to develop to the blastocyst stage.

3.6. Transfer of Embryos In Vivo

The most definite measure of pluripotency is the subsequent development of the embryo generated by either of the methods previously described: fertilization or cloning. One way to test for development is to transfer the embryos to the genital tract of pseudopregnant females. Embryo transfer is performed by surgery under general anesthesia (*see* **Note 18**) and involves the transfer of up to 30 embryos per side (1-2-4 cell stage embryos into 0.5-dpc oviduct; morula/blastocyst-stage embryos into 2.5-dpc uterus). Spare embryos can be maintained in vitro in α-MEM medium to ascertain blastocyst formation. Blastocysts are scored on developmental d 4 to be either fixed or seeded on a feeder layer of mouse fibroblasts (*see* **Note 19**) as a way of testing the competence of the blastocyst for implantation.

3.7. Viewing GFP in Live Cells and Tissues

GFP expression is characterized visually by examining the tissues with a fluorescence microscope. Embryos, fetuses, and gonads are scored for GFP using an inverted fluorescence microscope equipped with appropriate filters. The most common filter set used for GFP is the one designed for FITC (*see* **Note 20**). Because the blue excitation wavelength for GFP/FITC is close to that of UV light, in order to preserve cell viability, the light source should be attenuated by using the microscope built-in neutral density filters (ND4 + ND8). Bright field and GFP images can be captured with a commercial digital camera and saved in the JPEG format.

4. Notes

1. LacZ vs GFP. LacZ has also become amenable to viable detection. The recent application of the fluorogenic substrate 3-carboxy umbelliferyl-D-galactopyranoside (CUG) combined with fluorescence activated cell sorting (FACS) analysis has been shown to be several orders of magnitude more sensitive than the traditional βGal + XGal detection. Additionally, because of its high water solubility and detection limits, the CUG substrate has found extensive use in automated enzyme-linked immunosorbent assay (ELISA)-type systems. The conversion of the fluorogenic substrate CUG releases the fluorophore 7-hydroxycoumarin-3-carboxylic acid (CU); the emission of this highly fluorescent product is monitored at 460 nm using excitation at 390 nm ***(37,38)***.

2. Emergence and allocation of the germline visualized by *Oct4*/GFP (*Oct4*-ΔPE-GFP). Analysis of various stages of germline development was performed by fluorescence microscopy *in situ*. A progressive decline in GFP activity occurs in the epiblast, the first derivative of the ICM, from 5.5 dpc onward. Embryos recovered from 6–8 dpc show lower but significant levels of GFP because the *Oct4*-ΔPE promoter is silenced in the epiblast on its way to becoming active only in PGCs, but the protein persists longer than the mRNA. *Oct4*-ΔPE-GFP identifies germ cells shortly after (8 dpc) they have formed (**Fig. 4**). The gonadal ridges were dissected from 11.5 to 16.5 dpc fetuses, and postmigratory germ cells recovered based on GFP. From 13.5 dpc onward, male and female gonadal ridges could be distinguished based on their "striped" and "dotted" appearance, respectively (**Fig. 6**). Until about the fourth day postpartum in males and 13–16 dpc in females (when germ cells enter meiosis according to their sex), GFP is well-detectable and expressed exclusively in germ cells. After this point, expression decreases to a minimum, a finding consistent with the endogenous Oct4 expression pattern, being restricted to spermatogonia in the male testicular cords and to primordial oocytes in the newborn ovary.
3. Germ cells in the gonadal ridge are organized in clusters. Postmigratory germ cells were examined at various stages from 11.5 to 16.5 dpc, with stage-related differences observed. In male (B6OG2)F1 gonadal ridges, early postmigratory germ cells were found mostly as individual GFP-positive cells, whereas aggregates (clusters) occurred more and more frequently from 13.5 dpc onward (**Fig. 6**). These aggregates are reminiscent of the "cysts" described by Pepling in the newborn mouse ovary after immunohistochemistry for Vasa ***(39)***. The number of cells within an aggregate was estimated to range from 2 to 10 by aspirating individual cysts in a nuclear transfer micropipet and counting the nuclei while flushing them out. If these structures found in the developing male gonad were cysts indeed, they may have to serve a different biological function than the one postulated by Pepling (i.e., elimination of defective mitochondria ***[39]***).
4. PGCs can be enriched to near-purity using Oct4-GFP. Purification of germ cells can be automated by FACS. Ninety-nine percent of the GFP-positive putative PGCs in the lower right quadrant before antibody staining shifted to the upper right quadrant after staining with an antibody directed against c-kit (**Fig. 7**). This indicated that the high GFP-positive population of cells within the gonad is also positive for c-kit. Most of these GFP-positive cells also coexpress additional markers (integrins α6, β1; data not shown). Therefore, they are *bona fide* germ cells. The stromal cells within the ridge also express c-kit receptor, and are likely to be Leydig cell precursors, which as germ cells are known to express the receptor. Using the same transgenic mouse line as we did (OG2), the degree of PGCs' purity in the GFP-positive cells sorted by flow cytometry was found to be high (96%) as measured by coexpression of GFP and classical PGC markers, such as SSEA-1 ***(36)***. If necessary, the enrichment can be further increased, i.e., the few GFP-negative cells can be sorted out by individual picking using the micromanipulator while viewing under blue light excitation (**Fig. 9**).

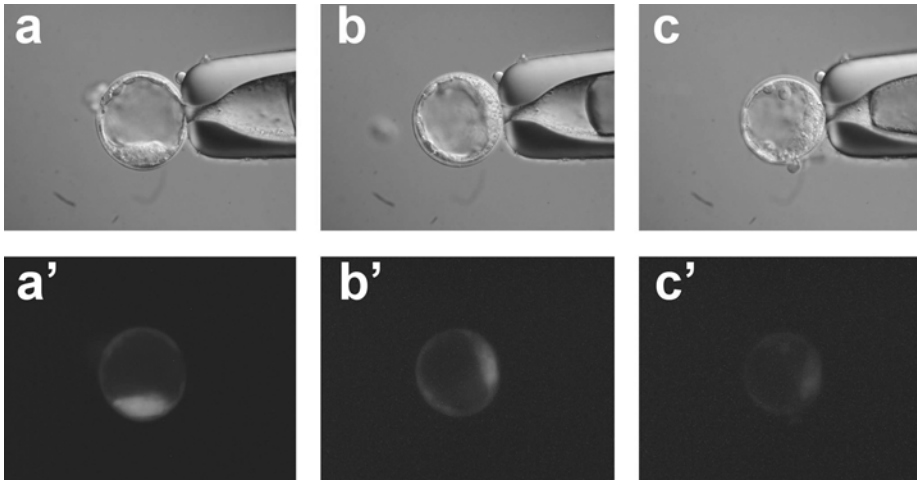

Fig. 10. Examples of Oct4-ΔPE/GFP levels and distributions in blastocysts. Although GFP is always localized to the ICM in fertilized blastocysts (**A**), abnormalities become evident as fertilization is by-passed by somatic cell nuclear transfer (**B**,**C**). **A–C**: DIC bright field; **A–C**: GFP fluorescence.

5. *Oct4*-GFP after fertilization. (B6OG2)F1 embryos were produced in vitro by in vitro fertilization (IVF) and examined for GFP expression as driven by the *Oct4*-ΔPE promoter. Feeble green fluorescence could be detected in some embryos at 48 h, suggesting the very onset of gene expression in asynchronous fast developers. GFP was first noted at the eight-cell stage, and its intensity peaked at the morula stage (**Fig. 8**). At 96 h of development, blastocysts were classified according to their GFP expression pattern as follows: blastocysts expressing GFP in all their cells, blastocysts with ICM-restricted expression, blastocysts without GFP expression, and mosaic expression in blastocysts (**Fig. 10**). Most fertilized blastocysts presented a ICM-restricted signal over a diffuse GFP background. Because the enhanced GFP used in this study is 35-fold stronger than the native GFP, this caused saturation of the green video channel so that ICM restriction could be appreciated only when the excitation was attenuated (32-fold; **Fig. 11**). Those IVF embryos that were scored as GFP-negative were presumed to be parthenotes (having formed by spontaneous oocyte activation in the presence of sperm) and consequently discarded. During postimplantation development, Oct4 expression is restricted to the epiblast. This is accomplished by the *Oct4*-ΔPE-GFP transgene as well, as described by Yeom et al. ***(18)***, but the signal then goes down, being the PE required for maintenance of expression in the epiblast (**Fig. 4**). However, Gertz ***(40)*** could not reproduce some of the observations noted by Yeom et al. ***(18)*** with respect to the spatial distribution of the transgene product in the primitive ectoderm and endoderm as compared to Oct4. Timing may be an

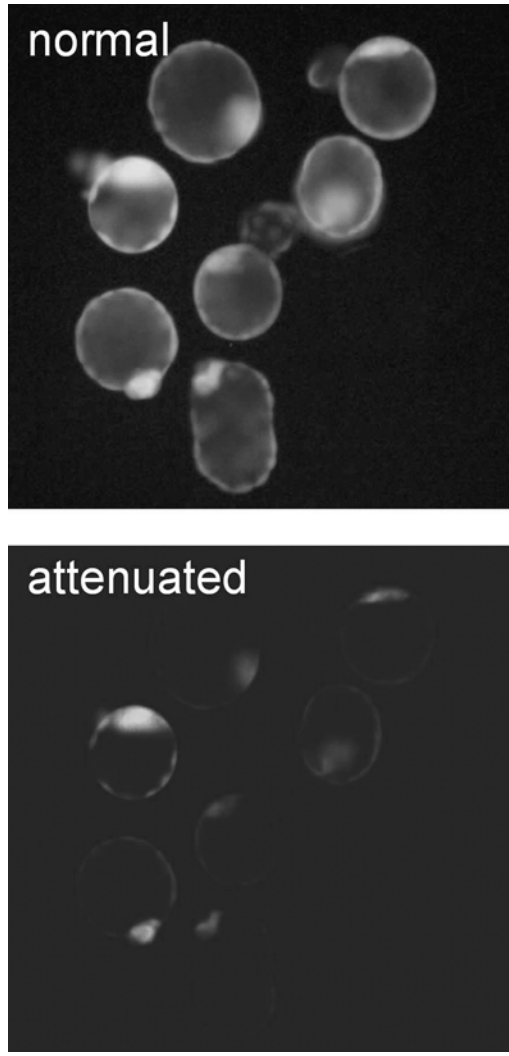

Fig. 11. The power of discrimination between the ICM and the trophectoderm of Oct4-ΔPE/GFP embryos becomes sharp after the excitation is dimmed. (Top) Normal excitation with no filters and no exposure correction (f-stop); (bottom) better signal/noise ratio after 32× filter attenuation.

issue, as it is known that Oct4 is transiently expressed in the primitive endoderm ***(41)***. Also, the different insertion locus may account, at least in part, for discrepancies observed in the *Oct4*/transgene expression patterns; reporter gene expression levels may slightly differ between different transgenic lines. The accurate

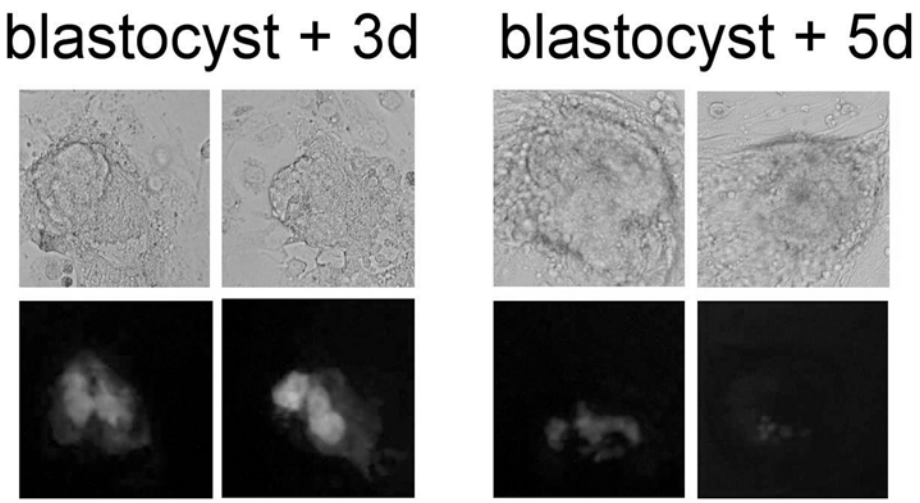

Fig. 12. Events occurring in the embryo around and immediately after the time of implantation can be modeled by allowing the blastocyst to attach to and spread on a feeder layer of embryonic fibroblasts. This is called outgrowth and is the first step toward the derivation of ES cells.

recovery of timed embryonic stages around implantation (4.5–5.5 dpc) is quite difficult. To circumvent this problem, these stages were studied by an in vitro outgrowth model and examined for expression of *Oct4*/GFP (**Fig. 12**). Briefly, blastocysts were plated on a feeder layer of mitomycin C-inactivated mouse fibroblasts, where they could attach, mimicking the process of implantation to the uterus. During 3–5 d coculturing, the trophectoderm of the blastocyst spreads on the feeder cells (trophoblast invasion), leaving the ICM cells behind to form a clump. *Oct4*-GFP lacking the PE is purported to be an exclusive marker for ES cells and, later on, PGCs. In fact, GFP almost completely vanishes in the spreading part of the outgrowth (derived from TE cells) to be maintained only in a cluster of cells growing from the ICM.

6. *Oct4*/GFP expression after transfer of somatic cell and PGC nuclei into oocytes. Using Oct4/GFP as a marker, germ cells now become amenable to viable selection and direct testing in biological assays. For instance, the developmental potential of germ cell nuclei after transfer into oocytes remains controversial. To date, gonadal cells bearing morphological resemblance to germ cells have been used or germ cells have been assessed for purity by AP staining and unprocessed sibling cells used for nuclear transfer (*see* **ref. *42*** and references therein). Given such experimental design, the identity of the nucleus donor cells is not known with any degree of certainty. In addition, because germ cells are maintained to undergo erasure of epigenetic imprints between 8.5 and 12.5 dpc in vivo, reports

on putative germ cell (13.5–16.5 dpc) nuclei that support the development of clones to midgestation are quite surprising. This prompted testing of certain (Oct4/GFP-positive) germ cells for developmental competence after nuclear transfer. The comparison between germ cell (*Oct4* on/open state) and cumulus cell (*Oct4* off/closed state) allows the testing of whether the silent state of Oct4 in somatic cells is mediated by a lack of transactivators or a physical modification of the gene locus.

Nuclei were transplanted from *Oct4*-ΔPE-GFP transgenic donor cells into oocytes to test for reactivation (cumulus cell) and maintenance (germ cell) of transgene expression and, hence, for pluripotency. The reconstructed oocytes were scored for GFP at the two-cell, four-cell, eight-cell, morula, and blastocyst stage. When expressed, Oct4-ΔPE/GFP was observed in clone embryos from about the eight-cell stage regardless of the nucleus source. Because the transgene was already functioning in germ cells at the time of nuclear transfer, as opposed to cumulus cells, this suggests that a physical modification (opening) of the gene locus is not required. Also, because GFP was not detectable through the one- and two-cell stages in both types of clones, this suggests that required transcription factors and/or coactivators are lacking during the first 40 h of development. In comparison to the fertilized embryos, GFP activity in clones was weak (**Fig. 10**), and clone blastocysts were scored with GFP distributed overall or "patchy" (GFP mosaicism). Because GFP is the same in fertilized and clone embryos (same transgene source, OG2), persistence of GFP expression in clones suggests a difference in gene expression (transcription, translation) rather than a longer half-life of the fluorescent protein in the clone cytoplasm environment. When clones were derived from male germ cell nuclei, the dynamics of GFP expression during preimplantation development were qualitatively the same as those for somatic cell nuclei; however, a much higher rate of blastocyst formation occurred using germ cell nuclei ***(35)***. It is very difficult to draw a correlation between the GFP pattern in blastocysts and their developmental potential. This would require transfer and follow-up of the blastocysts into the uteri of pseudopregnant females. Alternatively, the blastocysts could be assayed for outgrowth formation on a feeder layer, a well-established in vitro model for implantation. This was attempted on a limited number of NT blastocysts obtained from cumulus and germ cell nuclei. Outgrowths formed from cumulus cell-derived blastocysts regardless of the presence or absence of GFP; however, some GFP-positive blastocysts were found to have turned into GFP-negative outgrowths. The ability to derive some ES-like colonies suggested the presence of a pluripotent ICM. Although ES cell lines were established, this was the case for a scant minority of the blastocysts. The proportion of cumulus cell-derived blastocysts that gave rise to ES cell colonies was in line with the rate of postimplantation development attained in vivo. After embryo transfer of 472 cumulus cell-derived clones to uterus, five pups were recovered at 18.5 dpc by cesarean section. As expected, they were all phenotypic females with their gonads glowing green from the *Oct4*-GFP transgene activity (**Fig. 13**). In contrast to the cumulus cell-derived clones,

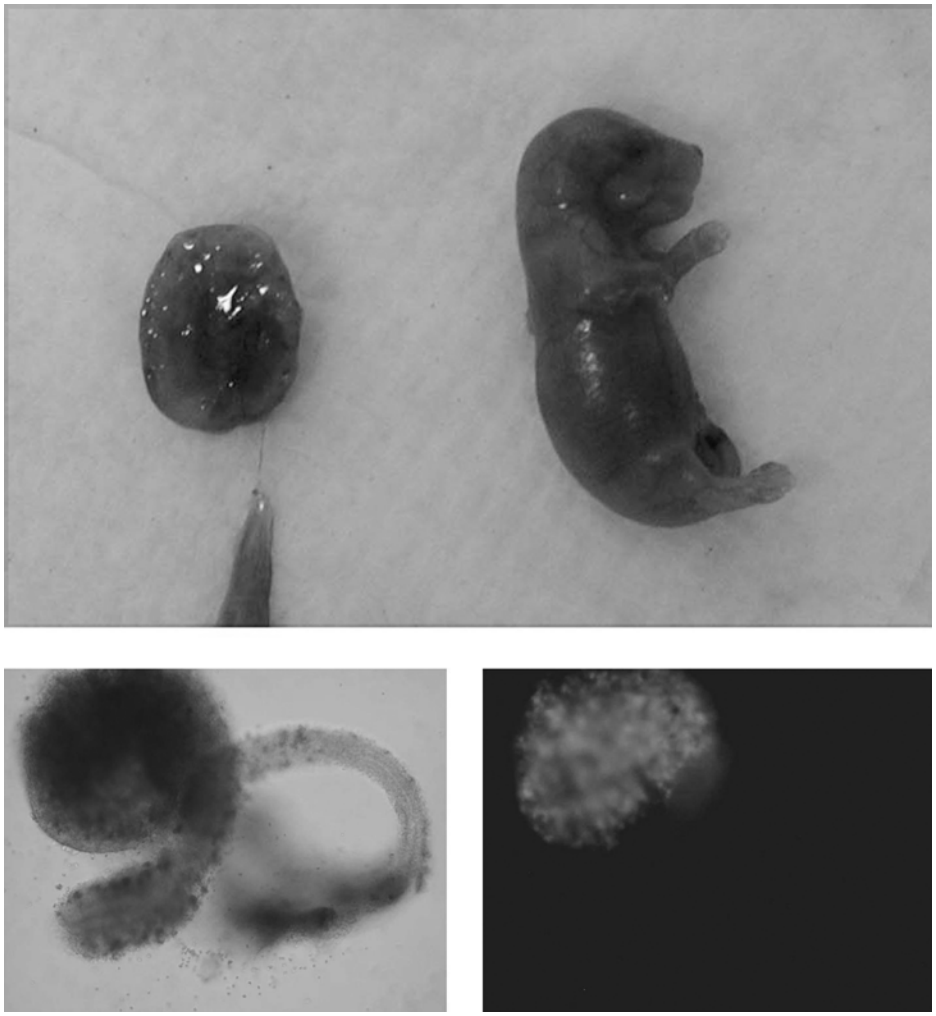

Fig. 13. A clone mouse pup (top) derived from an OG2 cumulus cell nucleus. The pup is therefore a female as indicated by the ovary as a gonad (bottom left). *Oct4*/GFP marks the germ cells (bottom right). Reproduced with permission from Cold Spring Harbor Press.

negligible postimplantation development and no ES colonies were obtained from 13.5 to 16.5 dpc germ cell-derived blastocysts and their outgrowths. To account for the reports of development to midgestation of putative germ cell-derived clones, we hypothesize that either gonadal cells other than germ cells may have been picked instead of true germ cells or that a subset of germ cells may not have undergone irreversible epigenetic reprogramming by 13.5–16.5 dpc. In summary,

Oct4 plays an essential role in the mechanism that ensures embryo viability. It is evident that Oct4 cannot account for all types of developmental failure in fertilized and clone embryos; faulty Oct4 downregulation at the blastocyst stage appears to be a weak explanation. The intrinsic deficiency in genome demethylation activity, which has been described recently ***(43)***, may be associated with the prior removal of the maternal genome from the oocyte. It follows Oct4/GFP can be used as a marker for reprogramming to exclude those embryos that will most likely fail at subsequent development based on their Oct4/GFP pattern. However, Oct4/GFP provides little or no information for those embryos that fail at later stages, possibly because of other defects.

7. Mouse welfare. Prerequisites for successful mouse studies include a good animal house environment and proper husbandry practices. In a noisy environment or in one with frequent animal access by caretakers, the outcome of animal-based research is compromised. As one environmental factor, the food diet has to be checked for phytosterols, which should be as low as possible in order not to interfere with the mouse female's own response to the hormones (gonadotropins) used for inducing ovulation (*see* **Note 8**). All mice used in this study were purchased from Taconic and fed with Harlan Teklad chow. The mouse colony room was kept under controlled temperature (20°C) and photoperiod (0800–2000 light hours). Animals were maintained and used according to the guidelines of the Internal Animal Care and Use Committee (IACUC) of the University of Pennsylvania.
8. Priming of females with hormones, sacrifice, and dissection. In vivo supermaturation and superovulation of mouse oocytes are to be induced with the lowest effective dose of gonadotropins (pregnant mare's serum gonadotropin [PMSG]; human chorionic gonadotropin [hCG]). More than 10 U of either PMSG or hCG per female significantly decreases oocyte quality; and, regardless of the dose, the uterine endometrium is negatively affected by PMSG, resulting in embryo difficulty at implanting ***(44)***. For this reason, reliable recovery of postimplantation material should follow natural mating. Different mouse strains are not equally sensitive or responsive to gonadotropins, therefore, the optimal dosage needs to be determined for the mouse strain concerned. In this study, PMSG and hCG have been purchased from Calbiochem (cat. nos. 367222, 230734) and reconstituted in PBS. For recovery of ovulated oocytes to be fertilized in vitro, 8–12 wk-old (B6C3)F1 females are administered with gonadotropins (7.5 U PMSG at 5:30 PM and 7.5 U hCG at 4:30 PM, 2 d apart) delivered intraperitoneally by a syringe fitted with a 1/2-inch 27-gauge needle. Fifteen hours after hCG injection, the females are sacrified by CO_2 inhalation followed by cervical dislocation. The oviducts are dissected, and the oocytes are isolated in HCZB medium (**Note 11**). To recover peri-implantation-stage embryos, 4–6 wk-old (B6C3)F1 females under PMSG + hCG stimulation are mated with OG2 stud males right after the hCG injection. The day of the vaginal plug is considered as 0.5 dpc (i.e., d 0.5 of development). The embryos are flushed from the

uterus at 3.5 dpc. To recover postimplantation stages, 4-wk-old (B6C3)F1 females are mated with OG2 stud males and the embryos or fetuses are recovered after 4.5 dpc.

9. Isolation and culture/storage of fetal germ cells. Germ cells do not exist as individual cells within the gonadal stroma, but are organized as aggregates or clusters. "Cysts" have been already described in the mouse ovary ***(39)***. Release of germ cells from the gonadal ridge by mechanical means disrupts the tissue macroarchitecture (i.e., testicular cords in the male), but leaves most germ cell–germ cell contacts in place, whereas exposure to EDTA frees germ cells from one another (loss of microarchitecture), while leaving the supporting matrix of the gonadal ridge intact. Prior to 13.5 dpc, mouse germ cells of either sex are engaged in active cell division that brings their number approximately from 300 (8.5 dpc) to 20,000 (13.5 dpc) approximately. This is relevant to the fine estimate of PGC numbers and the efficiency of cloning when germ cells are to be used as nucleus donors (**Note 17**); in fact, transfer of those nuclei frequently in S-phase results in oocyte fragmentation upon activation of the transplanted oocyte.
10. Collection of germ cells by FACS. The GFP expression in *Oct4* transgenic germ cells prevents the use of fluorescein–FITC conjugated primary antibodies. Although most commercial antibodies are offered conjugated with phycoerythrin, APC is another useful fluorochrome for performing multi-color FACS. Unlike phycoerythrin, APC's emission spectrum does not overlap with that of GFP or FITC, requiring no compensation between the FL1 and FL2 detectors. A good tip is to always perform a time-delay calibration using Calibrite-APC beads at the beginning of the experiment to synchronize the acquisition of events from both lasers. Many primary antibodies are now offered conjugated with APC. Alternatively, primary antibodies can be biotinylated and used with a strepavidin conjugated with either cychrome detected on FL3 or APC detected on FL4. When using biotinylated antibodies for FACS, substitute 10% BSA for the 20% FCS in the media used to inactivate the trypsin. Serum coats cells with large amounts of biotin that will bind the secondary strepavidin–fluorochrome conjugate. Always use an appropriate biotinylated isotype control to test for nonspecific binding of the primary antibody to the germ cells and to perform an initial antibody titration study.
11. Collection of adult oocytes. The cumuli oophori are dissected from the oviducts and hyaluronidase (50 U/mL in HCZB medium with 0.4% w/v BSA) is used to free the oocytes from their cumuli at room temperature. The commercial enzyme is often a crude preparation with many impurities and undesired activities. The commercial batch should be chosen with the highest specific activity (5000 U/mg or higher). In this study, hyaluronidase has been purchased from Calbiochem (cat. no. 38594). Low-working dosage (50 U/mL) and room temperature incubation are helpful in order to minimize the toxic effect of other activities (e.g., proteases) on oocytes. Do not incubate the hyaluronidase reaction at 37°C, as this would favor the activity of such proteases. Presence of albumin may quench the

effect of the residual protease activity. Therefore, pipet-transfer the cumuli oophori in a drop of 50 U/mL hyaluronidase solution. Leave for approx 10 min at room temperature, and remove the oocytes by a mouth-operated micropipet as soon as they are loose (**Note 13**).

12. Oocytes IVF. Sperm is isolated by swim-up from the cauda epididymis of mature OG2 males (2 mo or older) and capacitated in Whittingham medium (3% BSA, fraction V, Sigma, cat. no. A3311) at 37°C for 1.5 h prior to oocyte insemination. Sperm concentration is adjusted to 2×10^6 sperm/mL in the insemination drop. Fertilized oocytes are recovered from the insemination drop 2–3 h later. Composition of Whittingham medium is described in **Note 13**.
13. Oocyte and embryo culture. We obtained best results using α-MEM supplemented with specific brand and grade of BSA (ICN, cat. no. 103700; **Table 1**). Prepare the embryo culture dishes by placing drops of medium (20 µL) on a 35 mm suspension culture dish, overlay with silicon oil, and pre-equilibrate at 37°C in a 5% CO_2 incubator for at least 2 h. The best culture dishes are those designed for suspension cell culture (Corning cat. no. 430588), as their untreated (hydrophobic) surface keeps the culture drops rounder. The oil should be washed (*see* **Note 14**) and equilibrated with culture medium in the incubator at least overnight, prior to overlaying the drops. Embryos release autocrine and paracrine factors in the medium, which should therefore not be changed unless necessary when metabolic acidification compromises the buffer capacity of the medium. An efficient method to counteract the negative effects of metabolic acidification while preserving the growth factors released in the medium is to replenish the culture drop by adding fresh medium half way through the culture period.
14. Oil. Silicon oil (dimethylpolysiloxane [DMPS]) is used to overlay the drops of either manipulation or culture medium, and it comes in various grades of viscosity. The "thin" silicon oil (5–20 centistokes; Sigma, cat. no. DMPS-V, DMPS-2X) is more suitable to overlay the HCZB medium in the micromanipulation chamber; the "thick" silicon oil (100–200 centistokes; Sigma, cat. no. DMPS-1C, DMPS-2C) is more suitable to overlay the culture drops of α-MEM. Once the "thick" oil has been equilibrated in the CO_2 incubator, it will keep gas exchange between medium/air at a low rate as the dish is removed from the incubator. Instead, the "thin" oil does not have a strong buffer capacity for gas exchange, but makes it easier to move the needles in and out of the micromanipulation chamber. Some investigators sterilize the oil by autoclaving, whwewas others believe that this is unnecessary if antibiotics are present in the culture medium, and it may actually render the oil toxic. We recommend not to autoclave the oil and to minimize the use of antibiotics, but to shake the oil in a bottle with medium supplemented with antibiotics, then let the medium decant, and take the upper layer of oil for subsequent use.
15. Isolation and storage/culture of somatic cells. Cumulus cells are a very convenient source of somatic cells for cloning as they come with the oocyte and are arrested in G1/G0. However, they retain some ability to proliferate in the culture. To be used as nucleus donors, cumulus cells need to be prevented from attaching

to the substrate or entering the cell cycle. This is effectively accomplished by keeping the cell's drop of hyaluronidase in HCZB medium at 4°C, after adding 1/4–1/2 vol of Whittingham medium to inactivate the proteases that may have bound to the cell membrane. If the cumulus cells are to be used for cell culture, they need to be aspirated from the dish and transferred to a tube to be centrifuged/resuspended in proper medium.

16. Media for oocyte micromanipulation. Although a variety of media is commercially available from specialized vendors, we recommend producing them in-house using milli-Q water (**Table 1**). A protein-free, HEPES (ICN, cat. no. 1588413)-buffered, hypertonic CZB medium is used for all manipulations. A medium can be made hypertonic by adding sucrose (ICN, cat. no. 821713; 2.5% w/v final concentration) or by dissolving its components in less volume (95% of the standard volume). Here we recommend not adding any extra component, such as sucrose, but reconstituting the medium in less volume. Use of slightly hypertonic medium does not harm mouse development while it facilitates micromanipulations of oocytes owing to the enlarged perivitelline space. Medium supplementation with 0.1%–1% w/v PVP is aimed at counteracting stickiness in the absence of albumin. Substituting PVP (Calbiochem, cat. no. 529504) for albumin also takes care of the batch-to-batch variations in the quality of albumin. PVP is preferred over polyvinylalcohol, which is more difficult to dissolve (cold-water soluble, hence, problematic in warm media). A great deal of variability exists between different sources and batches of PVP (**Note 17**).
17. Nuclear transfer and oocyte activation. In our experimental setup, a manual micromanipulator (Narishige MN-188NE) and a piezo unit (PrimeTech PMAS-CT150) are coupled to an inverted microscope (Nikon Eclipse TE300) equipped with DIC long-working distance (ELWD) optics. Using such optics in association with glass-bottomed vessels, visible enucleation of mouse oocytes becomes possible without the need to displace the cytoplasmic granules by centrifugation or staining the chromosomes with a dye specific for DNA. The meiotic spindle is temperature-sensitive. In human oocytes, it starts disassembling soon at 1°C or 2°C below 37°C. In mouse oocytes, the spindle is not visible at or below 20°C. For these reasons, an intermediate temperature of 28°C was used in this study. The entire micromanipulation room was set as an environmentally controlled room warmed at 28°C. Although it is possible to remove the spindle as it is without altering the cytoskeleton, disassembling the actin network by exposure to cytochalasin D or preferably B (ICN, cat. no. 195119) facilitates the task. Cytochalasin B is different from cytochalasin D in that it acts faster, but it also inhibits the transport of glucose through the oocyte membrane. Cytochalasin B can de dissolved either in ethanol or in DMSO (Sigma, cat. no. D2650). It does not matter which carrier is used, provided its final concentration in the medium is kept low. Mouse oocytes should not become activated in the presence of minimal ethanol concentration, as they require up to 7% v/v for full activation; however, this may not apply to oocytes from other species. To assist oocyte enucleation, a stock solution of 5µg/µL cytochalasin B in DMSO is diluted to a final concentra-

tion of 1 μg/mL Cytochalasin B and 0.02% DMSO. It is important to let the enucleated oocytes recover for 1 or 2 h, as allowance of shorter time may result in a higher rate of lysis postinjection of the donor nucleus. For the nuclear transfer, there is no need to suspend the nucleus donor cells in 12% PVP as indicated in other studies. Besides, this is a very dense and viscous environment that does not permit the oocytes to be processed together with nucleus donor cells in the same drop of medium. We experienced a 1% PVP concentration that is compatible with oocyte viability and reduces the stickiness of exposed donor nuclei to a minimum. The PVP stock solution (16% w/v in water) must be made fresh every week and diluted to the working concentration each time micromanipulation is performed. We obtained consistently good results with PVP from Calbiochem (molecular weight 40 kDa), but not with other brands. Activation of the reconstructed mouse oocytes is best obtained using 10 m*M* $SrCl_2$, (Sigma, cat. no. 0390), according to reports, in the presence of Cytochalasin B to prevent pseudo polar body extrusion. A 1 *M* stock solution of $SrCl_2$ is prepared in water (do not use PBS as phosphates in conjunction with glucose, may cause a two-cell block of mouse development) and diluted 100 times in Ca-free M16 medium. A 1000X stock solution of cytochalasin B is diluted in Ca-free M16 medium to a final concentration of 5 μg/mL Cytochalasin B and 0.02% v/v carrier DMSO, which is increased up to 0.5% v/v DMSO, as this was empirically found to improve cleavage to two-cell. Great care must be taken to remove all Ca-containing medium from the activation drop by repetitive washes in predrops of activation medium. We found that 10 m*M* $SrCl_2$ may precipitate in a few hours at 37°C; therefore the activation drop should be inspected prior to transferring the oocytes, in order to clear. The timing of the preactivation and activation procedures has been empirically found to affect the outcome of the cloning experiment. Nuclear transfer would be best completed by noon, and the activation started by 2:00 PM. After activation, a single wash in Whittingham medium is enough to remove all the Cytochalasin B and enable cleavage, but more washes are helpful. Cytochalasin B is absorbed by albumin, which is present in large amounts in the Whittingham medium. Subsequent cleavage of the reconstructed oocytes is able to take place in α-MEM. Do not transfer embryos from drop to drop, but replenish the spent medium in the same drop.

18. Embryo transfer. The observation has been consistently made that the first round of plugs was not as good as the second or third in providing the best chances of pregnancy. For transfer of embryos in vivo, recipient females plugged by vasectomized males are anesthetized with a mixture of xylazine (Rompun) and ketamine (Ketalar), were the doses administered were 0.2 mg and 0.3 mg, respectively, per 10 g/body weight. Prior to transfer, embryos are rinsed in HCZB medium containing PVP to wash off the bovine albumin, which may trigger an immune response by the mother.
19. Blastocyst outgrowth formation. Feeder cells are required because the implanting embryo has an attachment/invasive behavior. STO (SNL) feeder cells, a murine embryonic fibroblast cell line expressing the recombinant leukemia-

inhibiting factor and G418 antibiotic resistance (neo), are a convenient source of feeder cells ***(45)***. Prior to seeding the blastocysts onto STO cells, feeders are mitotically inactivated by exposure to mitomycin C (10 µg/mL in Dulbecco's modified Eagle medium [DMEM]) for 2–3 h. After careful washing, feeders are maintained in DMEM medium with supplements (15% fetal bovine serum, 0.1 m*M* non-essential aminoacids, 2 m*M* L-glutamine, 0.1 m*M* mercaptoethanol, and 50 U/mL penicillin-streptomycin). Blastocysts are seeded on the feeder cells and their outgrowths are examined 3 d later, a time point equivalent to developmental d 7.

20. Imaging. Viable observations of cells or tissues are carried out while in a HCZB medium. HEPES is light-sensitive; therefore, illumination should not be protracted when unnecessary. GFP is excited with blue light (excitation range 470–480 nm; beam splitter range 495–505 nm; emission range 500–535 nm), which is akin to UV light. Some reports indicate that the toxic effect of GFP may actually be a consequence of irradiation rather than of the GFP itself. Here we recommend using neutral-density filters and long exposure to compensate for low intensity. Alternatively, the culture medium can be supplemented with 0.1 m*M* *N*-acetyl cysteine (NAC) to protect cells from the UV damage.

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2

Analysis of Germline Chromatin Silencing by Double-Stranded RNA-Mediated Interference (RNAi) in *Caenorhabditis elegans*

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1. Introduction

RNA interference is a simple, efficient, and highly scalable method for the analysis of gene functions in *Caenorhaditis elegans* independently of mutants availability ***(1,2)***. It is used to study individual genes, gene families, and also for genomewide screenings ***(3)***. In a RNAi experiment *C. elegans* is exposed to double-stranded RNA that corresponds to the sequence of a specific mRNA. This triggers a series of enzymatic processes, resulting in a specific degradation of this target mRNA ***(4–7)***. Consequently the animals display a phenotype that results from the depletion of the target protein. The RNAi phenotype can reach the severity of a null mutant ***(2)***. Although, generally RNAi is far more effective than antisense RNA ***(2,8)*** the effectiveness of a specific RNAi experiment depends on the target gene.

In germ cells of different organisms, the transcriptional activity is typically reduced by a variety of different molecular processes not yet completely understood ***(9)***. We used the RNAi approach to identify genes essential for the chromatin silencing in the germline of *C. elegans*, e.g., the linker histone isoform H1.1; ***(10)***.

1.1. Strategies of RNAi Interference in C. elegans

RNAi interference in *C. elegans* is a systemic response that results from exposure to specific dsRNA, which means that the interference spreads to all cells of the individual animal, regardless of the route of administration. Addi-

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tionally, the interference is passed on from the exposed hermaphrodite animal to the next generation (F1 animals), allowing two different kinds of experiments. Exposure of F0 hermaphrodites depletes maternal, as well as zygotic, mRNAs and also the mRNA of all later stages of the F1 generation. This allows a phenotypic analysis of the F1 generation if the RNAi is not embryonic lethal and does not result in larval arrest. Because the interference occurs in all life stages, it cannot be deduced when a certain transcript is functionally required.

Alternatively, L1 larvae or later-stage animals can be exposed to dsRNA and scored directly. This being used to study embryonic lethal or larval arrest genes and also demonstrating that a certain germline phenotype originates from the postembryonic depletion of mRNA.

Four different ways of exposing *C. elegans* to dsRNA have been introduced: feeding with dsRNA expressed in bacteria ***(11)***, soaking of worms in dsRNA ***(12,13)***, dsRNA microinjection, and expression of dsRNA by transgenes in *C. elegans* ***(14)***. The first two methods are currently used in genomewide screening experiments ***(3,13)***. Microinjection of dsRNA, however, occasionally gives stronger phenotypes, even to the extent that these phenotypes occur only in microinjection experiments, but not in soaking or RNAi feeding experiments. Therefore, we recommend to start the analysis of single genes by dsRNA microinjection. Microinjection also allows the mix of multiple different dsRNA species in order to achieve a combined depletion of different mRNA species, whereas a combined dsRNA feeding is not effective. Germline silencing in *C. elegans* is a function of temperature. Higher temperatures up to 25°C favor desilencing as well as RNAi efficacy and are used in the initial depletion experiments. Further biological characterization of phenotypes then includes experiments at lower temperatures, e.g., 16°C and 20°C.

1.2. Effector Sequence Design

The dsRNA sequence should not be longer than 2000 bp and no shorter than 500 bp. For larger sequences, the efficiency of T7 RNA polymerase transcription will be reduced, whereas shorter fragments generate reduced interference activity in *C. elegans*. When this is tolerable, fragments as short as 200 bp can be used. As interference originates from the degradation of the corresponding mRNA, the sequence needs to be deduced from cDNA or from genomic DNA containing mostly exons of a single gene. The specificity of the sequence can be controlled by a BLASTN analysis of the *C. elegans* genome (http://www.sanger.ac.uk/Projects/C_elegans/blast_server.shtml). Similar sequences with an identity of 70% or more that extend over regions longer than 100 bp potentially lead to crossreactivity of the RNAi, which is typical in gene families. By choosing appropriate cDNA fragments, the researcher can optimize

either crossreactivity or single-gene specificity. A public electronic resource is available for primer design (http://www.sanger.ac.uk/Projects/C_elegans/oligos.shtml). Public databases also contain examples of PCR primers for the amplification of any *C. elegans* gene for the purpose of RNAi interference (*see* **Subheading 2.6., item 4**). *C. elegans* cDNAs cloned in pBluescript II, e.g., yk85b12 encoding histone H1.1 ***(10)***, can be requested from the yk EST project, (http://nematode.lab.nig.ac.jp/index.htm) ***(13)***.

1.3. In Vitro Synthesis of dsRNA for Microinjection or Soaking

Highly concentrated double-stranded RNA is transcribed from a linear DNA template produced as a polymerase chain reaction (PCR) product that contains T7 RNA polymerase promotors in opposite orientations on both ends (**Fig. 1**). If PCR amplification is done using genomic template DNA the 3' end of both primers should be appended with the T7 RNA polymerase promotor sequence 5'-GTAATACGACTCACTATAGGG-3'. If the fragment is already cloned between the T3 and T7 polymerase promotors of pBluescript II (Stratagene), PCR amplification can be done with a mixture of a T7 primer and the primer T7–T3, which is a T3 primer appended with the T7–RNA polymerase promotor. For fragments cloned in the RNAi feeding vector L4440 PCR amplification with the T7 primer alone is sufficient.

1.4. RNA Interference by Microinjection

Any injection of dsRNA into *C. elegans* leads to a systemic interference reaction. For a maximum of interference in the next generation, a single dose of dsRNA is injected into the rachis of the syncytial gonad of L4 larvae or of young hermaphrodites (**Fig. 2A**). Alternatively, and far easier for the inexperienced operator the lumen of the intestine can also be injected. In a standard experiment, the F1 generation is scored according to germline silencing, morphology, behavior, and development. The occurrence of a RNAi phenotype, severity of this phenotype, and percentage of animals affected is a function of the time after injection. Typically, the animals from eggs laid in the first 6 h are relatively unaffected. Then, RNAi interference sets in and reaches a maximum penetrance in the F1 animals laid 16–30 h following the injection. Therefore, the injected animals are transferred every 6 h onto fresh plates in order to receive a batch of F1 animals with a maximum of phenotypic penetrance. Occasionally, RNAi penetrance can be critically low (≥5%). To enhance RNAi, efficacy the injected worms and their offspring can be fed with *Echerichia coli* HAT115 expressing the specific dsRNA. The resulting F1 animals can also receive an additional dose of injected RNAi before their adult phenotype is determined ***(10)***.

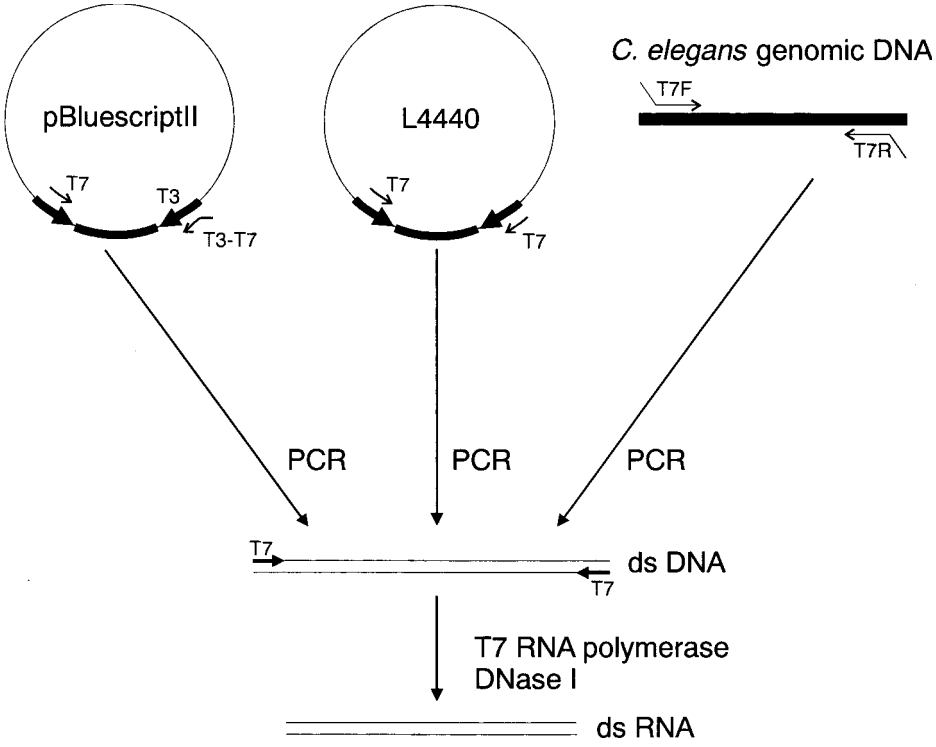

Fig. 1. In vitro synthesis of double-stranded RNA (dsRNA). *C. elegans* DNA is represented by a bold line. The fragment of interest is amplified from a feeding vector (L4440) derived plasmid with a T7 primer, from a pBluescript II clone with primers T7 and T3-T7, or from *C. elegans* genomic DNA with two specifically adapted T7 primers. Then, the PCR product is transcribed with T7 RNA polymerase. The template DNA is subsequently digested with RNase-free DNase I.

1.5. RNA Interference by Feeding

Systemic RNA interference can be achieved in *C. elegans* by feeding worms with transgenic *E. coli* bacteria producing specific dsRNA. The *E. coli* strain HT115 is used in combination with the expression vector L4440 (***11***; **Fig. 1**). In this system, IPTG induces T7–RNA polymerase expression and subsequent production of dsRNA, allowing continuous exposure of *C. elegans* populations to dsRNA. However, the efficiency of RNA interference achieved by feeding can be minor when compared to that of microinjection. RNA interference by feeding requires less technical skills than microinjection and can be used at far larger scales. Individual feeding clones are created by inserting genomic fragments or cDNAs into the L4440 vector. Alternatively, a full genome RNAi feeding library (approx 20,000 *E. coli* clones) has been created

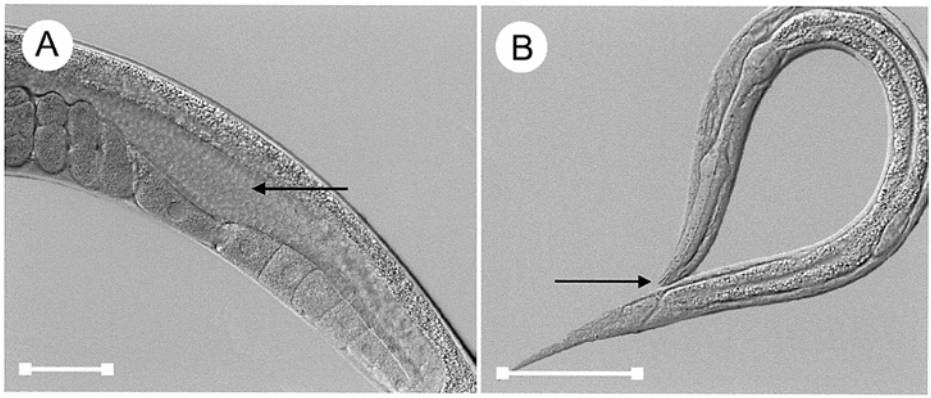

Fig. 2. Delivering dsRNA to Caenorhabditis elegans. **(A)** dsRNA is microinjected into the lumen of the syncytial gonad (the rachis) of hermaphrodites. The injection needle should be placed to where the tip of the arrow points. **(B)** Late L3/early L4-stage larvae are used for dsRNA feeding experiments and also for soaking. The arrow points to the mouth opening of the animal. The scale bars corresponds to 50 μm.

by Julie Ahringer's group (The Wellcome CRC Institute, University of Cambridge, UK) and is distributed by the MRC UK HGMP Resource Center ***(3)***(*see* **Subheading 2.6.**; **item 7**).

1.6. Analysis of Germline Silencing

Highly repetitive transgenes are efficiently silenced in the germline of *C. elegans*. Therefore repetitive green fluorescence protein (GFP) transgenes are used to monitor germline chromatin silencing in living *C. elegans*. Kelly and Fire ***(15,16)*** introduced *let-858::gfp* (plasmid BK48) for this purpose. We recommend using the *let-858::gfp* transgenic *C. elegans* strain PD7271 [genotype *pha-1(e2123ts) Ex 412.5/8 (ccEx7271)*] from W. G. Kelly, Emory University, Atlanta, GA. This strain needs to be maintained at 25°C in order to preserve the transgene. Alternatively, plasmid BK48 is also available in the Fire Lab 1997 Vector Kit Supplement and can be used for the production of transgenic reporter strains with standard techniques ***(17)***.

The loss of germline silencing has been implicated with severe cytological phenotypes in the germline of the *mes*-mutants: *mes-2*, *mes-4*, *mes-3*, and *mes-6* ***(18)***. Thus these *mes* mutants are useful to assess the cytological status of the germline in comparison to a RNAi phenotype of the gene under investigation. H1.1 RNAi and the *mes* mutants can be used as positive controls for a desilencing of the germline.

1.7. Analysis of the Depletion of the Target Protein

The easiest control of a RNA interference experiment is done in a transgenic *C. elegans* strain expressing a corresponding GFP reporter construct, which needs to contain the exons that are targeted by the RNAi. Such an expression construct is most easily generated by cloning a PCR product obtained from genomic DNA in pEGFP-N1 (Clontech). This is then used to transform *C. elegans* with standard techniques ***(17)***. GFP reporter strains, e.g., strain EC100 expressing H1.1::GFP can also be ordered from the *Caenorhabditis* Genetics Center (CGC). The fluorescence pattern in a RNAi experiment follows the time course, tissue specificity, and the intensity of the protein depletion. If an antibody specifically reacting with the target protein is available, immunofluorescence staining following standard protocols ***(19,20)*** can be used to analyze the protein depletion.

2. Materials

2.1. In Vitro Synthesis of dsRNA

1. Effector DNA sequence cloned in pBluescript II (Stratagene) or L4440.
2. Standard primers T7: 5´-GTAATACGACTCACTATAGGG-3', T7–T3:5'-CGC GCGTAATACGACTCACTATAGGGCGAATTGCCCTCACTAAAGGGA-3'.
3. Reagents: Diethylpyrocarbonate (DEPC)-treated plastic materials, gloves, Megascript T7 kit (Ambion, Cat no. 1334), dNTP, Taq-polymerase, Taq-buffer, $MgCl_2$, water-equilibrated phenol, ethanol, RNase-free water, 96:4 chloroform-isoamylalcohol, TE (1 m*M* EDTA, 10 m*M* Tris-HCl, pH 8.0), 3 *M* Na-acetate, pH 5.5.

2.2. RNA Interference by Microinjection

1. *C. elegans let-858::gfp* reporter strain e.g., PD7271, and *E. coli* OP50 CGC. Animals need to be young freshly grown hermaphrodites that have never been starved.
2. dsRNA of the gene of interest for control experiments: irrelevant dsRNA or M9 buffer (*see***Subheading 2.2.**; **item 5**).
3. Chemicals: agarose NEEO ultra-quality (Roth, Germany, cat. no. 2267.4), mineral oil (Sigma, cat. no. M-5904), 70% ethanol for sterilizing worm picks.
4. Equipment: 24 × 40 mm coverslips, worm picks (hairs from human eyebrows glued to toothpick), sterile injection needles Femtotips II (Eppendorf, cat. no. 5242 957.000), Microloader (Eppendorf, cat. no. 5242 956.003), a good binocular dissection microscope, inverted injection microscope (Zeiss Axiovert) equipped with a rotatable glide stage, differential interference contrast (DIC) optics, objective lenses with 10× and 40× magnification, mechanical micromanipulator, pressure system (the microinjection needle is connected via the output of a hand pistol to an adjustable 2000–5000 hPa pressurized air source). The

tubing connected to the pistols' outlet contains a single hole of 3-mm diameter, which can be closed with a finger when the pistol is triggered. This allows a very fast and fine-tuned microinjection of *C. elegans*.

5. Formulations: prepare M9 buffer by dissolving 3 g of KH_2PO_4, 6 g of Na_2HPO_4, 5 g of NaCl in 800 mL of H_2O, add 1 mL 1 *M* $MgSO_4$, add water to 1 L ***(21)***. Autoclave.
6. Preparation of seeded NGM worm plates: add 3 g of NaCl, 2.5 g of peptone, 17 g of agar, and 1 mL of a cholesterol solution (5 mg/mL in ethanol) to 975 mL of water (cholesterol is essential for the growth and development of *C. elegans*). Autoclave. After autoclaving, cool the molten agar to 55°C, and then add 1 mL of 1 *M* $CaCl_2$, 1 mL of 1 *M* $MgSO_4$ and 25 mL of 1 *M* potassium phosphate, pH 6.0, in single steps and in exactly that order to avoid precipitation (the $CaCl_2$, $MgSO_4$, and potassium phosphate solutions have to be autoclaved separately) before pouring the plates. Unseeded plates can be stored at 4°C for mo. Grow an overnight culture of *E. coli* OP50, dilute it with 2 vol of distilled water, and spread it onto the NGM plates (35 µL are sufficient for a 6-cm plate). Let the bacteria grow overnight at room temperature. Seeded NGM plates can be stored at 4°C for approx 1 mo.

2.3. RNA Interference by Feeding

1. *C. eleganslet-858::gfp* reporter strain e. g. PD7271 and *E. coli* OP50 (from The CGC).
2. *E. coli* strain HT115(DE3) transformed with the appropriate derivative of the RNA feeding vector L4440 (a modified version of pBluescript II with T7 promoters on both sides, which is available in the 1999 Fire lab vector kit, *see* **Subheading 2.6.**; **item 1** and **Subheading 2.6.**; **item 2**).
3. Chemicals: tetracycline (light sensitive; 12 mg/mL stock solution, (IPTG) isopropyl-β-D-thiogalactopyranosid, ampicillin, carbenicillin.
4. Equipment: incubators for 16°C, 20°C, and 25°C, worm picks (*see* **Subheding 2.2.**; **item 4**).
5. Formulations: prepare NGM plates as described in **Subheading 2.2.**; **item 6**. IPTG and antibiotics should be added to the 55°C warm molten agar immediately before pouring the plates to avoid degradation.
6. Prepare Luria–Bertani (LB) medium by dissolving 10 g peptone, 5 g yeast extract, 5 g NaCl in 700 mL of water, and adjust pH with 2 *M* NaOH to 7.2. Complete to a volume of 1 L by adding water. Autoclave.

2.4. RNA Interference by Soaking

1. 0.2 mL PCR plastic tubes.
2. 5X M9-Mg (M9 without Mg^{2+}): 3.4 g Na_2HPO_4, 1.5 g KH_2PO_4, 0.25 g NaCl, 0.5 g NH_4Cl per 100 mL, autoclaved.
3. 10X soaking buffer: 2.5 × M9-Mg, 30 m*M* spermidine (Sigma, cat. no. S2626), 0.5% gelatin; autoclaved and filtered.

2.5 Analysis of Germline Silencing

1. Obtain *C. elegans* strain PD7271 ([genotype *pha-1[e2123ts] Ex 412.5/8 [ccEx7271]*) from the CGC or from W. G. Kelly, Emory University, Atlanta, GA.
2. 4% agarose in M9 buffer, 20 m*M* sodium azide in H_2O.
3. A fluorescence microscope capable of the observation of GFP fluorescence equipped with DIC.

2.6 Material Resource and Electronic Information Sources

1. Fire Lab protocol and vector/RNAi information (http://www.ciwemb.edu/pages/firelab.html).
2. Fire Lab FTP site (ftp://www.ciwemb.edu/pub/FireLabInfo/).
3. The CGC (http://biosci.umn.edu/CGC/).
4. WORMBASE: http://www.wormbase.org/.
5. Program for oligonucleotide design: http://www.sanger.ac.uk/Projects/C_elegans/oligos.shtml.
6. Yuji Kohara's (yk) *C. elegans* EST project: http://nematode.lab.nig.ac.jp/index.html.
7. *C. elegans* RNAi libraries: http://www.hgmp.mrc.ac.uk/geneservice/reagents/products/descriptions/Celegans.shtml ***(3)***.

3. Methods

3.1. Synthesis of dsRNA (see *Note 1)*

1. Always wear gloves; use only DEPC-treated plastic materials and Rnase-free chemicals.
2. Add the following amounts of the indicated reagents in the order shown to a 0.5-mL PCR tube: 2 µL T7 primer (100 pmol/µL), 0.4 µL T7-T3 primer (100 pmol/µL), 4 µL plasmid DNA (10 ng/µL), 4 µL 2.5 m*M* dNTP, 20 µL *Taq* buffer without $MgCl_2$, 10 µL 15 m*M* $MgCl_2$, and 155 µL H_2O. Mix well and add 4 µL *Taq*-polymerase (1 U/µL).
3. Overlay with mineral oil if your thermocycler requires it.
4. Use the following PCR protocol: once at 95°C for 5 min; once at 45°C for 20 s (required only for the T7–T3 primer); and 30 cycles at 51°C for 60 s, 72°C for 90 s, and 94°C for 60 s.
5. Check the PCR product with an analytical agarose gel, remove the mineral oil (*see* **Note 2**), and extract with 80 µL chloroform/isoamylalcohol. Precipitate with 20 µL 3 *M* Na-acetate, pH 5.5 and 400 µL ethanol during 3 h at –20°C. Centrifuge for 30 min at maximum speed to pellet DNA. Wash the DNA pellet with 70% cold ethanol and dissolve it in 30 µL TE.
6. Transcribe 1 µg of the DNA with a T7 polymerase kit (Megascript T7, Ambion, cat. no. 1334) to produce the dsRNA in a single reaction. Use a reaction time of 6–12 h (*see* **Note 3**).
7. Determine the integrity of the RNA on a gel (*see* **Note 4**) and quantify it by UV

spectrophotometry.
8. Store the RNA at –80°C (*see* **Note 5**).

3.2. RNAi by Microinjection (see Note 6)

1. Prepare injection pads (50–100 at once). Place drops of 0.15% agarose molten in water in the middle of a 24 × 40-mm coverslip. Dry the pads on the bench overnight (*see* **Notes 7–9**).
2. Load needles with 1–2 (L dsRNA using the microloader. Keep the remaining dsRNA stock on ice (*see* **Notes 10–12).**
3. Mount the loaded needle on the micromanipulator so that the tip of the needle is in optical axis of the microscope. Always start with 100× magnification.
4. Check the flow rate of the needle. Increase the flow rate by increasing the injection pressure up to 5000 hPa. If this is in sufficient, break the tip of the needle by pushing it against the edge or corner of a coverslip. Use 400× magnification.
5. Place a drop of oil onto an agarose pad. Use a worm pick to transfer 1–2 young hermaphrodites onto the agar surface under the oil. Work under a binocular (*see* **Note 13**).
6. Mount the coverslip with the worms onto the microscope. Two small drops of distilled water are sufficient to adhere the coverslip to the stage. Focus onto the worm and center it together with the tip of the needle in the optical axis using 100× magnification.
7. Locate the syncytial region of one gonad arm. Search for a sausage-shaped clear area (*see* **Fig. 2A**).
8. Rotate the worm to allow a 45° entry angle of the needle. Then switch to 400× magnification. You need to see the germ nuclei now. Focus on the center region of the syncytial region of one gonad arm (*see* **Fig. 2A**).
9. Bring the tip of the needle into the focal plane.
10. Inject into the rachis.
11. You must ensure that the syncytial gonad fills with liquid.
12. Retract the microinjection needle before the gonad bursts. Remove the agarose pad from the microscope (*see* **Notes 14**).
13. Add 2 µL of M9 buffer to the worm to rehydrate it.
14. Pick the injected worms onto seeded NGM plates (*see* **Note 15).**
15. Incubate the plates at 25°C.
16. Transfer the animals every 6–12 h onto fresh plates.
17. Screen the F1 generation for RNA interference phenotypes.

3.3. RNAi Feeding (see Note 16)

1. Clone the DNA fragment of interest between the T7 promoters of the vector L4440 and transform the construct into *E. coli* strain HT115 using ampicillin selection (*see* **Notes 17–19**).
2. Raise an overnight culture in LB + AT (LB with 12.5 µg/mL tetracycline and 100 µg/mL ampicillin) at 37°C (*see* **Note 20**).

3. Dilute the overnight culture 1:100 in LB + AT and grow the culture to an optical density of OD_{600} = 0.4–0.5.
4. Induce dsRNA production by adding IPTG (0.4 m*M* final concentration) and continue the culture at 37°C for 4 h (*see* **Note 21**).
5. Add a second dose of both antibiotics and of IPTG to the culture in order to double the original concentration. Incubate for another 30 min.
6. Harvest the induced cells by centrifugation and spread them onto NGM plates, which contain 1 m*M* IPTG, 100 µg/mL ampicillin and 12.5 µg/mL tetracycline (*see* **Note 22).**
7. Pick *C. elegans* larvae (**Fig. 2B**) or eggs of the reporter strain used (e.g., PD7271) onto the plates and incubate at temperatures between 15 and 25°C (*see* **Notes 23–25**).
8. Remove parental worms after they have laid eggs and score the phenotypes of the adult F1 generation by fluorescence microscopy of the germ nuclei. Worms can be transferred onto fresh feeding plates at any time (*see* **Note 26**).

Alternative method (simpler, without tetracycline, *see* **Note 27**):

1. Grow *E. coli* HT115 with the plasmid of interest in LB with 50 µg/mL ampicillin overnight at 37°C.
2. Spread 4 µL culture onto a 6-cm NGM plate containing 25 µg/mL carbenicillin and 1 m*M* IPTG.
3. Leave the plates for 5 h at room temperature.
4. Pick four M9-washed L3-stage hermaphrodites (**Fig. 2B**) of the reporter strain used (e.g., PD7271) onto the plates and incubate at temperatures between 15–25°C (*see* **Notes 23–25**).
5. Remove the F0 animals after they have laid 20–60 eggs.
6. Score the phenotypes of the adult F1 animals by fluorescence microscopy of the germ nuclei.

*3.4. RNAi Soaking (***see** *Note 28)*

1. Collect 10–20 gravid hermaphrodites of the reporter strain used (e.g., PD7271) and wash them with M9 that contains 0.05% gelatin.
2. Cut them with two crossed injection needles in two halves and collect the emerging embryos.
3. Transfer the embryos in M9 to a 1.5-mL tube and incubate them at 25°C overnight.
4. Transfer the hatched L1 worms (*see* **Note 29**) to a fresh NGM plate without bacteria and let them crawl for several minutes to clean them.
5. Supplement 3.6 µL dsRNA solution with 0.4 µL 10X soaking buffer in a 200-µL PCR tube.
6. Insert 15–20 worms into the buffered RNA solution and incubate them at 20°C for 24 h (*see* **Note 30**).
7. Transfer the animals on to seeded NGM plates or RNA feeding plates.
8. Score the phenotypes of the adult animals.

3.5 Analysis of Germline Silencing

1. Produce agarose pads by dropping molten 4% agarose in M9 buffer on to a glass slide laying on a flat surface. Place two glass slides covered with two layers of transparent Scotch tape in parallel beside the first glass slide. Press a fourth glass slide in an orthogonal orientation onto the liquid agarose drop on the first slide. Wait until agarose solidifies. The resulting agarose pad has the exact thickness of two layers of Scotch tape. Release it from the top covering slide by shifting it away.
2. Add 5 µL of 20 m*M* sodium azide onto the agarose pad.
3. Use a worm pick to transfer adult F1 generation animals into the sodium azide drop.
4. Cover worms with a coverslip.
   Record DIC and green fluorescent images. When desilencing occurs, the germ nuclei (**Fig. 2A**) will be green fluorescent. If desilencing is observed, additionally characterize the cytological status of the germline by identifying oocytes and developing embryos. Also count the number of germ nuclei in young hermaphrodites of a defined age.

4. Notes

1. In our hands, PCR fragments of this kind are superior templates for dsRNA synthesis. We prefer to use T7 promotors on both ends because T7 RNA polymerase has the highest synthesis activity.
2. Remaining mineral oil can be removed by absorption to parafilm.
3. Longer reaction times considerably increase the dsRNA yield.
4. A standard nondenaturing gel is sufficient.
5. Otherwise, it will slowly degrade.
6. Learning to microinject *C. elegans* commonly is a frustrating experience intially. Expect to practice a few times. Typically after 4–10 sessions, everyone can learn.
7. The agarose pads produced with 0.15% agarose in water will work for the cited material. Agarose from other distributors (DNA separation quality) does work as well, but the necessary concentration has to be determined experimentally. To do this, test pads have to be produced with agarose concentrations ranging from 2% to 0.07%. If the pads are too thick, the worms die too quickly from dehydration. If the pads are too thin, the worms will not be immobilized.
8. It is convenient to use thicker agarose pads (0.15% agarose) for hermaphrodites and thinner pads (0.07% agarose) for younger animals, which generate lower muscle forces, but dehydrate faster.
9. The pads can be stored at room temperature for months in the original coverslip box.
10. The needles contain an internal glass filament that will slowly transport the liquid to the tip by capillary forces without producing air bubbles. If you fill the tip of the needle directly with the microloader, you will trap air in it.
11. To release the cap of the needle, point it exactly downward and let go. This will remove the cap without breaking the needle.

12. dsRNA must be particle-free, completely dissolved, and very pure. Particularly, it may not contain traces of phenol originating from the extraction procedure. The concentration of dsRNA can be very critical. Although some proteins can be efficiently depleted with RNA concentration below 1 μg/μL, other depletion experiments show phenotypes only when the RNA concentration exceeds a certain threshold. Therefore, initial dsRNA concentrations of 4–5 μg/μL are used.
13. Experienced operators can handle more worms on one pad. Worms can be cleaned from adhering bacteria by transferring them onto a nonseeded NGM agar plate.
14. When the needle is retracted, no internal organs may protrude through the puncture. If this consistently happens, the needle is too thick and needs to be replaced by a thinner one.
15. A fast-working cycle is essential for animal survival. Vitality can be assessed by monitoring the locomotor activity of the animals.
16. The first feeding protocol is from Lisa Timmons ***(11)***, Carnegie Institution of Washington, whereas the second alternative feeding protocol is from Julie Ahringer (WellcomeTrust/Cancer Research UK Institute, University of Cambridge, UK; ***3***).
17. The cloning should be performed in a standard *E. coli* cloning strain (e.g., DH5α), and the plasmid is then transferred to *E. coli* HT115(DE3). Plasmid DNA prepared from HT115(DE3) does not have the same quality as typical plasmid preparations and should be not used for further cloning steps.
18. *E. coli* HT115(DE3) has IPTG-inducible T7 polymerase activity. Competent cells can be made by using the standard methods. The strain is selectable with tetracycline. When there seems to be something wrong with the HT115(DE3) cells, test for the presence of the DE3 lysogen by PCR.
19. Julie Ahringer's feeding libraries can be obtained from the MRC UK HGMP Resource Center (*see* **Subheading 2.6.**; **item 5**).
20. Some researchers believe that the tetracycline decreases the RNAi efficacy in feeding experiments.
21. Different duration of induction time can be tested.
22. For 30 plates, a 30 mL culture is enough.
23. The ratio of worms to the bacteria is very important. Too many worms will deplete the bacteria and starve. Then, RNAi will not work.
24. *C. elegans* can be cleaned from adhering bacteria by washing worms in M9 on unseeded NGM plates or in 1.5-mL plastic tubes.
25. Initially, 25°C should be used because this enhances RNAi efficacy and germline desilencing.
26. Fresh cells work better. The feeding plates can be stored at 4°C for a maximum of 1 wk.
27. It may be necessary to test which method works best in a given environment.
28. This protocol is from Ikuma Maeda and Asako Sugimoto ***(13)***. We recommend soaking especially for the analysis of postembryonic development because it allows a very long exposure of L1 larvae to dsRNA. We suggest to always do this

in comparison with L1 feeding experiments, because for a given gene of interest, typically, one of the two methods works considerably better.

29. Alternatively, L1 animals may also be obtained from a clean starved plate.
30. For the analysis of the embryonic phenotype, four L4-stage larvae may be used instead.

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3

cDNA Subtraction and Cloning in the Field of Trophoblast/Placental Development

Myriam Hemberger

1. Introduction

1.1. General Considerations for the Applicability Of cDNA Subtraction

cDNA subtractive hybridization is a very powerful method to compare gene expression profiles between two cell or tissue samples of interest. The sample in which differentially expressed transcripts are to be found is usually referred to as "tester," whereas the reference sample is called "driver." A cDNA subtraction experiment should always be performed in both directions, with each sample serving as the tester and the driver in separate reactions. The general requirements to perform a cDNA subtraction and cloning procedure encompass the isolation of total RNA or mRNA from the specimens of interest, followed by reverse transcription of the mRNA into cDNA. The concept of cDNA subtraction is based on the hybridization of reverse-transcribed mRNAs present in both experimental samples. These sequences can form double-stranded hybrids that are then removed from the reaction. Only cDNAs from the tester population that remain single-stranded are further amplified, thus representing the pool of differentially expressed genes. This pool enriched for differentially expressed sequences can easily be cloned to generate a subtraction library. The analysis of subtraction efficiency is a crucial step after the procedure in order to rely on the results obtained. The subtractive hybridization is very valuable to achieve insights into genes differentially regulated between two samples of interest. Importantly, however, the generated cDNA pools enriched for genes overexpressed in either of the two tissue or cell specimens are also excellent

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probes for array hybridization applications—timely methods to identify gene expression changes on a large scale.

Although it is clear from the general concept of cDNA subtraction that only genes expressed in both samples are successfully eliminated from the tester cDNA pool, one of the most common problems in subtractive hybridization experiments is the nonequality of starting samples. Therefore, it should be stressed that extreme care must be taken in the selection and preparation of the initial tester and driver material to minimize cloning of differentially expressed genes owing to the presence or absence of contaminating tissue of no interest in the experimental design.

1.2. Specific Considerations for Application of cDNA Subtraction on Trophoblast/Placental Material

The above-mentioned importance of the quality of the starting material specifically applies for extraembryonic tissues. In the case of mouse trophoblast samples, early stages have to be carefully dissected to remove all decidual and embryonic tissue. Strategies to isolate the egg cylinder from within the thick layer of decidualizing uterine stromal tissue are either to cut the embryo in halves along the longitudinal axis (**Fig. 1A**) or to open up the conceptus from the mesometrial side mainly with the force of tweezers (**Fig. 1B**) and to lift the egg cylinder out of the implantation cavity (**Fig. 1C**; *see also (1)*. The egg cylinder can then be easily divided into the proximal (extraembryonic) and the distal (embryonic) parts (**Fig. 1D**).

For later postmidgestational stages, considerations for placental samples to be compared in a cDNA subtraction procedure include the presence or absence of decidual tissue and the extent of yolk sac remnants left attached to the chorionic plate. Differences in these components can again cause cloning of false-positives in the subtractive hybridization.

Furthermore, it is generally advantageous to pool several tissue samples for both the tester and driver material to rule out aberrant effects of individual samples. For mouse tissues, it is also recommended to isolate the specimens from the same preferrably inbred strain to rule out any potential inter- or intersubspecific differences.

Although described here for mouse trophoblast, the general idea of these considerations for sample choice and preparation can be easily extrapolated not only to trophoblast samples from other species, but also to any other tissue or organ material that is compared in a subtraction procedure. Many of these precautions should also be taken into account when comparing cell culture material. Although contaminating tissue is much less important in that case, tissue culture samples should otherwise be treated as equally as possible, because slight changes in culturing conditions (e.g., cell density, media, sol-

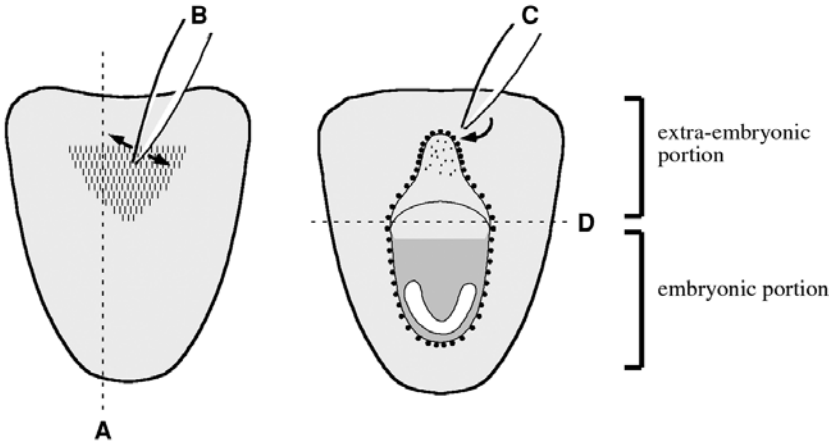

Fig. 1. Dissection strategies for E7.5–E8.5 mouse embryos.

vents of additives, and so on.) again can superimpose nondesired expression profile changes.

2. Materials

2.1. RNA Purification

1. DEPC-H_2O: 0.1% (100 µL/L) diethyl-pyrocarbonate (DEPC) in distilled water, mix vigorously, let stand overnight in fumehood, and autoclave (twice if necessary; DEPC disintegrates during heating). DEPC is extremely toxic and should not be inhaled.
2. Phosphate-buffered saline (PBS): 0.01 *M* Na_2HPO_4/KH_2PO_4, 0.15 *M* NaCl/KCl, pH 7.3 (8 g NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4, 0.2 g KH_2PO_4 per liter). Make up with DEPC-H_2O in RNase-free bottle or jar.
3. Glassware can be cleaned either by baking for approx 8 h at 200°C (remove all plastic components, e.g., lids) or by rinsing with DEPC-H_2O.
4. Trizol® Reagent (Invitrogen, Carlsbad, CA).
5. Chloroform.
6. Isopranol (2-Propanol).
7. 70% Ethanol.
8. 3 *M* Na-acetate, pH 5.2. Adjust pH with acetic acid.
9. 20 mg/mL Glycogen solution (Roche Applied Science, Laval, QC, Canada).
10. Special equipment if applicable: tissue homogenizer (e.g., Polytron).

2.2 cDNA Synthesis

1. SMART™ PCR cDNA synthesis kit (Clontech, Palo Alto, CA).
2. RNaseH-deficient reverse transcriptase (Superscript II) and 5X transcription buffer are available from (Invitrogen).

3. 0.1 *M* Dithiothreitol (DTT).
4. Deoxynucleotide mix of 10 m*M* each of dATP, dCTP, dGTP, and dTTP made up in DEPC-H_2O.
5. RNase inhibitor (Roche Applied Science).
6. TE buffer: 10 m*M* Tris-HCl, 1 m*M* ethylendiamine-tetraacetic acid, disodium salt (EDTA), pH 7.5.
7. *Taq* DNA polymerase is available from Clontech (Palo Alto, CA).
8. Anchoring primers as provided in the SMART™ PCR cDNA synthesis kit.
9. 0.5 *M* EDTA, pH 8.0.
10. Phenol:chlorofrom:isoamylalcohol as 25:24:1 mix.
11. Microcon YM-30 spin columns (Millipore Corporation, Bedford, MA).
12. Size fractionation columns (CHROMA SPIN-1000) as provided in the SMART™ PCR cDNA synthesis kit (Clontech).
13. TNE buffer: 10 m*M* Tris-HCl, 10 m*M* NaCl, 0.1 m*M* EDTA, pH 8.0.
14. 100% and 70% ethanol.
15. Restriction enzyme to reduce size of cDNAs for subtraction, e.g., *Rsa*I (Invitrogen).
16. 7.5 *M* Ammonium acetate, pH 7.0 (NaOAc). Adjust pH with acetic acid.
17. Special equipment: PCR thermocycler.

2.3 cDNA Subtraction

1. PCR-Select™ cDNA Subtraction Kit (Clontech). When this kit is used, **items 2–8** are not needed.
2. Two sets of appropriate adaptor molecules (as supplied with the PCR-Select™ cDNA Subtraction Kit) that contain divergent "inner" sequences and an identical "outer" stretch (*see* **Fig. 2**).
3. Oligonucleotide primers to the outer and inner regions of the adaptors (**Fig. 2**).
4. PCR primers to the housekeeping gene glycerinealdehyde-3-phosphate-dehydrogenase (*Gapd*).
5. *Taq* DNA polymerase (Clontech).
6. Deoxynucleotide mix of 10 m*M* each of dATP, dCTP, dGTP, and dTTP. AdNTP set is available from (Amersham).
7. 4X hybridization buffer: 200 m*M* HEPES-HCl, pH 8.3, 2 *M* NaCl, 0.08 m*M* EDTA pH 8.0, 40% (w/v) PEG 8000.
8. cDNA subtraction dilution buffer: 20 m*M* HEPES-HCl, pH 8.3, 50 m*M* NaCl, 0.2 m*M* EDTA, pH 8.0.
9. T4 DNA ligase and 10X ligation buffer (New England Biolabs, Beverly, MA).
10. Mineral oil.
11. Special equipment: cooling water bath at 16°C for the adapator ligation reaction.

2.4. Cloning of Amplified, Subtracted cDNA Pools

1. T/A PCR cloning kit (Promega, Madison, WI).
2. Electrocompetent cells from *E.coli* strain DH10B (Invitrogen).
3. 0.1-cm Electroporation cuvets.

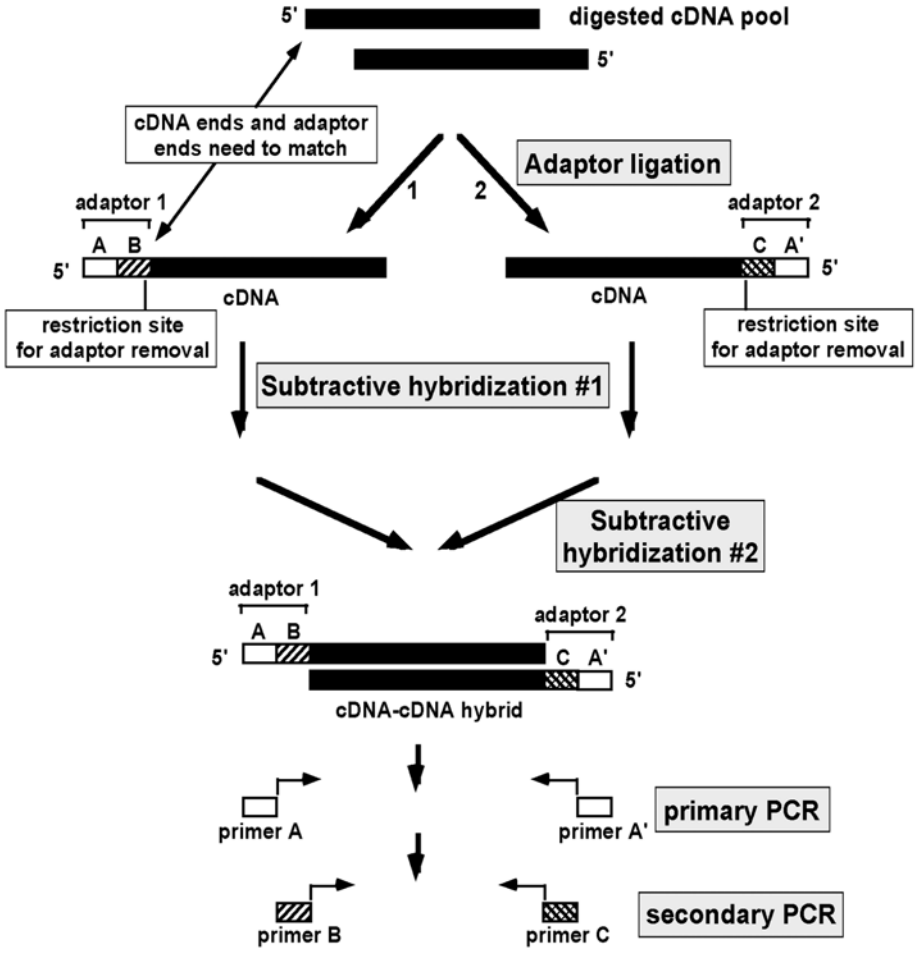

Fig. 2. Schematic representation of subtractive hybridization procedure. The diagram shows outer adaptor regions A/A' that may, but do not have to be, identical. Inner regions B and C differ from each other to identify cDNA hybrid molecules with two different ends. These are preferentially amplified in two rounds of PCR amplification. Adaptors should not be phosphorylated to allow ligation only to the 5'-ends of cDNA fragments. Furthermore, they should contain at least one restriction site in proximity to the cDNA that allows their removal during the differential screening procedure.

4. SOB medium: 20 g bacto-tryptone, 5 g yeast extract, 0.5 g NaCl per liter of H_2O, Autoclave.
5. SOC medium: 20 m*M* glucose (1 mL/100 mL from 2 *M* stock) in SOB medium. Sterile filtrate glucose solution. Do not autoclave!

6. Luria–Bertani (LB)-Amp/X-Gal/isopropyl-β-D-thiogalactopyranosid (IPTG) agar plates: 10 g bacto-trypyone, 5 g yeast extract, 10 g NaCl, 15 g bacto-agar per liter of H_2O. Autoclave. After autoclaving, add 100 µg/mL ampicillin, 45 µg/mL X-Gal, and 45 µg/mL IPTG.
7. LB-Amp medium: 10 g bacto-trypyone, 5 g yeast extract, 10 g NaCl per liter of H_2O. Autoclave.
8. Glycerol.
9. Special equipment: electroporator for bacterial transformation.

2.5 Analysis of Subtraction Library

1. PCR primers to the housekeeping gene *Gapd*.
2. *Taq* DNA polymerase (Clontech).
3. Deoxynucleotide mix of 10 m*M* each of dATP, dCTP, dGTP, and dTTP. dNTPs are available from Amersham.
4. QiaQuick PCR purification columns are (Qiagen, Valencia, CA).
5. Restriction endonuclease to cut off adaptor molecules (*see* **Fig. 2**).
6. 0.6 *M* NaOH.
7. Hybond Nylon membrane (Amersham).
8. 3 M*M* chromatographic paper (Whatman, Maidstone, England, UK).
9. 0.5 *M* Tris-HCl, pH 7.5.
10. Random hexamers (Invitrogen).
11. 10 µCi/µL ^{32}P-α-dCTP (Amersham).
12. 10X labeling buffer: 900 m*M* HEPES-HCl, pH 6.6, 100 m*M* $MgCl_2$, 20 m*M* dithiothreitol, 3 m*M* each of dTTP, dATP, and dGTP.
13. Klenow large fragment DNA polymerase (Invitrogen).
14. 1 *M* Na-phosphate buffer: make up 1 *M* Na_2HPO_4 and 1 *M* NaH_2PO_4 solutions. Adjust pH of Na_2HPO_4 to pH 7.2 with NaH_2PO_4.
15. Church hybridization buffer: 0.5 *M* phosphate buffer, 7% (w/v) sodium dodecylsulfate (SDS), 1 m*M* EDTA, pH 7.5, 1% (w/v) bovine serum albumine (BSA), grade V.
16. 20X SSC: 3 *M* NaCl (175.3 g/L), 0.3 *M* sodium citrate (88.2 g/L), pH 7.0.
17. 20% (w/v) sodium dodecyl sulfate (SDS).
18. Autoradiographic films and cassettes (Amersham).
19. Special equipment: hybridization oven.

3. Methods

3.1. RNA Purification

During RNA handling and cDNA synthesis, make sure to provide RNase-free conditions during all steps by using RNase/DNase-free plasticware, glassware baked for approx 8h at 200°C and always wearing gloves.

1. Carefully dissect and isolate tissues/cells/organs to be analyzed. During dissections, use cold PBS and handle specimens quickly. Samples should be kept at 0–4°C during dissection and then directly processed for RNA isolation; tissues can be stored at -80°C for at least 6 mo after immediate freezing in liquid nitrogen.

2. For RNA isolation, use excess amounts of Trizol® Reagent (at least 1 mL/100 mg tissue). Completely disaggregate tissue by homogenizing. Make sure samples do not heat up (>30°C) during procedure.
3. Add 1/5 vol of chloroform (200 µL/mL Trizol® solution) and mix thoroughly.
4. Spin for 15 min at 10,500*g* and transfer upper aqueous phase into new tube.
5. Precipitate the RNA by adding 1 vol 100% 2-propanol and 1/10 vol 3 *M* NaOAc, pH 5.2. If starting tissue material is very small, add 1 µL of 20 mg/mL glycogen solution to the 2-propanol precipitation (*see* **Note 1**).
6. Wash the RNA pellet with 200 µL of 70% ethanol.
7. Remove all residual ethanol and dissolve the pellet in DEPC-H_2O. Adjust concentration to 0.5–1 µg/µL. Keep dissolved RNA on ice all the time. Samples can be stored at -80°C for > 3 mo.
8. Check quality of total RNA by loading 500 ng of each sample on a standard 0.8% agarose gel ***(2)***. 28S and 18S rRNA bands should be visible as sharply defined bands. If smearing appears, RNA quality is not optimal and the samples should not be used for subtraction, if possible. The intensity of the larger 28S rRNA band should be approx 2–3 times higher than the intensity of the 18S rRNA band.

Also use agarose control gel to compare RNA amounts between samples and readjust if necessary.

3.2. cDNA Synthesis

The cDNA synthesis and amplification technology (as based on the SMART™ PCR cDNA synthesis kit [Clontech]) starts with an oligo(dT)-primed first-strand cDNA synthesis reaction. Only when the reverse transcriptase (RT) reaches the 5'-end of the mRNA, its terminal transferase activity adds a few nucleotides to the synthesized cDNA strand, preferentially deoxycytidine residues ***(3,4)***. This tailing reaction can serve to provide a binding site for an oligo(dG)-containing oligonucleotide which then defines the 5'-end of the synthesized cDNA ***(3,4)***. Because the terminal transferase activity of the RT is far more efficient at the ends of mRNA–cDNA hybrids than at internal RT stopping or pausing sites, this technology results in generation of full-length cDNAs that are characterized by the 5'-oligo(dG)-oligonucleotide and the 3'-oligo(dT)-cDNA synthesis primer. It should be noted that the cDNA "capping" also takes place, however, at premature mRNA breakpoints stressing the importance of high-quality RNA (as judged by the criteria described previously). After generating the flanked double-stranded cDNAs, sequences of the anchoring primers can be used to further amplify the full-length products in a PCR reaction step.

3.2.1. First-Strand cDNA Synthesis

1. Ideally start with 0.5–1 µg of total RNA (*see* **Note 2**). Make sure to start with the same amounts of RNA for both samples. Mix RNA with oligo(dT)-cDNA synthesis primer and oligo(dG)-oligonucleotide (10 pmol each).

2. Denature 5 min at 70°C.
3. Place on ice and spin down briefly.
4. Add 2 µL of 5X first strand buffer to 1X final concentration:, 1 µL dNTP mix, 0.2 µL 100 m*M* DTT, and 200 U RT. Also add 10 U RNase inhibitor and adjust total volume to 10 µL with DEPC-H_2O.
5. Incubate at 42°C in an air incubator for 1–1.5 h. Note that the use of a water bath or heating block leads to increased evaporation. Cover reaction with mineral oil if needed.
6. Add 40 µL of TE buffer and heat tubes to 70°C for 10 min to stop the RT reaction.
7. Store samples at –80°C.

3.2.2. Second-Strand cDNA Synthesis and PCR Amplification

1. Use 2 µL of single-strand cDNA mix from **Subheading 3.2.**; **item1**.
2. Set up at least four PCR reactions for each cDNA (one test tube and three sample tubes; *see* **Note 3**). Prepare a master mix for 100 µL reactions with 20 nmol dNTP's (2 µL from 10 m*M* stock), 20 pmol of both flanking primers, 1X final concentration of PCR buffer, and 1 U *Taq* polymerase (*see* **Note 4**). Aliquot 98 µL into PCR tubes and add 2 µL of cDNA.
3. Place reaction tubes in a PCR thermocycler with short denaturing (5 s at 94°C) and annealing (15 s at 65°C) phases, but a long extension phase (6 min at 68°C) for 15 cycles.
4. Remove sample tubes and place at 4°C. Transfer 10 µL from test reaction tubes into a fresh microfuge tube and continue PCR of the remaining 90 µL for three additional cycles. Repeat transfer/cycling three more times so that 10 µL PCR reaction aliquots are present after 15, 18, 21, 24, and 27 cycles.
5. Run 5 µL of these 10-µL test reactions on a 1% agarose gel. Determine the optimal number of PCR cycles by the appearance of a smear between approx 1 and 6 kb. Overcycling can be determined by a lack of further product increase and/or by a size shift of the smear. Use one or two cycles less than the determined optimal cycle number (*see* **Note 5**).
6. Place sample tubes for tester and driver cDNA synthesis back into the thermocycler for the additional cycles as determined in the optimization step.
7. Stop the reaction by adding 2 µL of 0.5 *M* EDTA.
8. PCR reactions can be stored at -80°C for at least 6 mo and are a very valuable source of full-length cDNA templates.

3.2.3 Purification of PCR-Amplified cDNA Pools

1. For every experimental sample to be analyzed by subtraction, combine two to four PCR reactions. The total amount of cDNA should be 2 µg. If too less cDNA is used, the number of clones retrieved after subtraction will be small; on the other hand, a more than twofold excess of cDNA leads to poor subtractive hybridization efficiency.
2. Save 10 µL of this raw cDNA pool for comparative analysis after purification.

3. Adjust total volume of combined PCR reactions to 400 µL. Add equal volume of phenol:chloroform:isoamylalkohol mix. Vortex thoroughly and centrifuge at 15,000*g* for 10 min.
4. Transfer upper aqueous phase on top of Microcon spin columns. Centrifuge at 12,000 rpm for 8 min.
5. Add 400 µL of H_2O and spin again at 15,000*g* for 8 min.
6. Add 50 µL of H_2O on top of the filter and mix by carefully pipetting up and down. Then transfer the 50–70 µL from the top of the filter into a new microfuge tube (*see* **Note 6**).
7. Size fractionate the cDNA pool by applying it onto a CHROMA SPIN-1000 size fractionation column.
   Flip column to completely resuspend the matrix. Remove top and bottom caps. Add 1.5 mL of TNE buffer and let the column drain by gravity flow.
8. Add the cDNA to the center of the column. Add 25 µL TNE buffer and let column drain completely. Add another 150 µL of TNE buffer, drain again.
9. Place columns in fresh microcentrifuge tubes. Add 350 µL of TNE buffer and collect as the purified cDNA.
10. Apply another 75 µL of TNE buffer to the column and collect eluate in a separate tube as the small cDNA fraction.
11. Run 10 µL of the raw cDNA (**Subheading 3.2.3., step 2**) and 10 µL of the 350 µL and 75 µL elutions on a 1% agarose gel to confirm that the major fraction of the cDNA is contained in the 350 µL eluate (*see* **Note 7**).

3.2.4. Restriction Digest of the cDNA Pools

1. Digest the size-fractionated cDNA (350 µL eluate) with 30 U restriction enzyme (*see* **Note 8**) in a total volume of 400 µL using the appropriate digestion buffer. Incubate digest for more than 3h or overnight.
2. Purify the digest by phenol:chloroform extraction.
3. Ethanol-precipitate the cDNA with 2 vol (800 µL) ethanol and 1/2 vol (200 µL) 7.5 *M* NaOAc in the presence of glycogen (1 µL of 20 mg/mL solution; *see* **Note 1**).
4. Wash the cDNA pellet with 200 µL 70% ethanol.
5. Redissolve the pellet in 5.5 µL H_2O.

3.3 cDNA Subtraction

The cDNA subtraction procedure itself (as based on the Clontech PCR-Select™ cDNA Subtraction Kit (***5***,***6***) requires the ligation of two different adaptor molecules to the tester cDNA population in separate reactions (**Fig. 2**). Both reactions are incubated with an excess of driver cDNA to obtain a first subtractive hybridization. Then, both tester pools are mixed together without further denaturation in the presence of new driver cDNA. In this second hybridization step, the type of cDNA hybrid is formed that is subsequently further amplified: these molecules contain two different adaptors at their ends

(**Fig. 2**). This type of hybrid can only form between cDNA strands that remained single stranded in both primary hybridization reactions. Because reaction kinetics lead to a self-hybridization of highly abundant transcripts during the first hybridization, this method results in an equalization of high- and low-abundance sequences. The amplification of weakly expressed transcripts is an extremely valuable feature, as it can lead to the identification of novel genes in a small-cell population and reduces the redundancy of high abundance sequences in the subtraction library.

After two rounds of subtractive hybridization against excess amounts of driver cDNA, the reaction mix is subjected to two PCR amplification steps. In the primary PCR, only the above-mentioned molecules carrying both types of adaptors at each end are amplified. This selection is achieved by self-pairing and looping of cDNAs with identical adaptor molecules. The principle of this technique is known as "suppression subtractive hybridization" as described by Diatchenko et al. ***(5)***. The secondary PCR is a simple reamplification of cDNA hybrids selected in the first PCR step to further enrich for the differentially expressed sequences (*see* **Note 9**).

3.3.1 Adaptor Ligation

1. Dilute 1 µL of the digested tester cDNA with 5 µL H_2O.
2. Prepare two separate ligation reactions for each experimental tester cDNA: mix 2 µL of diluted tester with 20 pmol of adaptor 1, and another 2 µL of diluted tester with 20 pmol of adaptor 2; 1X ligation buffer and 200 U T4 DNA ligase in a total volume of 10 µL.
3. Mix 2 µL of both, tester-adaptor 1 and tester-adaptor 2 ligations in a fresh tube as an unsubtracted tester control.
4. Incubate the ligation reactions overnight at 16°C in a water bath.
5. Store reactions at –20°C (*see* **Note 10**).

3.3.2. Subtractive Hybridization #1

1. In separate 0.2 mL reaction tubes, mix 1.5 µL of each adaptor-ligated tester with 1.5 µL of undiluted, digested driver cDNA from **Subheading 3.2.4.**: H1, tester-adaptor 1 plus driver and H2, tester-adaptor 2 plus driver.
2. Add 1 µL of 4X hybridization buffer to yield a total volume of 4 µL.
3. Overlay the mix with one drop of mineral oil to avoid evaporation.
4. Heat denature the samples at 98°C for 2 min.
5. Place samples at 68°C and incubate for 8–12 h.

3.3.3. Subtractive Hybridization #2

1. Denature fresh driver: Mix 1 µL of driver cDNA (**Subheading 3.2.4.**) with 1 µL of 4X hybridization buffer and 2 µL H_2O. Place 1 µL of this mix into a fresh tube and overlay it with mineral oil. Incubate for 2 min at 98°C.

2. Collect the 4 µL of hybridization sample H2 and the freshly denatured driver in the same pipet tip.
3. Add this mix to hybridization sample H1, thereby mixing the separate adaptor-ligated tester populations plus new driver. The total volume of the reaction is now 9 µL.
4. Spin down briefly if necessary.
5. Incubate mix again at 68°C for 12–16 h.
6. Add 200 µL of cDNA subtraction dilution buffer and heat the mix to 68°C for 10 min.

3.3.4. Primary PCR Amplification

1. Prepare a master mix for 25 µL PCR reactions containing each: 1X reaction buffer, 5 nmol dNTPs (0.5 µL from 10 m*M* stock), 10 pmol adaptor primer A/A' and 1 U *Taq* polymerase per reaction.
2. Add 1 µL of the diluted, subtracted cDNA from **Subheading 3.3.3.** to the mix. Also perform the PCR with the unsubtracted tester control from **Subheading 3.3.1.** (1 µL from 1:1000 dilution).
3. Incubate the reaction mix at 75°C for 5 min in a thermal cycler.
4. Commence PCR cycling for 27 cycles with 15 s at 94°C, 30 s 66°C and 1.5 min 72°C (*see* **Note 11**).

3.3.5. Secondary PCR Amplification

1. Dilute the primary PCR reactions 1:10 by pipetting 2 µL into 18 µL of H_2O.
2. Use 1 µL of this dilution for the secondary PCR. Prepare master mix as in **Subheading 3.3.4.** (1X reaction buffer, 5 nmol dNTPs, 1 U *Taq*, total volume: 25 µL), but now containing 10 pMol of each adaptor primer B and C.
3. Start PCR cycling using 15 s 94°C, 30 s 68°C and 1.5 min 72°C for 12 cycles.
4. Analyze 8 µL of the primary (3.3.5) and secondary (3.3.6) PCR products on a 1% agarose gel.

3.4. Cloning of Amplified, Subtracted cDNA Pools

Easiest cloning of the PCR products is achieved by using a conventional T/A cloning kit that results in high-efficiency broad-spectrum cloning of the fragment mix. Furthermore, routine blue/white selection of bacterial colonies is helpful to analyze only successfully ligated plasmids.

1. Use 1 µL of the secondary PCR and mix with 50 ng of T/A cloning vector.
2. Add 3 U of T4 DNA ligase and 1 µL of 10X ligation buffer (as provided with the ligase) in a total volume of 10 µL.
3. Incubate reaction at 16°C overnight in a water bath.
4. Transform 1 µL of the ligation into 20 µL DH10B electrocompetent cells (*see* **Note 12**) by electroporation in 0.1-cm electroporation cuvets at 1.7 kV. Immediately add 1 mL SOC medium and shake at 37°C for 1 h.

5. Plate 10 µL and 20 µL onto LB-Amp/X-Gal/IPTG agar plates.
6. Incubate overnight at 37°C.
7. Pick white colonies with toothpicks or pipet tips into 100 µL of LB-Amp medium in 96-well plates and incubate for 6–12 h at 37°C.
8. Use colony cultures for insert amplification (**Subheading 3.5.2.2.**) and store at –80°C after adding 50 % glycerol to each well.

3.5. Analysis of Subtraction Library

3.5.1. Subtraction Efficiency

As a first assessment of the subtractive hybridization efficiency, elimination of a housekeeping gene should be determined.

1. Dilute unsubtracted and subtracted secondary PCRs (**Subheading 3.3.5.**) 1:10 in H_2O.
2. Use 1 µL of these dilutions in 25-µL PCR reactions containing 1X reaction buffer, 5 nmol dNTPs (0.5 µL from 10 m*M* stock), 10 pmol *Gapd* primers (*see* **Note 13**), and 1 U *Taq* polymerase.
3. Commence thermal cycling for 20 cycles with 30 s at 94°C, 30 s at 63°C and 1 min at 72°C.
4. Analyze 5 µL on a 1% agarose gel. Complete or near-complete absence of PCR products should be observed for all subtracted samples.

3.5.2. Secondary Screening of cDNA Subtraction Clones

Despite the preferential amplification of differentially expressed transcripts in the subtraction procedure, a secondary large-scale screening of the clones retrieved is advisable (*see* **Note 14**).

3.5.2.1. Probe Preparation

1. Repeat the secondary PCR using 1 µL of the 1:10 diluted primary PCRs from **Subheading 3.3.4.** Perform duplicate reactions for subtracted and unsubtracted tester samples.
2. Prepare a master mix for 25-µL PCR reactions containing each: 1X reaction buffer, 5 nmol dNTPs (0.5 µL from 10 m*M* stock), 10 pmol adaptor primers B and C, and 1 U *Taq* polymerase per reaction.
3. Commence PCR cycling using 15 s at 94°C, 30 s at 68°C and 1.5 min at 72°C for 12 cycles.
4. Pool the two identical PCR reactions of each tester.
5. Using PCR purification columns, add 250 µL of PB buffer provided with the kit.
6. Apply samples onto the columns and spin at 12,000 rpm for 30 s.
7. Discard the flow-through and add 750 µL of PE buffer.
8. Spin again at 15,000 *g* for 30 s.
9. Discard flow-through and repeat centrifugation at 12,000 rpm for 1 min.

10. Place columns in fresh centrifuge tubes. Add 30 µL of elution buffer (10 m*M* Tris pH 8.5) to the center of each column. Spin at 15,000*g* for 1 min.
11. Digest the eluted probes with the appropriate restriction enzymes to cut off the adaptor sequences (**Fig. 2**) in a total volume of 50 µL by adding 5 µL of 10X reaction buffer (1X final concentration) and 30 U restriction enzyme. Incubate digests for more than 3 h.
12. Purify the digested probes again using the PCR purification columns by repeating steps 5–10.

3.5.2.2. Clone Array Preparation

1. Set-up a standard PCR master mix for clone screening: 1X reaction buffer, 5 nmol dNTPs, 10 pmol adaptor primers B and C (*see* **Note 15**), 1 U *Taq*,; total volume: 25 µL. Aliquot into 96-well PCR reaction tubes.
2. Using a multichannel pipet, transfer 1–2 µL of the bacterial cultures (from **Subheading 3.4.**) into the PCR reactions.
4. Commence thermal cycling with an initial boiling phase: 5 min at 94°C to break up bacteria, followed by 30 cycles of 15 s at 94°C, 30 s at 58°C and 1.5 min at 72°C.
5. Analyze 8 µL of the PCR reactions on a 1% agarose gel. Single bands should be abundantly visible in each lane.
6. Mix 5 µL of the PCR reactions with 5 µL 0.6 *M* NaOH.
7. Cut 2 Nylon membranes in the size of a 96-well microtiter plate for each clone set.
8. Again using the multichannel pipet, spot 1.5 µL of the PCR/NaOH mix onto Nylon membranes. Prepare duplicate sets of arrays with the identical spotting pattern.
9. On the still wet membrane, mark rows and columns of the spotted array with a pencil.
10. Neutralize membranes by placing them on Whatman paper soaked with 0.5 *M* Tris, pH 7.5, for 2 min.
11. Place membranes on Whatman paper soaked in H_2O for 1 min.
12. Briefly air-dry the Nylon membrane and UV crosslink.

3.5.2.3 Clone Array Hybridization

1. Radioactively label 10 µL of the digested purified cDNA pools (subtracted and unsubtracted) with ^{32}P-dCTP. Use 50 µCi for the labeling reaction in the presence of 100 ng random hexamers, 1X labelling buffer, and 2 U Klenow large-fragment DNA polymerase ***(2)***. Incubate the reactions 1–2 h at room temperature.
2. Prehybridize membrane arrays in Church hybridization buffer at 65°C.
3. Change hybridization buffer when labeling reaction is finished, and add the labelled probe. Hybridize each duplicate filter set with the corresponding subtracted and unsubtracted cDNA probes.
4. Incubate the hybridizations overnight at 65°C in a rotating hybridization oven.
5. Remove membranes from hybridization tubes and wash them twice in 2X SSC, 0.1 SDS at 65°C for 10 min in a water bath, followed by two times in 0.5X SSC, 0.1 SDS at 65°C for 10 min.

6. Expose membranes to an autoradiographic film overnight or longer if required.
7. Select clones with clearly visible hybridization differences between the subtracted and unsubtracted cDNA hybridization probes (three or more times). These clones are thereby confirmed to be enriched in the subtraction pool and can be further analyzed by sequencing and Northern blot hybridizations.

3.5.3 Outlook: Independent Verification and Suitability of cDNA Subtraction for Microarray Applications

Further analysis of the subtraction clones first starts with sequencing. Select the verified differentially expressed clones from **Subheading 3.5.2.**, grow the corresponding bacterial cultures for a routine plasmid preparation, and subject them to DNA sequencing ***(2)***. The amount of clones sequenced is dependent on the facilities available and can, if possible, be done in a 96-well format.

A cDNA subtraction library always requires independent verification by Northern blot analysis, quantitative RT-PCR, and so on. An easy method to verify cDNA subtraction clones is to use excess RNA from the tissue preparation (**Subheading 3.1.**) for multiple two-lane routine Northern blots ***(2)*** and hybridization with individual inserts that can be recovered from the insert amplification PCRs (**Subheading 3.5.2.2.**). The independent verification of subtraction clones should also be performed on different RNA samples that are derived from comparable stages, conditions, and so on. This step is necessary to rule out effects specific to the individual samples used for the subtraction. See also the initial remarks to minimize the probability of these unwanted "secondary" effects.

With the availability of cDNA microarrays covering the majority of all genes of a genome, the subtraction library is extremely useful for hybridization on these arrays and to identify +/– all sequences present in the subtraction pool. Although the independent verification of differential expression still has to be performed for a representative amount of clones, this application skips the individual clone sequencing and gives a fast overview of differentially expressed genes. In fact, when two samples are differentially analyzed by microarray analysis, the preceding performance of a cDNA subtraction is strongly recommended. The advantages of this intervening step are (1) elimination of all housekeeping and nonregulated genes before array hybridization; (2) conspicuous reduction of the hybridization spots that have to be analyzed; and (3) importantly, because of the equalization step within the subtraction, an increase of low-abundance transcripts that are otherwise lost in the overall hybridization noise.

4. Notes

1. Glycogen generally helps to precipitate small quantities of nucleic acids. Furthermore, it also results in formation of a clearly visible pellet and therefore

facilitates precipitation procedures in reducing the danger of losing tiny RNA or DNA pellets.

2. When the starting material is unlimiting and more than100 mg total RNA can be easily obtained, a poly A^+ RNA-isolation step can be carried out before starting a standard first- and second-strand cDNA synthesis. Excellent quality and yield of poly A^+ RNA can be obtained using the Qiagen Oligotex mRNA isolation kit (Qiagen, Valencia, CA). Poly A^+ RNA isolation is inherently less successful when directly using tissue specimens, and the total RNA isolation step should always be carried out first.
3. PCR conditions and optimization are extremely critical for the quality of the generated cDNA. Set up one test tube for optimizing cycle numbers and at least three tubes for both driver and tester samples. Because the amplified cDNA is very useful in further applications (e.g., full-length cDNA cloning approaches), it is recommended to run 6–7 PCR reactions for each sample as a future resource. These PCR reactions should be prepared simultaneously to minimize amplification variability. If reamplification is necessary, always run the optimization reaction again.
4. Clontech's Advantage *Taq* polymerase mix, provided in the SMART™ cDNA synthesis kit, gives very reliable results and is also extremely useful for further downstream applications.
5. The observed smear in the cDNA amplification step can vary considerably between different tissue samples. Always determine the optimal number of PCR cycles by comparing each sample individually, not by comparing different samples.
6. Concentration of cDNA pools can also be achieved by adding approx 4 vol of *n*-butanol. Butanol serves to extract water from the mix so that the adjustment to a total volume of 50–70 µL can be achieved. However, visibility of the organic phase border is often difficult, and the Microcon spin columns are much easier to handle for probe concentration procedures. Note that centrifugation time to spin down 400 µL may vary between centrifuges and should be individually determined. Filters should not fall completely dry, but the residual volume after centrifugation on top of the filter should be less than 20 µL.
7. Make sure to collect the complete cDNA size range except for products less than 300 bp. Although this step will remove very small transcripts from the pool, most of the small-sized fragments are truncated products that accumulate as a result of PCR amplification.
8. As DNA hybridization is a crucial factor for the efficiency in the subtraction procedure, the cDNA fragments should be reduced to a common size of 200–500 bp. Useful restriction endonucleases that yield this size range are typically 4-bp cutters (e.g., *Rsa*I). When using an enzyme different from *Rsa*I, make absolutely sure that the generated ends are compatible with the adaptors that are to be ligated in **Subheading 3.3.1.** *Rsa*I is a blunt-end cutter and is very useful for conventional adaptor ligation.
9. It is extremely advisable to perform a control subtraction in parallel with the experimental samples. A control can be easily introduced by mixing one cDNA

sample with 0.2% of a marker DNA ladder. The subtraction procedure is then carried out using the same cDNA (without marker DNA) as the driver. Subtractive hybridization should result in the appearance of clear marker bands that can be nicely shown side-by-side with standard marker DNA loading in the final control gel of **Subheading 3.3.5.** to prove subtraction efficiency.

10. Ligation efficiency test: The efficiency of the ligation reaction can be easily tested in a PCR reaction spanning the adaptor-cDNA junction. As adaptor-specific primers, the outer regions of the adaptor molecules can be used (binding to region A/A' in **Fig. 2**). As gene-specific oligonucleotides, primers to any housekeeping gene (e.g., *Gapd* or β-*actin* can be used. Four reactions should be set up and subjected to 25 PCR cycles:
    A: Tester with adaptor 1 + 1 housekeeping gene primer (5' or 3') + adaptor primer A')
    B: Tester with adaptor 1 + both houskeeping gene primers (5' and 3')
    C: Tester with adaptor 2 + 1 housekeeping gene primer (5' or 3') + adaptor primer A'
    D: Tester with adaptor 2 + both houskeeping gene primers (5' and 3')

    By comparing the intensities of PCR products between reactions A-B and C-D, the efficiency of the adaptor ligation can be determined. A/B and C/D intensity ratios may range between 20–100%. Importantly, the adaptor ligation test should readily detect products spanning the adaptor-cDNA junction even if their abundance is considerably lower than the gene-specific product. If this is not the case, repeat the adaptor ligation step, control activity of the T4 DNA ligase, and ensure presence of ATP in the ligation reaction. ATP is usually contained in the ligation buffer, but this might vary and should be confirmed for each supplier. Repeated thawing-freezing cycles of the ligation buffer can inactivate ATP. When inherently low-ligation efficiency is observed, a freshly prepared buffer should be used and distributed into aliquots to prevent decay.
11. Owing to the restriction digest that reduced the cDNA length to an average of approx 500 bp, the extension phase of the PCR reaction does not have to be longer than 1.5 min. These conditions were set up using *Rsa*I for cDNA cleavage. If another restriction endonuclease is chosen that produces longer average-length fragments, the elongation time has to be readjusted.
12. The exact strain of the competent bacteria is not important for routine cloning procedures. However, the method of transformation affects cloning efficiency, and the use of electrocompetent cells is recommended in library construction applications because of the higher transformation yield when compared to $CaCl_2$-competent bacterial cells.
13. In generating primer oligonucleotides for the subtraction efficiency test, any housekeeping gene can be used. However, make sure that the amplified product does not contain a restriction site for the enzyme used to reduce cDNA size (i.e., for example, *Rsa*I).
14. The procedure described here is an easy and quick method to spot clones and screen them by hybridization with the subtracted and unsubtracted cDNA pools. When a robotic spotting facility is available, this step can be modified to accom-

modate the needs of the robot, but otherwise relies on the same experimental principles.

15. Alternatively, vector specific primers such as T7/T3 or M13 Universal and reverse primers can be used for insert amplification.

Acknowledgments

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4

PCR-Based Cloning and Differential Screening of RNAs from *Xenopus* Primordial Germ Cells

Cloning Uniquely Expressed RNAs from Rare Cells

Thiagarajan Venkataraman, Elio Dancausse, and Mary Lou King

1. Introduction

Primordial germ cells (PGCs) are a small population of unique cells from which all germ cells arise in an organism. In this sense, PGCs can be considered the stem cells of the species. An important characteristic of PGCs is their ability to remain developmentally totipotent,whereas somatic cells become restricted in their fates. Understanding the genetic program that underlies the retention of totipotency is a major goal in the stem cell field. To accomplish this goal, methods must be considered for both the isolation of these cells and the purification of the RNAs they express. The isolation of PGCs from any organism presents certain challenges. Because PGCs arise outside the gonad during early embryogenesis, their exact location within a germ layer is unknown. In addition, PGCs are relatively rare in number compared to somatic cells (on the order of 0.05% or less). This chapter presents detailed procedures for isolating live PGCs from *Xenopus laevis* embryos and for cloning their expressed genes, should be applicable to other organisms that have PGCs rich in mitochondria.

1.1. Formation of Xenopus *Primordial Germ Cells*

As a general rule, PGCs are specified in a cell-autonomous fashion by the inheritance of germ plasm if they are set aside before the primary germ layers are formed and, if afterwards by induction events. Germ plasm is a cytologi-

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cally distinct cytoplasm rich in mitochondria, unique RNAs, proteins, and electron-dense germinal granules. In the *Xenopus* egg, germ plasm is found as yolk-free cytoplasmic islands distributed broadly within the vegetal subcortex. Beginning shortly after fertilization and through the early cleavage divisions, the germ plasm undergoes extensive cytoskeletal-dependent aggregation into a smaller number of large "islands." By the 32-cell stage, the germ plasm has "disappeared" into the interior of the embryo ***(1)***. By the blastula stage, the germ plasm is concentrated within four to five PGCs within the endodermal mass. Here the PGCs remain dividing a few times until the tailbud stage when they begin to migrate out of the endoderm, along the dorsal mesentery (tadpole), and into the somatic gonad—their final destination. Therefore, the best time to vitally stain germ plasm is at the four-cell stage while it is aggregating and still near the embryo surface.

1.2. Selectively Labeling Primordial Germ Cells

To distinguish PGCs from somatic cells, we took advantage of the extremely high content of mitochondria in germ plasm and the fluorescent vital dye $DiOC_6(3)$ (3,3'-dihexyloxacarbocyanine). The spectra exhibited by $DiOC_6(3)$ is similar to that of fluorescein. $DiOC_6(3)$ is a carbocyanine dye with a short alkyl chain that stains mitochondria in live cells when used at low concentrations. At higher concentrations, other membrane systems may also be stained. The uptake of $DiOC_6(3)$ is dependent on the mitochondrial membrane potential. $DiOC_6(3)$ accumulates on hyperpolarized membranes and is translocated into the lipid bilayer ***(2)***. Although fluorescence is not lost in fixation, the dye is no longer concentrated within mitochondria and diffuses throughout the cell. To circumvent this possible drawback, Molecular Probes has developed mitochondrion-selective dyes that retain their organelle localization upon fixation, such as MitoTracker Green FM and MitoFluor™. However, in our experience, using $DiOC_6(3)$ resulted in lower background staining and hence, a better contrasting signal between somatic cells and PGCs (**Fig. 1**).

A potential drawback to using carbocyanine dyes is that they have been reported to interfere with respiration ***(3)***. Embryos treated with $DiOC_6(3)$ must be raised in the dark to protect against phototoxicity of fluorescently-labeled mitochondria, which is especially important for long-term viability. Although we have not raised embryos stained with $DiOC_6(3)$ to sexual maturity and therefore cannot confirm their fertility, we have assayed tadpoles for proper PGC migration into the dorsal mesentery. Migration was unaffected at the concentrations used, suggesting that the vital dye does not interfere with normal developmental events.

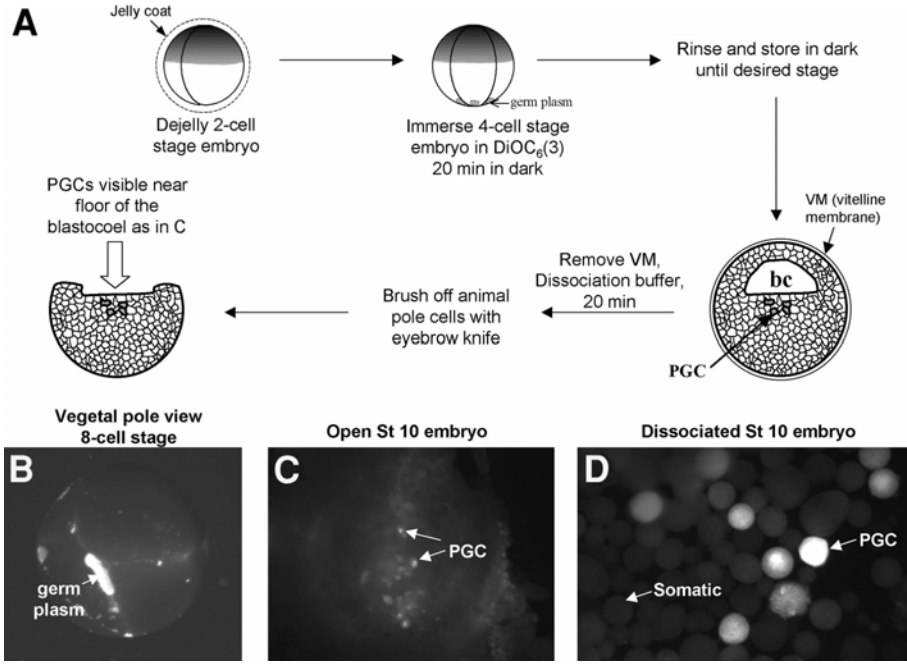

Fig. 1. PGC isolation from St 10 embryos. **(A)** Schematic diagram of $DiOC_6(3)$ staining and PGC isolation. The shaded cells show the location of primordial germ cells (near the floor of the blastocoel [bc]). The Vitelline Membrane (VM) is removed manually with a watchmaker's forceps. The embryo is then immersed in dissociation buffer for 20 min. The animal pole cells are then brushed off using an eyebrow knife. PGCs are visible near the floor of the blastocoel as in panel C. Stereofluorescence microscopy showing $DiOC_6(3)$ staining of **(B)** germ plasm in an eight-cell stage embryo; **(C)** PGCs visible near the floor of the blastocoel; **(D)** isolated dissociated cells. Both PGCs and somatic cells can be seen. PGCs can be differentiated based on the distinctive fluorescent staining of the germ plasm.

1.3. Isolating Primordial Germ Cells from Different Staged Embryos

All donor embryos were dejellied and labeled at the four-cell stage by immersion in $DiOC_6(3)$ for 20 min. We found that $DiOC_6(3)$ staining of germ plasm was evident in embryos through stage 42—the last stage tested. At the desired stage, the vitelline membranes are removed by poking a small hole through the animal cap and peeling the membrane off with forceps. In this way, any possible damage to the PGCs during this procedure is avoided as PGCs are found in the endoderm away from the animal pole. Furthermore, the

hole in the blastocele-facilitates the cellular dissociation of the embryo as the dissociation buffer is now freely accessible to the internal endoderm cells. PGCs are close to the floor of the blastocele within the endodermal mass of early staged embryos,and they are fragile cells and easily broken. Using an eyebrow knife to gently brush off the animal cells during the dissociation step, works best to expose the labeled PGCs. PGCs can then be viewed by fluorescence stereomicroscopy and manually selected for further analysis (**Fig. 1C**).

1.4. Strategy for Cloning Primordial Germ Cell Specific cDNAs

We estimate that one PGC contains approx 100–250 pg of total RNA. Starting with 50 PGCs, we could routinely achieve efficient polymerase chain reaction (PCR) amplification and a size distribution of cDNAs from 0.5–5 kb. By using specific oligo d(T) primers during the reverse transcriptase (RT) reaction and amplification process, products were generated that could be used either in library construction or suppression subtractive hybridization (SSH). A number of PGC-specific markers are available to verify the isolation procedure at a molecular level. For example, *Xpat* ***(4)*** and *Xcat2* RNAs ***(5)*** are unique to PGCs. Primers specific to these can be used in RT-PCR analysis (see example **Fig. 3**).

For library construction, RT primers were designed with specific restriction sites for cloning into any desired vector system. *Sfi*I is a good restriction enzyme to use for directional cloning since it has a degenerate site of recognition (GGCCNNNN/GGCC). Upstream and downstream primers can have *Sfi*I sites with different degenerate cutting sites. Either lambda-based (e.g., lambda gt11™, ZAPII™ Stratagene) or plasmid vector systems (eg. CREATOR™ library system, Clontech) can be used.

Representational difference analysis (RDA) is a powerful PCR-based method, originally to clone differences between genomes. It has been used successfully with cDNAs to study gene expression ***(6)***. RDA has been designed specifically to amplify the differences between two cDNA populations. The PCR amplification step allows the products identified to be directly cloned and characterized by standard methods. SSH is a variation on this technique, modified specifically for very small amounts of starting material ***(7)***. First, mRNA is reverse transcribed into cDNA. The cDNA is then converted to double stranded DNA and cut into smaller pieces with a frequent cutter such as *Rsa*I or *Dpn*II. The population of ds cDNAs from which differential sequences are desired is designated the "Tester", and the population that is subtracted against is called the "Driver". The digested tester DNA is ligated to two different unique adaptors, adaptor 1 and 2. Two separate hybridizations are carried out with adaptor 1 and 2 carrying testers and an excess of driver DNA. All common sequences between the tester and driver crosshybridize. Only the

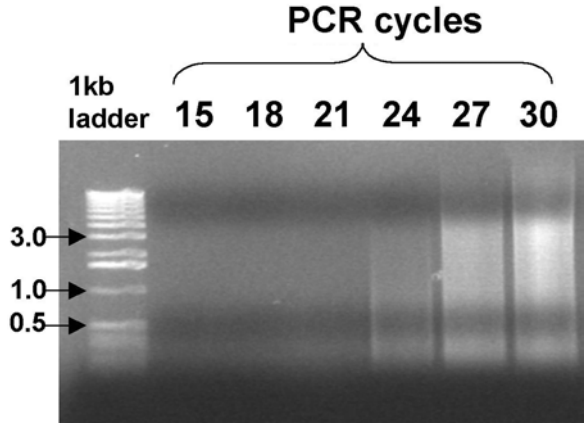

Fig. 2. Optimization of PCR cycle number during cDNA amplification. Refer to **Subheading 3.4.**, **step 5** for details. The optimum number of cycles determined in this case was 27. Linearity was reached after cycle 29.

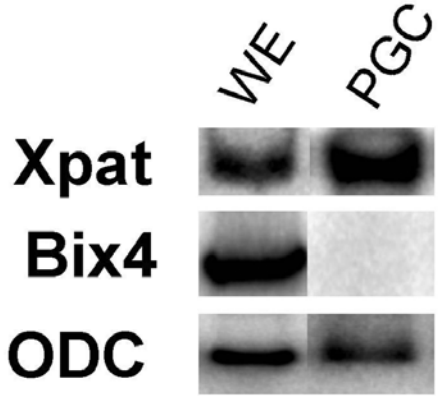

Fig. 3. Molecular verification of PGC isolation by RT-PCR. *Xpat* is a specific marker for PGCs, *Bix4* is an endodermal specific marker, and *ODC* is a general housekeeping gene present in all cells. Note that the PGC sample does not contain *Bix4*, but does contain *Xpat* RNA.

unique sequences are left with an adaptor at both ends. The two hybridization reactions are mixed with each other, and this mixture is allowed to further hybridize. A third of all unique sequences now have both the adaptors flanking it and all common sequences have one adaptor on one side. PCR amplification with primers designed against the adaptor regions specifically amplify the unique sequences (*see* **Fig. 2**) in Diatchenko et al. *(7)*].

2. Materials

2.1. Isolation of Xenopus PGCs

1. Stage 8 (blastula), 10 (gastrula), and 14 (neurula) embryos (*see* **ref. 8**).
2. Ca^{2+}, Mg^{2+}-free medium (CMFM): 50.3 m*M* NaCl, 0.7 m*M* KCl, 9.2 m*M* Na_2HPO_4, 0.9 m*M* KH_2PO_4, 2.4 m*M* $NaHCO_3$, 1.0 m*M* EDTA, pH 7.3.
3. 35 × 10 mm plastic culture dishes. Falcon, Cat. no. 351008.
4. 2% Agarose in CMFM medium.
5. Sharp, fine forceps for removing vitelline membrane from embryos (Fine Science Tools, Foster City, CA, cat. no. 11252-20).
6. Eyebrow knife. This is made by inserting an eyebrow hair into the narrow end of a Pasteur pipet and sealed in place with melted paraffin (for detailed instructions *see* **ref. *8)***.
7. Plastic transfer pipets (SARSTEDT, cat. no. 86.1172). Pipet tips are drawn out over heat and will be used to select individual PGCs.
8. Marc's Modified Ringers (MMR) buffer: 0.1 *M* NaCl, 2 m*M* KCl, 1 m*M* $MgSO_4$, 2 m*M* $CaCl_2$, 5 m*M* HEPES, pH 7.8, 0.1 m*M* EDTA. Most current formulations of MMR omit EDTA and are adjusted to pH 7.4.
9. 1.25% Cysteine Y in 0.1X MMR, pH 8.0 (Sigma, cat. no. C-7352).
10. $DiOC_6(3)$ stock solution 2:1000 v/v, made from a saturated solution of DiOC6(3) in 0.1(MMR buffer. (Molecular Probes cat. no. D-273.)
11. 10X MOPS buffer: 0.2 *M* MOPS, pH 7.0, 20 m*M* sodium acetate, 10 m*M* EDTA, (pH 8.0).
12. 5X MEM Buffer: 0.5X MOPS buffer, 10.0 m*M* EGTA, 5.0 m*M* $MgSO_4$, pH 7.5.
13. MEMFA fixative freshly made out of 5X MEM buffer and 37% Formaldehyde Sigma, cat. no. F-1268). MEMFA is 1X MEM + 1 part of formaldehyde.
14. 10X Phosphate-buffered saline (PBS) buffer: 80 g NaCl, 2 g KCl, 14.4 g Na_2HPO_4, 2.4g KH_2PO_4, 800-mL distilled H_2O, pH 7.4. Make up to 1000 mL.
15. PBT: PBS + 2 mg/mL BSA+ 0.1% Triton X-100 + 1% saponin (optional, may improve permeability).
16. PTW: PBS + 0.1% Tween 20.
17. Fluorescent stereo microscope (magnifications 1.6× PF, 16-100 zoom).

2.2. Extraction of RNA from PGCs

1. RNA extraction was performed using Stratagene's ABSOLUTELY RNA microprep kit (cat. no. 400805). This is a spin column-based method. Lysis is performed in guanidium isothiocyanide (GITC) containing buffer. All required components are included in the kit.
2. RNA quantitation: Ribogreen dye (Molecular Probes, cat. no. R-11491).
3. Phosphorimager with ImageQuant Software (Molecular Dynamics).

2.3. First-Strand Synthesis from RNA

2.3.1. Primer Design for cDNA Synthesis and SSH

1. Oligo d(T) primers are made with AdX-GTAC$(T)_{30}$VN. V is A,G, or C, and N is A, T, G, or C. Adaptor X (AdX) refers to a 20–25-nt long custom region,

which is designed based on the cloning vector to be used. GTAC is a *Rsa*I restriction site.
2. 5' Oligo adaptor: synthesized as AdX-GTAC-GCGGG.
3. PCR primer: synthesized as AdX (*see* **Notes 5–9**).
4. Clontech's Super SMART PCR cDNA Synthesis Kit (cat. no. K1054-1).

2.3.2. cDNA Synthesis

1. RNA synthesis was performed using Clontech's PowerScript Reverse Transcriptase (cat. no. 8460-2).
2. dNTPs (10 m*M* each, PCR grade).
3. 100 m*M* Dithiothreitol(DTT).
4. RNase Inhibitor: SUPERase•In (Ambion cat. no. 2696). Other RNase inhibitors like Promega's rRNasin also work well.

2.3.3. cDNA purification

Clontech's Nucleospin Extraction kit (cat. no. K3051-1).

2.4. cDNA Amplification by PCR

Clontech's Advantage 2 polymerase kit (cat. no. K1910-y).

2.5. DNA Purification and Size Selection

Clontech's Chroma Spin-1000 columns (cat. no. K1334-1) and Chroma Spin-400 columns (cat. no. 1323-2).

2.6. Suppression Subtractive Hybridization (or Subtractive PCR)

Clontech's PCR-select cDNA Subtraction Kit (cat. no. K1804-2).

3. Methods

3.1. Isolation of PGCs

1. Follow the Cold Spring Harbor Laboratory Manual ***(8)*** procedures for producing embryos at the desired stage.
2. Dejelly embryos at two-cell stage and rinse 10 times with 0.1X MMR.
3. Stain embryos at four-cell stage with a solution of 0.1X MMR buffer and 0.08:1000v/v $DiOC_6(3)$ from a stock solution of 2:1000 v/v. Stain in the dark for 20 min and then rinse five times by moving the embryos to dishes with fresh 0.1X MMR. All subsequent procedures should be conducted in the dark unless otherwise indicated (*see* **Note 1**).
4. Incubate stained embryos coming from the same female (collected at different times) at different temperatures (e.g., 17–18°C and room temperature) to have a range of stages available the following morning (*see* **Note 2)**.
5. Wash them 2 times in dissociation medium, then transfer single embryos to 35 × 10-mm plastic Petri dishes containing 2% agarose in CMFM medium.
6. Remove the vitelline membrane by opening a hole in the animal pole using forceps, then pull the membrane off. This step is done using a regular stereomicroscope. Act quickly to reduce the exposure of embryos to light.

7. Incubate for 20–30 min at room temperature in the dark. After this time, the embryos will appear intact, but their blastomeres will be very loosely associated.
8. Working with a stereofluorescence microscope, use an eyebrow knife to brush off the cells on top of the PGCs until they are revealed. PGCs from stages 6.5 to 10.5–11 can be distinguished from somatic cells by an intense and discreet green fluorescent signal. At later stages, the signal is less discrete, but clear.
9. Move the PGCs away from the mass of cells and transfer them to a new small agarose Petri dish containing 0.1 MMR using a fine tipped plastic pipet.
10. Transfer groups of PGCs into nuclease-free 1.5-mL tubes in minimum buffer and snap-freeze in a dry-ice/ethanol bath. These tubes will be used for RNA extraction and cDNA synthesis.
11. For immunostaining steps (if desired), transfer PGCs in the same manner to a new Petri dish containing MEMFA fixative and fix for 1 h. Transfer cells to a 24-well multiwell previously coated with PBT (*see* **Note 3**).

3.2. Extraction of RNA from PGCs

1. For RNA extraction from PGCs, use Stratagene's ABSOLUTELY RNA microprep kit. Detailed instructions are provided in the kit. Briefly, cells are lysed in a GITC based buffer. RNA is bound to a spin column and washed twice with a buffer provided in the kit to which ethanol is added.
2. DNase I treatment is carried out in the column.
3. Elute sample in 30 (L of elution buffer: 10 m*M* Tris-HCl, pH 7.5. Repeat elution step with 20 (L to obtain RNA in a final volume of approx 50 µL (*see* **Note 4**).

3.3. First-Strand Synthesis from RNA

3.3.1. RT Reaction (see ***Note 5*** and ***6***)

1. Quantitate RNA using the Ribogreen method (refer to the protocol provided by Molecular Probes). Use approx 2–10 ng of RNA(*see* **Note 7**). For SSH, at least 10 ng is recommended. It is always advisable to begin with as much starting material as possible. Usually RNA extracted from approx 50–70 cells is sufficient for one cDNA synthesis reaction.
2. RNA should be in a maximum total volume of 50 µL.
3. Mix RNA (10–1000 ng) and 7 µL of each primer [Oligo d(T) and 5' oligo adaptor primer] to a final volume of 64 µL with nuclease-free water.
4. Prepare RT master mix in a tube on ice. Master mix: 20 µL 5X first strand buffer, 2 µL 100 m*M* DTT, 10 µL 10 m*M* each dNTP, 5 µL 20 U/µL RNase Inhibitor, 5 µL PowerScript RT enzyme.
5. Heat RNA–primer mix at 65(C for 2 min and put on ice for 5 min.
6. Immediately add master mix to the denatured RNA and transfer the tube to a preheated PCR block at 42(C for 90 min. Use a heated-lid cycler to prevent evaporation during cDNA synthesis.

*3.3.2. cDNA Purification (*see ***Notes 8*** *and* ***9****)*

1. After first-strand synthesis, the cDNA must be purified for further steps. For this purpose, use Clontech's Nucleospin Extraction kit. This step is required to ensure that there is no carryover of primers, dNTP, or buffer from the previous steps. The purification method is based on a DNA-binding spin column that binds single-stranded cDNA. The column is washed to remove impurities and pure cDNA is eluted in milli-Q-H_2O.
2. Elution is done in two steps, in a total of approx 85 µL of Milli-Q-H_2O.

3.4. cDNA Amplification by PCR

1. Use a high-fidelity DNA polymerase for the PCR. Clontech's cDNA subtraction kit contains its own polymerase and PCR components.
2. Make a master mix for each amplification reaction as follows. 172 µL Milli-Q-H_2O, 30 µL 10X buffer, 6 µL 50X dNTP, 6 µL PCR primer, 6 µL Polymerase.
3. Add 220 µL of master mix to the purified cDNA from **Subheading 3.3.2.** The total volume of the mix is 300 µL.
4. Split the above mix into three tubes, each carrying 100 µL, and label them A, B, and C.
5. Optimization of PCR: a typical PCR program is 95°C for 1 min, 95°C for 15 s, 65°C for 30 s and 68°C for 3 min times "X" cycles. "X" refers to the maximum number of cycles required to stay within the exponential phase of amplification to avoid overcycling (*see* **Note 10**). Optimization is done as follows. Cycle all three tubes for 15 cycles. Store them at 4°C. Transfer 30 µL from tube C into a separate tube marked "optimization." Transfer 5 µL from this tube to a fresh tube marked "15 cycles". Return the other 25 µL to the thermal cycler and continue the PCR. After every three cycles, remove an aliquot of 5 µL to a fresh tube with the total number of cycles marked on it.
6. Analyze the saved aliquots on a 1% agarose gel and determine the total number of cycles required to reach the linear phase, meaning the amount of cDNA remains constant (**Fig. 2**). The optimum number of cycles will be 2 less than this number.

*3.5. DNA Purification and Size Selection (***see *Notes 11 and 12****)***

1. At this point, combine the contents from all three tubes.
2. Add an equal volume of phenol-chloroform and vortex. Centrifuge at 18,000*g* and transfer the aqueous phase to a fresh tube.
3. Concentrate the DNA by mixing it with an equal volume of *n*-butanol. Transfer the aqueous phase to a new tube. Repeat this step until the volume is approx 40–70 µL.
4. Next, size select the DNA using column chromatography (**Fig. 3**).
5. For SSH, cDNA above 1kb is required. Clontech's Chroma Spin-1000 columns are ideal for size selection in this range.

6. If the cDNA will be used for the construction of libraries, then size selection can be carried out using Clontech's Chroma Spin-400 columns.

3.6. Suppression Subtractive Hybridization (or Subtractive PCR)

cDNA subtraction was performed using Clontech's PCR-Select cDNA subtraction Kit.

4. Notes

4.1. Isolation of PGCs

1. Use shallow containers with lids and large plastic Petri dishes. Wrap containers in aluminum foil to protect embryos from the light.
2. Suggested time table for collecting embryos: keep two-thirds of the stained embryos at 17–18(C and one third at room temperature overnight. The next morning, collect the desired number of stage 8, and leave the rest at room temperature to reach stage 10 later that day. The embryos left at room temperature overnight should be at stage 14 in the morning. The temporal order of dissociation for embryos collected using this time table would be Stages 8, 14, and 10. Changes can be made in the schedule according to time available and number of embryos required. For dissociation, select only embryos of the best quality.
3. If PGCs will be used for immunostaining procedures, do not use agarose-coated chambers. It appears that the antibody is absorbed by the agarose. Do not use gelatin or dishes treated for tissue culture for the same reason.

4.2. Extraction of RNA from PGCs

4. The elution buffer provided with the kit works well and is compatible with the steps that follow for cDNA synthesis. Standard precautions when handling RNA should be observed. RNA samples must be stored at (80°C if not used immediately in the next step.

4.3. First-Strand Synthesis from RNA

5. All primers are provided in Clontech's Super SMART PCR cDNA Synthesis kit.
6. Procedure is adapted from Clonetech's Super SMART kit.
7. Use as much RNA as possible (not exceeding 1 μg per reaction) to obtain the best results: lower background, well-defined bands, and efficient subtraction in SSH.
8. The cDNA purification step is critical for the success of the following steps. Before purification, the cDNA can be stored at –20°C. RT-PCR with gene-specific primers works better on cDNA after purification.
9. The PowerScript RT is a modified Moloney Murine Leukimia Virus (MMLV) RT with a higher efficiency and has the ability to add a short Oligo C tail to full length cDNA. The 5' Oligo adaptor pairs with the oligo C stretch. The PowerScript RT extends the cDNA with the 5' Oligo adaptor serving as a template. This results in cDNAs with two adaptors, one on either side. In the case of SSH, primers are designed with the same adaptor for each side. If the cDNA is to

be used in cloning, two adaptors, AdX and AdY, can be used to differentiate the 5' and 3' sides of the cDNA for directional cloning purposes.

4.4. cDNA Amplification by PCR

10. It is important that cDNA amplification stays within the exponential phase. Representation will be lost if the cDNA is overcycled. Artifacts and false-positives will result if this step is not optimized correctly.

4.5. SSH (or Subtractive PCR)

11. One of the most important conditions for success when performing this procedure is to make sure that the primers used for the PCR and RT steps are ultrapure. Reverse-phase high performance chromatography (HPLC) purification of the primers reduces the number of false positives generated and increases the sensitivity of the procedure ***(9)***.
12. At least 10 ng of RNA is recommended as starting material. In our case, approx 50 PGC equivalence of total RNA worked well.

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5

Differentiating Embryonic Stem Cells into Embryoid Bodies

Gisele Höpfl, Max Gassmann, and Isabelle Desbaillets

1. Introduction

Since the early 1980s, embryonic stem (ES) cells have been isolated from the inner cell mass of the preimplantation blastocyst ***(1–3)***. They can be maintained in a pluripotent state for indefinite periods of time in the presence of the leukemia inhibitory factor (LIF) or in coculture with mouse embryonic fibroblasts (MEFs). When cultured in the absence of LIF, ES cells differentiate spontaneously, forming three-dimensional (3D) aggregates called *embryoid bodies* (EBs, **Fig. 1**). EBs recapitulate many aspects of cell differentiation during early mammalian embryogenesis ***(4,5)*** and give rise to mature cells of all three germ layers (reviewed in **ref. *6***). The EB system is an extremely valuable tool for the investigation of embryonic development in vitro because it is challenging and time-consuming to isolate early-stage cells from developing embryos. Moreover, EBs can be used when difficulties arise to determine the cellular basis for embryonic defects or the interpretation of knockout phenotypes, especially those complicated by early embryonic lethality. In addition, great efforts are made to define protocols to preferentially direct ES cell differentiation as EBs toward one single cell fate, therefore providing a source of normal cells and raising opportunities for therapeutic interventions to correct cell/tissue damage and/or dysfunction.

A significant amount of data has accumulated recently so that a detailed discussion of the differentiation methods used would go beyond the purposes of this chapter. The methods described in this chapter build the basis for these techniques and allow an immediate start, provided the adequate factors are added. In order to facilitate experimental onset, a summary of the most impor-

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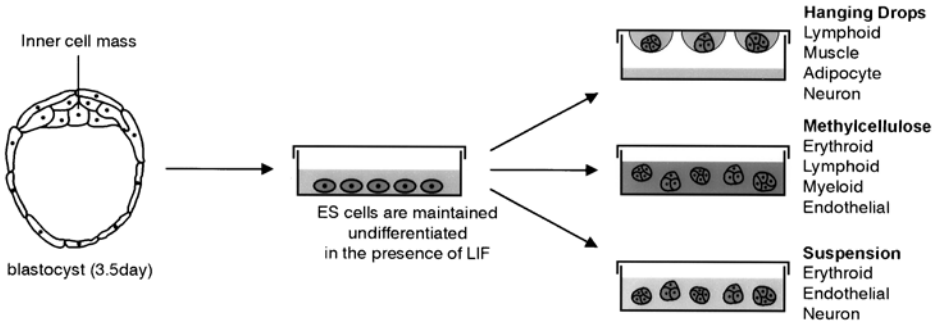

Fig. 1: Methods for the in vitro differentiation of ES cells into EBs: ES cells are obtained from the inner cell mass of the preimplantation blastocyst and cultured in the presence of LIF to retain their pluripotency. ES cells give rise to EBs when cultured in suspension, hanging drops or MC medium in the absence of LIF. A few examples of resulting cell lineages are given for each EB formation culture type.

tant differentiation protocols, including growth factors, is given. Furthermore, examples are presented for the use of these techniques. For instance, hematopoietic differentiation is described as a classic example for the use of methylcellulose (MC) medium. Also, a few neural differentiation protocols (some of which are currently used in our laboratory) were chosen to exemplify the methods, demonstrating the usage and usefulness of a combination of techniques, focusing those ones that seem most promising in terms of in vivo application.

2. Materials

2.1. Cell Culture Equipment

1. 90-mm Cell culture dishes (Sarstedt, Nümbrecht, Germany).
2. 90-mm Bacterial-grade Petri dishes (Sterilin, Stone, Staffordshire, UK).
3. 140 × 20 mm Bacterial-grade Petri dishes (Nunc GMBH, Wiesbaden, Germany).
4. 10- and 50-mL sterile polypropylene tubes (Falcon, Becton Dickinson Labware, USA).
5. Sterile pipets.
6. Sterile 1.5-mL Eppendorf tubes.

2.2. Cell Culture Media and Solutions

1. DMEM-FCS medium: Dulbecco's modified Eagle's medium (DMEM high-glucose), 10% or 15% heat-inactivated fetal calf serum (FCS; (Gibco-BRL, *see* **Notes 1** and **2**), 1.7 µL/500 mL, 2-Mercaptoethanol (Fluka; 0.05 m*M* final concentration) 100 IU/mL penicillin (Sigma), 0.1 mg/mL streptomycin (Sigma), and, where indicated 103 U/mL LIF (ESGRO, Chemicon international; *see* **Note 3**).
2. Iscove's modified Dulbecco's medium (IMDM): always prepared fresh from powder.

3. 4% MC solution: 16 g MC (Methocel®, Fluka) dissolved in ddH_2O made as described in **Subheading 3.3.3.**
4. 2% Methylcellulose stock (MCM stock): 4% MC solution diluted 1:1 with 2X concentrated IMDM medium.
5. Complete 1% MCM: 2% MCM stock diluted 1:1 with IMDM and supplemented with 15% heat inactivated FCS, 450 µ*M* monothioglycerol (Sigma), 10 µg/mL insulin (Sigma), and 300 µg/mL transferrin (Roche applied biosciences, all components of this medium—except for FCS and MCM stock—have to be freshly prepared; otherwise, the EB yield decreases.
6. Phosphate-buffered saline (PBS).

3. Methods

3.1. Culture of MEFs

1. Culture MEFs in 90-mm cell culture dishes using DMEM supplemented with 10% FCS. Addition of 2-mercaptoethanol to the medium is not necessary. Culture cells in a humidified atmosphere containing 5% CO_2 at 37°C.
2. Trypsinize cells twice a week (e.g., on Mondays and Fridays) and split in a 1:4 or 1:5 ratio.

3.1.1. Mitomycin C Treatment of MEFs

1. Coat 90 mm cell culture dishes with 0.75% gelatin (type A from porcine skin;) solution for at least 30 min at room temperature. After removal of the gelatin solution, plates can be used for 1–2 d if kept under sterile conditions.
2. On d 1 (usually Mondays), trypsinize one 90-mm cell culture dish. (Usually there are approx 4×10^6 MEFs in a full dish).
3. Count the cells and add 10^6 MEFs/90-mm gelatin-coated cell culture dish containing DMEM-10% FCS without 2-mercaptoethanol.
4. On d 2 (usually Tuesdays), incubate the cells with a final concentration of 10 µg/mL of mitomycin C (usually 400 µL of a 0.2 mg/mL mitomycin C [Sigma] stock solution in PBS) in 8 mL DMEM–10% FCS for 3 h at 37°C.
5. Remove the medium and wash the cells two or three times with PBS.
6. Add DMEM–10% FCS without 2-mercaptoethanol. These cells can be used for 1 wk of ES cell culture.
7. Change medium twice a week.

3.2. ES Cell Culture

3.2.1. ES Cell Culture with MEFs

1. Add ES cells to Petri dishes containing MEFs and cultivate them in DMEM–15% FCS with 10^3 U/mL LIF. Culture cells in a humidified atmosphere containing 5% CO_2 at 37°C.
2. To split ES cells, wash once with PBS and detach the cells using 1.5 mL trypsin/EDTA (Gibco-BRL) for 5 min at 37°C.

3. Resuspend well with a 5-mL pipet and replate the ES cells on a new culture of mitomycin C-inactivated MEFs.

3.2.2. ES Cell Culture on Gelatin-Coated Plates

1. Culture ES cells on gelatin-coated Petri dishes (coating described in **Subheading 3.1.1., step 1**) in DMEM–15% FCS supplemented with 10^3 U/mL LIF. Be careful to keep the amount of cells seeded low (between 40% and 50% confluence) because high-seeding numbers favor differentiation.
2. Passage ES cells every 2 d. These conditions ensure that ES cells retain their pluripotency.

3.3. Embryoid Body Formation (see Note 4)

3.3.1. Liquid Suspension

1. Trypsinize ES cells and make a single-cell suspension by pipetting the cells up and down with a 5- or 10-mL sterile pipet at least 10 times (*see* **Note 5**).
2. Count the cells and add the appropriate amount to obtain the end concentration desired (*see* **Table 3**) to DMEM–15% FCS medium without LIF. Culture in bacterial-grade culture dishes to prevent adherence of ES cells (*see* **Note 6**). HM-1 cells used in our laboratory are plated at 10^4 –5×10^4 cells/mL. Exchange culture medium two or three times a week.

3.3.2. Hanging Drop Cultures

1. Plate 20–30 µL drops containing 400–1000 ES cells on the lids of Petri dishes filled with PBS (*see* **Table 3**) to prevent the drops from drying out (*see* **Notes 7** and **8**).
2. Harvest EBs and subsequently cultivate in suspension in bacterial-grade Petri dishes in DMEM–15% FCS medium without LIF or plate directly onto 0.75% gelatin-coated tissue culture plates (*see* **Table 3**).

3.3.3. MC Culture

1. Add 16 g of MC to 400 mL boiling ddH_2O to prepare a 4% solution. To reduce risk of contamination, start with 400 mL water that has been autoclaved together with the magnetic stirrer in a 1000-mL bottle. MC should be added slowly spoon-by-spoon under vigorous stirring, to avoid clumping of the medium.
2. Sterilize the solution by at least 15 min of additional boiling with continuous stirring. The resulting solution is turbid white.
3. Cool the MC down to 37°C, add 400 mL freshly prepared 2X concentrated IMDM (*see* **Table 1**) and mix thoroughly (the solution then turns turbid orange) to prepare the 2% MCM stock. Stirring should not be stopped during cooling or after addition of IMDM. In our laboratory, the whole procedure (except for the cooling procedure, which is done under continuous stirring in a water bath) is under a sterile hood.

Table 1
IMDM Components[a]

End volume	400 mL (2X)	100 mL (1X)
IMDM powder	14.16 g	1.77 g
$NaHCO_3$ (7.5% in water)	32 mL	4 mL
100X MEM	8 mL	1 mL
100X Penicillin/Streptomycin	8 mL	1 mL
H_2O	352 mL	94 mL

[a]Filter under sterile conditions. Always prepare fresh.
1X, standard concentration; 2X, double concentration.

4. Aliquot the medium immediately after adding of IMDM while the mixture is still warm. Aliquot by pouring the desired amounts of warm MCM (*see* **Table 2**; 2% MCM stock) into sterile plastic tubes. For example, for 50 mL of final solution, pour 21.5 mL 2% MCM stock into 50-mL tubes (*see* **Table 2**).
5. Mix the solution by inverting the tubes before freezing. The aliquots must be mixed at least twice by inversion during freezing (every 5 min for 15 min); otherwise the MC will sediment at the bottom of the tubes. Upon freezing, MCM becomes a transparent and viscous medium.
6. Store the aliquots at –20°C until use.
7. At the time of use, thaw the 2% MCM stock and dilute it with an equal volume of freshly prepared 1x IMDM (**Table 1**). To prepare complete 1% MCM, use the amounts indicated in **Table 2** (*see* **Notes 9** and **10**).
8. Thoroughly mix this complete medium for 5 min until a homogeneous solution is formed *(7)*.
9. Add ES cells (prepared as described in **Subheading 3.3.1.**, **step 1**) to the complete 1% MCM. Mix carefully by inversion for 5 min to ensure single-cell suspension and dispense into 90-mm bacterial-grade Petri dishes to avoid attachment (*see* **Notes 11** and **12**). Never pipet medium containing MC. Owing to its viscosity, it adheres to the inner surface of the pipet, resulting in EB yield losses.

3.3.4. Complete 1% MCM Exchange (***Note 13***)

1. Replace the complete 1% MCM twice weekly (e.g., on Mondays and Fridays) to avoid medium exhaustion.
2. To wash the complete 1% MCM out, use a 10-mL pipet that should not be filled with more than 5 mL complete 1% MCM at a time. Always pipet slowly so that EBs and medium do not adhere to the walls of the pipet.
3. Put the recovered medium in a 50-mL tube.
4. After removing all the complete 1% MCM, wash the plate at least twice with the whole pipet volume using IMDM without changing the pipet and add the washing medium to the 50-mL tube. This procedure avoids losses due to adherence of the EBs to the pipet.

Table 2
Components of Complete 1% MCM[a]

	20 mL	50 mL	80 mL	Final concentration
2% MCM stock	8.5 mL	21.5 mL	34 mL	–
Fresh IMDM	8.5 mL	21.5 mL	34 mL	–
Heat-inactivated FCS	3 mL	7.5 mL	12 mL	15%
Diluted 1:100 MTG	75 µL	187.5 µL	300 µL	450 µ*M*
2 mg/mL Insulin	100 µL	250 µL	400 µL	10 µg/mL
30 mg/mL Transferrin	200 µL	500 µL	800 µL	300 µg/mL

[a]All ingredients—except for MCM and FCS—have to be prepared fresh. MTG, Monothioglycerol.

5. The mixture of complete 1% MCM and high amounts of medium is centrifuged at 150–180*g*, 4°C for 10 min.
6. Remove the excess medium and leave the pellet in a maximum of 500-µL medium.
7. Add the recovered pellet to a tube containing complete 1% MCM.
8. Mix carefully for 5 min, avoiding bubble formation.
9. Pour (do not pipet!) into 90-mm bacterial-grade Petri dishes to avoid attachment.

3.4. Embryoid Body Dissociation

1. Recover the EBs as described in **steps 1–7** of **Subheading 3.3.4.**
2. Put the pellet in a 1.5-mL sterile Eppendorf tube. Total volume should not be greater than 50 µL. If the pellet volume is greater than 50 µL, make smaller aliquots before adding the collagenase solution.
3. Add 1 mL of 0.25% collagenase (Sigma) supplemented with 20% FCS in PBS previously heated to 37°C to dissociate EBs (**Note 14**).
4. Mix the solution using a 100–200-µL pipet every 15 min for 1 h at 37°C (***8***,***9***; *see* **Notes 15** and **16**). Ideally, this procedure is done with gentle rotation.
5. Wash the cells gently with PBS.
6. Replate the cells as described in **steps 7–9** of the previous subheading.

3.5. Summary of ES Cell Differentiation Protocols

Table 3 summarizes the ES cell differentiation protocols.

3.6. Examples

3.6.1. Hematopoietic Differentiation

The MCM culture method described above is classically used for the study of hematopoietic differentiation in EBs based on the pioneer work of several groups ***(9***,***11***,***39)***. After EB differentiation (ranging from 5 to 10 d), EBs are disrupted, and the cells are replated in complete 1% MCM enriched with ap-

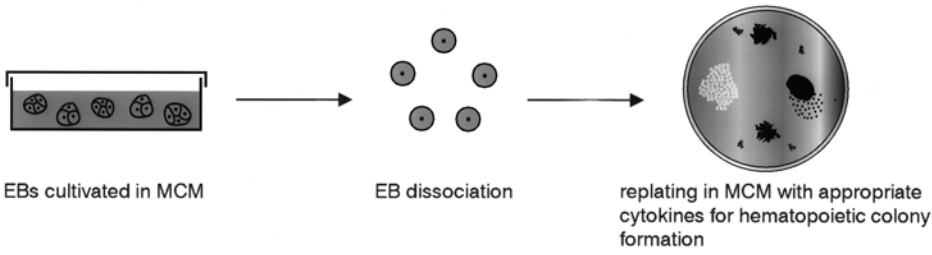

Fig. 2. Two-step hematopoietic progenitor assay: this two-step assay consists of the formation of EBs in complete 1% MCM, dissociation of the EBs, and replating in complete 1% MCM with the adequate cytokines (**Table 3**; *see* **Note 17**).

propriate cytokines to perform colony formation assays (**Fig. 2**, **Table 3**). This two-step method is especially interesting because one can score the number of colonies formed, observe their morphology, and follow their differentiation. Alternatively, gene expression and gene expression modulators can be studied during the differentiation period of the EBs. This method is important, e.g., to gain insights about the GATA-1 and GATA-2 transcription factors, which are central for hematopoietic differentiation ***(11,40)***.

A recent report ***(41)*** comparing the efficiency of EB formation and differentiation of the haematopoietic system showed no differences between the three methods currently used to form EBs (suspension, hanging drop, or MC). Interestingly, regardless of being generated from single-cells in MC, collision in suspension or from a defined number of cells, such as in hanging drops, EBs grew to a similar maximum cell number (approx 3×10^4 cells/EB). This study also reinforced the importance of a 3D system for the ES cell differentiation because hematopoietic differentiation was significantly impaired in two-dimensional (2D)-attached cultures. A dramatic reduction was observed in hematopoietic development when EBs formed in liquid suspension were left to attach in the first 4 d of culture, which means that hematopoietic commitment requires signals present in 3D cultures that are lost or absent in 2D cultures. Also of note is the fact that EBs are efficiently oxygenated ***(42)***.

3.6.2. Neural Induction

Differentiation of EBs toward neuronal fates can be achieved either by growth factor or retinoic acid (RA) stimulation. Growth factor promoted neural induction was first developed by Okabe et al ***(31)*** and modified by others ***(28,29,32,43)***. This protocol includes: (1) differentiation of EBs into all three germ layers in suspension during 4 d; (2) EB attachment and promotion selection of neuroectodermal differentiation selection through growth factor removal (serum-free medium): (3) proliferation and maintenance of neural pre-

Table 3
ES Cell Differentiation Protocols

Cell or tissue type	ES cell lines	Protocols (differentiation days: EB formation culture; number of cells)	Growth factors and others	Ref.
Hematopoietic precursors	CCEG2, D3	6, 10, or 14 d: (0.9–1.5 × 10^3 c/mL of CCEG2) MCM or (1–2 × 10^3 c/mL of D3) MCM, EB dissociation: colonies scored after 7–8 d (=MCM–two step assay)	2 U/mL EPO, 2 U/mL IL-3, 10^3 U/mL IL-1α; EB dissociation: 0.25% collagenase 1 h/37°C in PBS–20% FCS	***(9,10)***
Erythroid primitive precursors	CCE	6 d: MCM; two-step assay as in **refs. *7*** and ***27***	2 U/mL EPO	***(11)***
Erythroid definitive precursors	CCE	6, 10, or 14d: MCM; two step assay as ***(9, 10)*** SCF (250 ng/mL)	2 U/mL EPO,	***(11)***
Erythroid definitive precursors, macrophages and mixed colonies	CCE	6, 10, or 14,d: MCM; two step assay as in **refs. *7*** and ***27*** 10^3 U/mL IL-1, 10^2 U/mL IL-3, 10^3 U/mL) G-CSF, 15 U/mL GM-CSF, 100 U/mL M-CSF	2 U/mL EPO, 250 ng/mL SCF,	***(11)***
Lymphoid precursors	BL/6 III	Up to 20 d: 2–3 × 10^3 c/mL MCM or (1.5–2.5 × 10^3 c/mL) suspension; dissociation (0.8 U/mg) 4 h/4°C	7.5% CO_2/5% O_2 Dissociation: 0.1 U/mg collagenase/dispase	***(12)***

	BL/6-III/D3	d 1–15: 15–20 c/20 µL HD or suspension ($0.8–1.4 \times 10^4$/mL), dissociation as in **ref. *9***	7.5% CO_2/5% O_2	***(13)***
Endothelial cells	CCE, CJ7	Up to 11 d: (1.25×10^3 c/mL or 2.5×10^3 c/mL) MCM bFGF, 10 g/mL IL-6	50 ng/mL VEGF, 2 U/mL EPO, 100 ng/mL	***(14)***
	W4	d 1–2: 400 c/30 µL HD; d 3–5: suspension	20% FCS in culture medium, 1 ng/mL TGF-β1	***(15)***
Cardiomyocytes	D3	d 1–2: 400 c/20 µL HD; d 3–7: suspension, TC		***(16,17)***
	D3	d 1–2: 400 c/20 µL HD; d 3–7: suspension, TC 10^{-7} *M* RA	15% Dextran-coated charcoal FCS; d 5–7:	***(18)***
Adipocytes	ZIN 40, E14TG2a, CGR8	d 1–2: 10^3 c/20 µL HD; d 3–5: suspension + additives; d 6–7: suspension w/o additives, TC insulin, 2 n*M* triiodothyronine	3 d suspension with 10^{-8} *M* RA; TC medium with 85 n*M*	***(19)***
Skeletal muscle	BLC6	d 1–2: 800 c/20 µL HD; d 3–5: suspension, TC charcoal	FCS treated with Dextran-coated	***(20)***
	D3	d 1–2: 400 c/20 µL HD; d 3–7: suspension, TC	15% Dextran-coated charcoal FCS; d 2–5: 10^{+8} *M* RA	***(18)***
Vascular smooth muscle	D 3	d 1–2: 800 c/20 µL HD; d 3–7: suspension, TC and 0.5×10^{-3} *M* db-cAMP	7 d–11: 10–8 *M* RA	***(21)***

continued

Table 3 (*Continued*)
ES Cell Differentiation Protocols

Cell or tissue type	ES cell lines	Protocols (differentiation days: EB formation culture; number of cells)	Growth factors and others	Ref.
Neuronal and glial cells	BLC6	d 1–2: 400 c/20 µL HD; d 3–4: suspension, TC	HD with 10–7M RA	***(22–24)***
	CRG8	d 1–2: (5×10^5 c/10 cm dish) suspension, TC	d 1–2: 1 µ*M* RA	***(25)***
	D3	d 1–4: suspension; d 5–8: suspension + RA; TC or dissociation	d 5–8: 0.5 µ*M* RA	***(26,27)***
	J1	d 1–4: suspension; d 5–12: d ITSF medium	ITSF medium	***(28)***
	R1	d 1–2: (100, 200, 400 c/drop); d 3–4: suspension M1; d 5:attachment; d 6–12: TC; EB dissociation; d 13–18: TC M2; d 18–48: M3	TC: M1; 6 d: dissociation 0.1% trypsin/0.08% EDTA, 1 min; replating: M2; M3	***(29)***
	R1 and MPI-II	d1-4: (1.5×10^6 c/10 cm) suspension; d 5–13: TC	Suspension:1 µ*M* RA and 1% FCS; TC with 1% FCS	***(30)***
	J1, CJ7, D3, R1	d 1–4: suspension, 24 h TC; d 6–8 ITSF medium; EB dissociation d 9–15: ($0.5–2 \times 10^5$ c/cm^2) M4;	Dissociation with 0.05% trypsin/0.04% EDTA; M4	***(31,32)***

		d 16–20: M4 w/o bFGF; long-term neurobasal medium + B27 suplement + 5% FCS		
Osteoblasts	CGR8	d 1–2: 10^3 c/20 µL HD; d 3–5: suspension + additives, TC: M5; matrix mineralization increased by M5+	Additives: 10^{-7} *M* RA; TC: M5; M5+: 10 ng/mL BMP-2 or 2.5 µ*M* compactin	***(33)***
Chondrocytes	D3	d 1–2: 800 c/20 µL HD; d 3–5: suspension with BMP-2, TC	2 ng/mL BMP-2 during suspension	***(34)***
Mast cells	W9.5	d 1–7: 2×10^3 c/1.5 mL MCM; d 8–14: 2×10^3 c/2.5 mL MCM; suspension: M7	d 1–7: 50 ng/mL SCF; 5 ng/mL IL-11, d 8–14: +1 mL M6; suspension: M7	***(35)***
Dendritic cells	ESF116	d 1–14: suspension (4×10^5 c/90-mm dish), TC	TC with 25 ng/mL GM-CSF and 10^3 U IL-3/mL	***(36)***
Mature hepatocytes	R1, SEK1, W9.5	d1–2: 10^3 c/30 µL HD; d 3–5: suspension; d 5–18: TC (collagen coated dishes)	d 9–12: 100 ng/mL aFGF; d 13–18: 20 ng/mL HGF; d 15–18: M8	***(37)***
Pancreatic islets	E14.1, B5	d 1–4: suspension; d 5–10/11: TC-ITSF; d 12–18: M9; d 18–24: M10	M9; M10	***(38)***

Abbreviations c, cell(s); HD, hanging drop; TC, tissue culture; MCM, 0.9–1% MC medium; w/o, without; EPO, erythropoietin; IL, interleukin; SCF, stem cell factor; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte macrophage colony stimulating factor; M-CSF, macrophage colony-stimulating factor; VEGF, vascular endothelial growth factor; RA all *trans* retinoic acid; db-cAMP, dibutyryl-cyclic adenosine monophosphate; bFGF, basic fibroblast growth factor; BMP-2, bone morphogenetic-2, aFGF, acidic fibroblast growth factor; HGF, hepatocyte growth factor; ITSF, DMEM/F12 (1:1), 5 µg/mL insulin, 50 µg/mL transferrin, 30 n*M* selenium chloride, 5 µg/mL fibronectin.

M1: DMEM/F12 (1:1), 5 µg/mL insulin, 50 µg/mL transferrin, 30 n*M* sodium selenite, 5 µg/mL fibronectin.

M2: DMEM/F12, 20 ng progesterone, 100 µ*M* putrescine, 1 µg/mL laminin 25 µg/mL insulin, 50 µg/mL transferrin, 30 n*M* sodium selenite, 10 ng/mL bFGF, and 20 ng/mL epidermal growth factor in polyornithine/laminin coated dishes.

Table 3 (*Continued*)
ES Cell Differentiation Protocols

Cell or tissue type	ES cell lines	Protocols (differentiation days: EB formation culture; number of cells)	Growth factors and others	Ref.

M3: Neurobasal medium plus 2% B27, 200 pg/mL IL-1β (added daily), 700 µmol db-cAMP (every 4 d starting d 18), 2 ng/mL TGF-β_3 (start d 25), 2 ng/mL glial cell line-derived neurotrophic factor (start d 22), 10ng/mL Neurturin (start d 25). Medium change together with growth factor took place every 3 d.

M4: DMEM/F12, 20 ng progesterone, 100 µ*M* putrescine, 1 µg/mL laminin, 25 µg/mL insulin, 50 µg/mL transferrin, 30 n*M* selenium chloride, 5 ng/mL bFGF in polyornithine/laminin-coated dishes (coated with 15 µg/mL polyornithine, 1 µg/mL laminin).

M5: Glasgow MEM/BHK12 medium enriched with 50 µg/mL ascorbic acid phosphate, 10 m*M* β-glicerophosphate.

M5+: M5 medium enriched with 10 ng/mL BMP-2 or 2.5 µ*M* compactin.

M6: Complete 1% MCM with 60 ng/mL SCF; 30 ng/mL IL-3; 30 ng/mL IL-6.

M7: DMEM 20%v/v WEHI-3 conditioned medium, 50 ng/mL SCF.

M8: DMEM + 10 ng/mL oncostatin M, 10^{-7} *M* dexamethasone, 5 mg/mL insulin and transferrin, 5 µg/mL selenious acid.

M9: N2 medium + B27 supplement (Gibco-BRL) and 10 ng/mL bFGF.

M10: N2 medium + B27 supplement (Gibco-BRL) + 10 m*M* nicotinamide.

cursor cells through addition of basic fibroblast growth factor (bFGF); and (4) induction of neuronal and glial maturation by bFGF withdrawal and addition of neuronal differentiation and survival-promoting factors (**Fig. 3**; **Table 3**). Functional neurons and glia obtained through this method differentiated into dopaminergic and serotonergic neurons and participated in brain development in vivo. Other reports pointed out the possibility of using these neurons as a possible source of myelinating transplants ***(29,32)***.

RA is also known to strongly induce neuronal differentiation ***(44)***. RA-promoted neural differentiation is time- and concentration-dependent (**Table 3**). Two major protocols have been used: one was originally developed by Bain et al. ***(26)*** and includes an 8-d induction period in which RA induction ($5 \times 10–7$ *M*) takes place from d 5 to d 8 (also called 4–/4+ protocol). Neural cells obtained through this method were also able to myelinate axons in host animals and promote recovery after spinal cord injury ***(45,46)***. In the second protocol, EBs (usually cultivated as hanging drops) are cultured with lower RA concentrations (10–7 *M*) during the first 2 d of differentiation. RA is then washed out, and EBs continue their differentiation for two further days in suspension ***(23,24,48)***.

Neuronal differentiation from EBs is one of the most successful types of ES cell differentiation in terms of in vivo application. Both RA and growth factor induction are able to give rise to neuronal cells which are capable of restoring functional losses of the central nervous system.

4. Notes

1. An important factor to be observed in EB culture is the choice of FCS because concentrations of growth factors, such as BMP-4 and TGF-β that influence differentiation may vary from batch to batch ***(48)***. To circumvent this problem, use the same batch of high-quality serum (special ES cell sera are now commercially available) for all the experiments performed. An even better solution is to culture in serum-free media in combination with known amounts of growth factors, a combination tried successfully by Johansson and Wiles in suspension culture ***(48)***. Adelman et al. wished to develop of serum-free MCM. They tested two sources of commercially available serum substitutes—Knockout SR (Life Technologies) and BIT 9500 (Stem Cell Technologies; ***49***). They found that although EB formation efficiency, size, and morphology were comparable between serum containing media and the knockout SR media (but not the BIT 9500) EBs did not express committed erythroid markers, EKLF and GATA-1, or terminally differentiated globin markers under serum-free conditions. Addition of BMP-4 (5 ng/mL from R&D Systems) to the medium recovered the expression of EKLF and GATA-1. In addition to testing different types of MCM serum-free conditions, this study demonstrated that EB differentiation can be manipulated to study induction of specific genes expressed early within a lineage ***(49)***.
2. Conditions for serum heat inactivation: 57°C for 30 min.

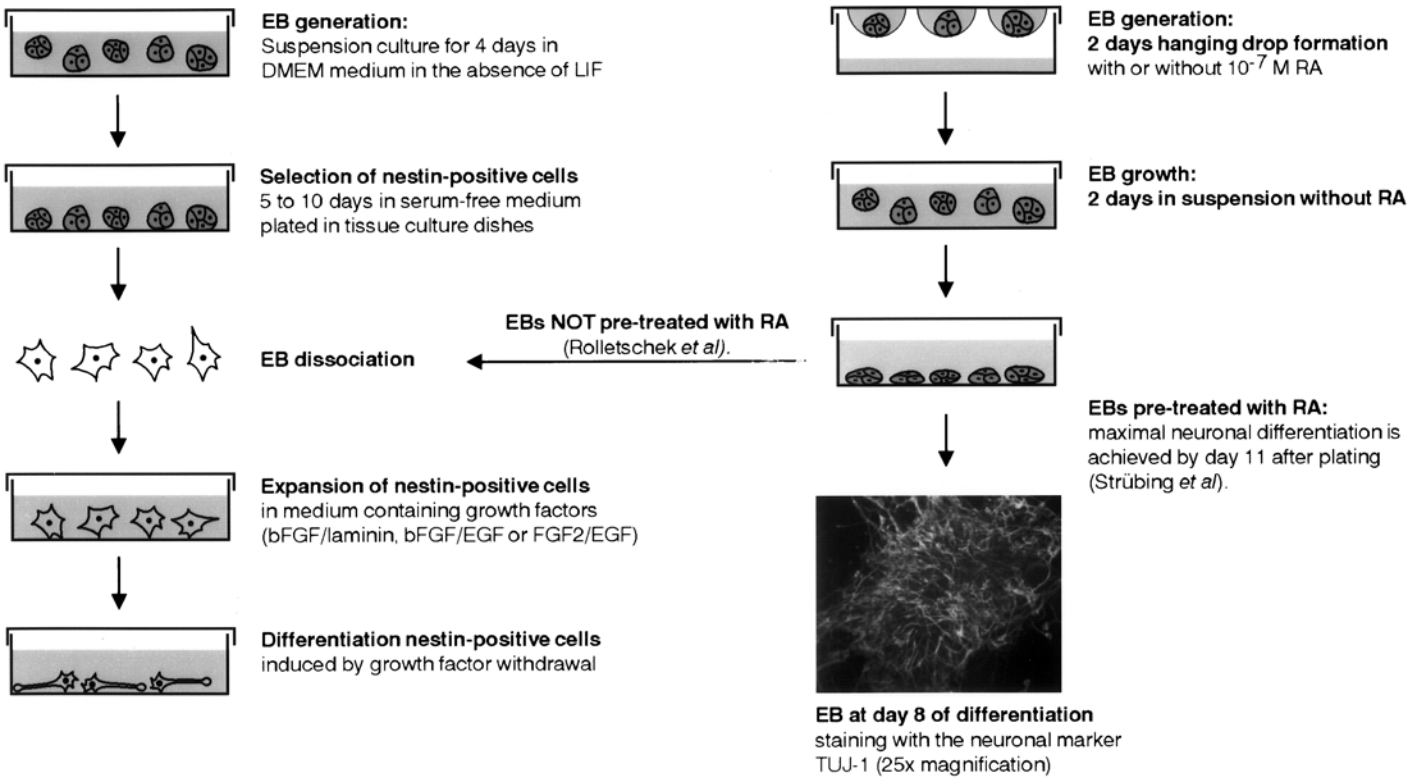

Fig. 3. Neuronal differentiation can be achieved using different protocols: The differentiation protocol developed by Okabe et al. ***(31)*** and modified by others ***(32,43)*** is shown on the left hand side. A similar protocol was used recently to obtain transplantable neural precursors from human embryonic stem cells ***(53)***. On the right side, the protocol of Rolletschek et al. ***(29)***, which is also a modification from former protocols, and the alternative from Strübing et al. ***(22)*** using RA-induced neuronal differentiation. The picture shown on the bottom right was taken in our laboratory using this technique.

3. LIF is diluted in medium without 2-mercaptoethanol and kept at –20°C in 500-µL aliquots. Aliquots are thawed only once and kept at 4°C.
4. Each type of EB culture has advantages and disadvantages. The MCM method induces the formation of a microenvironment surrounding the aggregates through local accumulation of factors. Furthermore, this culture method favors the formation of synchronized clonal aggregates because EB collision is reduced as a result of the viscosity of the medium. Additionally reproducibility of temporal development occurs more synchronously than in suspension. A disadvantage is that it is technically more difficult than the other methods. The suspension method is technically the easiest to perform, but has the disadvantage of forming EBs of irregular sizes owing to increased collision that which lead to reduced efficiency in EB formation with higher numbers of cells. Technically speaking, the hanging drop method stands, between the suspension and the MCM in terms of difficulty. The EBs obtained have a much more regular size and are formed from a defined number of cells, which may be an advantage when compared to the suspension methods.
5. We always trypsinize and replate the ES cells the day before the experiment, regardless of the type of EB culture used. In this way, it is easier to obtain a single-cell suspension on the experimental day. Also, if this precaution is taken the EB yield is increased and the experimental conditions normalized.
6. Dang et al. ***(41)*** showed, using the CCE ES cell line, that EB forming efficiency remained constant in cultures initiated with 10^3 ES cells/mL, whereas it gradually decreased in cultures initiated with 10^4 and 10^5 ES cells/mL, but this effect was less pronounced when MC medium was used.
7. As an alternative to using the lids as a pipetting surface, one can actually use the bottom of the plates (140 Y 20-mm Nalge Nunc International), and turn the plate up-side down. To prevent the hanging drops from drying out, we put the lid of a 10-cm tissue culture Petri dish filled with 15-mL PBS inside the plate. There are two advantages with this method: first, there is less danger of contamination when compared with the pipetting on the lid itself, and, second, if suspension culture follows the hanging drop, simply fill the plate with the culture medium. Avoid culturing the hanging drops for more than 1–2 d, because the drops will dry and the medium will be exhausted.
8. Differentiating agents, such as RA (*see* **Table 3**) cause apoptosis in several cells in addition to their differentiating effects. To obtain a high EB yield, start with large numbers of drops, which is best achieved with a multichannel pipet.
9. Insulin is water-soluble only at a low pH. Add one drop of 10 *M* HCl to the solution before adjusting the volume. Always prepare fresh.
10. Best mixing results are obtained if MCM, IMDM, and FCS are preheated to 37°C.
11. To circumvent plating efficiency problems in MC medium, the ES cell line SQ1.2S8 was grown in suspension for 48 h in IMDM supplemented with 10% FCS and 0.45 m*M* of monothioglycerol prior to MCM culture ***(10)***. Plating efficiency could also be further increased by culturing EBs under low oxygen conditions ***(7)***.

12. The seeding density of ES cells depends on their plating efficiency, but also on the differentiation day the growing EBs are required to reach. ES cells used in our laboratory (CCE, HM-1 and R-1) have a good plating efficiency under the conditions described above, when seeded directly into MCM. The number of cells used for MCM differentiation are given in **Table 3**. This number also depends on the duration of the experiment. For example, for a 16-d experiment using the HM-1 cell line where RNA extraction was required, we plated 5×10^3 cells/mL (20 and 10 plates, respectively) for d 2 and 4 of differentiation, 10^3 cells/mL (6–7 plates/d) for d 5 and 6 of differentiation, and 500 cells/mL (5 plates/d) from d 7 to 16. The total volume in each plate was always 10 mL/90 mm plates ***(50)***.
13. It is quite difficult to exchange complete 1% MCM due to its viscosity. For this reason, and to improve the experimental outcome, the exchange of this medium is described in detail.
14. Usually, the less EBs in the solution, the better the outcome tends to be. The amount is difficult to define because the size and amount of the EBs vary in the different differentiation days in which this procedure can be applied.
15. EB dissociation is not always easy. Avoid being harsh to the cells to prevent decreased plating efficiency. Gentle agitation and mechanical shearing (100-µL pipet tip and/or a 21-gauge needle) during the incubation period improves the outcome. Usually, a step with a cell strainer (e.g., 100 µm, Falcon Becton Dickinson Labware) at the end of the procedure is included to reduce the amount of cell debris or nondissociated EBs.
16. Alternatives are incubation with cell dissociation buffer (Gibco-BRL) for 30 min at 37°C ***(51)***, trypsin/EDTA, or a mixture of collagenase/dispase (*see* **Table 3**).
17. This assay can also be used to analyze cells obtained from yolk sac dissociation ***(52)***.

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6

The Derivation of Murine Embryonic Stem and Embryonic Germ Cells by Selective Ablation

Edward J. Gallagher and Jim McWhir

1. Introduction

Embryonic stem (ES) cells have the developmental capability to contribute to every cell type of the adult mouse when reintroduced into the blastocyst embryo ***(1,2)***. They can be easily manipulated in vitro to produce specific genetic alterations for the study of gene function and the biological basis of the diseased state. More recently, human ES ***(3–5)*** and embryonic germ ***(6)*** (EG) cells have been isolated; these cells have the potential to provide new therapeutic treatments in a broad range of diseases ***(7)***. The methods used for human ES isolation are similar to those used for mouse ES derivation ***(8)***. Although ES-like cells have been reported in rabbit ***(9)***, pig ***(10)***, cattle ***(11)***, rhesus monkey ***(12)***, and human ***(3–5)***, as yet, only a few strains of mice (predominantly, strain 129 and C57BL/6) ***(13)*** have successfully produced totipotent ES cells capable of colonizing the germline. There is an apparent genetic variation even among mouse strains to the relative ease with which ES cells can be isolated ***(14)***.

We have shown that it is possible to isolate ES cell lines from the CBA mouse strain, which is otherwise refractory to ES isolation by traditional means ***(15)***. Transgenic mice were generated, which expressed the selectable neomycin phosphotransferase (*neo*) gene under the transcriptional control of a 1.9-kb proximal *Oct3/4* promoter that is active only in the ES and EG cells and their precursors ***(16)***. Differentiating cells in an explanted embryo downregulate

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expression of the *oct/neo* transgene, rendering them susceptible to the neomycin analogue G418. Using this selective ablation approach, 87.5% and 94% CBA ES lines could be generated with similar frequency to ES lines from the permissive 129 strain. We concluded that the removal of differentiating lineages relaxes inhibition of the stem cell population, permitting the isolation of ES cells from an otherwise nonpermissive genotype, (*see* **Fig. 1** for a schematic overview). Gardner and colleagues have shown that CBA ES lines can be isolated following microdissection to remove ES progenitors from the influence of other embryonic lineages ***(14)***. The dependence of ES isolation in some genotypes on physical- or selection-mediated removal of other lineages is consistent with the view that the strain barrier arises from differences in the extent to which the embryo/explant exerts control over the proliferation of ES precursor cells.

We have extended the selective ablation approach to isolate EG cells from 94% CBA mouse embryos. EG cells are derived from the primordial germ cells of mouse embryos either during their migratory phase or from the genital ridges ***(17,18)***. Mouse EG cells are similar to ES cells in many ways: they are pluripotent; they can be propagated indefinitely in culture; they maintain a stable karyotype; and they are morphologically indistinguishable. The only apparent difference is in the expression of some of their imprinted genes. The loss of imprint may explain why the lines we derived from embryos after d 11 did not display germline competence ***(19)***. In our hands, we were unable to derive EG cells from 94% CBA embryos using conventional methods, although we could derive EG lines from strain 129 embryos. However, when we apply selection against differentiating cells (selective ablation) CBA embryos did yield EG lines.

Selective ablation can overcome the apparent strain barrier that exists for ES isolation between mouse strains. The next challenge is to adapt this method for the derivation of ES lines from other species, presuming the barriers to ES isolation are similar.

2. Materials

2.1. Isolation of ES Cells by Selective Ablation

All procedures were possible are performed under sterile conditions in a laminar flow hood. This is not possible when collecting and dissecting embryos. To minimize the risk of contamination, we supplement our media with antibiotics (penicillin and streptomycin). Established cell lines do not need to be maintained in the medium containing antibiotics. We further reduce the contamination risk by dissecting the embryos under a binocular microscope placed within a still air hood. The hood is a purpose-built Perspex box around

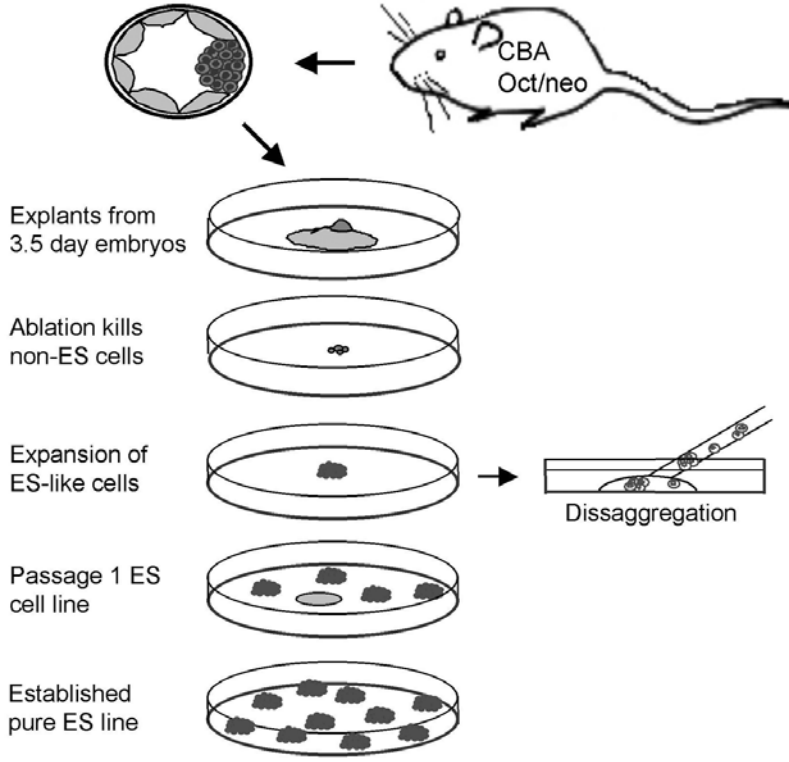


Fig. 1. ES Isolation by selective ablation. Step 1: blastocyst stage embryos were collected and explanted onto inactivated STO feeder cells in the presence of ES isolation media supplemented with G418. Step 2: over a period of 2–3 wk of selection, the non-ES cells are removed from the explant, resulting in the formation of a primary ES-like colony. Step 3: the primary ES colony is carefully removed and disaggregated in the presence of trypsin and transferred to fresh feeders. Step 4: after a further 7–10 d of selection, numerous ES colonies are present with a limited amount of differentiating cell types. Step 5: the ES-like colonies are trypsinized and passaged until a "pure" ES-like cell line is established, typically 5–6wk for the complete procedure.

the microscope with approx 40 cm of working space either side; only the eye pieces protrude from the hood.

2.2. Maintenance of ES Cells

1. ES medium: Glasgow's minimum essential medium (GMEM; Sigma) supplemented with 5% newborn calf serum (NCS) and 5% FCS (FCS) (Globepharm), 0.1 m*M* non-essential amino acids (Life technologies), 0.1 m*M* β-mercaptoethanol, 1 m*M* sodium pyruvate (Life Technologies), 2 m*M* L-glutamine (Life

Technologies), and 500 U/mL recombinant murine leukemia inhibitory factor (LIF; Life technologies). ES isolation media consisted of ES maintenance media supplemented with FCS to a final concentration of 10% and 50–200 μg/mL G-418 sulfate (geneticin). EG isolation medium differs from ES isolation medium in that it also contains basic fibroblast growth factor (bFGF) to final concentration of 20 ng/mL. Penicillin–streptomycin is added to the media at 1X concentration when isolating primary lines, (100X stock consists of 10,000 U/mL penicillin G sodium and 10,000 μg/mL streptomycin sulfate in 0.85% saline).

2. 1X PBS: 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, and 0.24 g KH_2PO_4 in 800 mL double-distilled water and adjusting the pH to 7.4 with HCl. Make the volume to 1 L with double-distilled water and autoclave.
3. TEG: 6.3 g NaCl, 0.12 g Na_2HPO_4, 0.216 g KH_2PO_4, 0.333 g KCl, 0.9 g D-glucose, 2.7 g Tris, and 0.9 mL 1% phenol red dissolved in 800 mL Analar water. Then add 100 mL 2.5 % trypsin, 0.4 g EGTA, and 0.1 g polyvinyl alcohol. Adjust pH to 7.6 and make 1 L. Filter through a sterile 0.2 μm filter, aliquot, and store at –20°C.
4. Bench-top centrifuge.
5. Inverted phase contrast microscope.
6. Tissue culture flasks (25 cm^2, 75 cm^2, and 175 cm^2), 6-well and 24-well plates.
7. Sterile 15-mL Falcon tubes and 2-mL cryopreservation vials.

2.3. Generation of Transgenic ES Lines

The *oct/neo* transgene and the plasmid DNA (pOctneo1) can be obtained from Dr. Jim McWhir of the Roslin Institute, Roslin, Midlothian, EH25 9PS, UK.

2.4. Preparation of STO Feeder Layers

1. STO fibroblasts (American Type Culture Collection).
2. DMEM supplemented with 10% NCS.
3. Prepare 50X stock of mitomycin C by adding 4 mL of 1X PBS to 2 mg mitomycin C. Store in the dark at 4°C for no longer than 1 mo.

2.5. Collection of Murine Blastocyst Embryos

1. Transgenic *oct/neo* female mice naturally mated to provide d 3.5 embryos.
2. Dissecting microscope.
3. Dissecting equipment: scissors and blunt forceps.
4. Hypodermic needle (25-gauge) and 2-mL syringes.
5. ES Isolation medium supplemented with HEPES buffer to a final concentration of 20 m*M*.
6. 6 cm Petri dishes.
7. Embryo-grade mineral oil.
8. Dissecting microscope.
9. Pulled Pasteur pipet with connected rubber tubing to allow mouth pipetting.

2.6. Selective Ablation of Mouse Explants

1. Transgenic *oct/neo* blastocyst embryos.
2. G-418 sulfate for ES isolation medium.
3. 24-well plates preseeded with inactivated STO fibroblasts.
4. ES media (*see* **Subheading 2.2.**).

2.7. Freezing and Storage of ES Cells

Freezing medium: ES medium supplemented with 10% dimethyl sulfoxide (DMSO).

2.8. Preparation of m220 Feeder Cell Layers

The materials required are detailed in **Subheading 2.4.** with the exception of the cells; Sl^4-m220 can be obtained from Dr. David Williams of the Howard Hughes Medical Institute, Indiana University Medical School.

2.9. Collection of PGCs for EG Isolation

1. Transgenic *oct/neo* female mice naturally mated to provide d 8.5 embryos and d 12.5 embryos.
2. Dissecting microscope.
3. Dissecting equipment: scissors, blunt fine forceps, no. 5.
4. EG isolation medium supplemented with HEPES buffer to a final concentration of 20 m*M*.
5. 6-cm Petri dishes.
6. Dissecting microscope.

3. Methods

3.1. Isolation of ES Cells by Selective Ablation, a General Introduction

The traditional method of mouse ES cell isolation routinely generates ES cell lines at 10–30% efficiency from the 129 mouse strain ***(8)***. The selective ablation procedure has a comparable frequency of ES isolation from the CBA strain ***(15,19)***. Overall selective ablation requires less expertise at the level of embryo culture as the critical timing decision of inner cell mass explant dissociation is avoided.

3.2. Maintenance of ES Cells

ES cells are cultured on plastic tissue culture flasks that have been pretreated with gelatin in standard ES medium (*see* **Note 1**) that is normally changed daily. Cells are passaged every 2–3 d at a subculture density of 1:6 or 1:12.

1. A confluent or nearly confluent flask of cells is washed twice with 1X PBS
2. To the washed cells, add trypsin–EGTA (TEG) solution to dissociate the cells for approx 2 min (*see* **Note 2**).

3. Stop the reaction by adding medium containing serum (ES medium), and pellet the cells by spinning at 200*g* for 5 min.
4. Resuspend in ES medium and seed onto freshly gelatin-coated flasks. A 0.1% gelatin solution is applied to flasks at room temperature for a minimum of 5 min; prior to their use, remove the gelatin.

3.3. Generation of Transgenic ES Lines

1. Generate transgenic mice by pronuclear microinjection of the *oct/neo* transgene (*see* **Fig. 2**) pOctneo1 DNA one-cell embryos by standard methods ***(20)***. We used F1 embryos from CBA mice crossed to C57BL/6. The transgenic *oct/neo* line 72–49 ***(15)*** was further backcrossed with the CBA mouse strain to produce 94% CBA, 6% C57BL/6 mice hemizygous, and homozygous for the *oct/neo* transgene.

3.4. Preparation of STO Feeder Layers

STO fibroblasts are mitotically inactivated with mitomycin C for use as feeder layers during the isolation of ES cells. The inactivated STO cells are plated prior to the day ofuse.

1. Culture STO fibroblasts to near confluence in Dulbecco's modified Eagle's medium (DMEM) medium supplemented with 10% NCS, *see* **Note 3**.
2. Replace the growth medium with fresh medium containing 1X mitomycin C stock and incubate for 2–3 h.
3. Harvest cells by washing 2 times with 1X phosphate-buffered saline (PBS), trypsinize with TEG, stop reaction with media, and spin down at 200*g*.
4. STO feeders can be used directly or stored in liquid nitrogen for future use (*see* **Subheading 3.7.**).
5. Seed cells at a density to produce a subconfluent monolayer. This is approx 2×10^5 cells/well of a 24-well tissue culture plate. A 175 cm^2 flask of STO cells can be frozen into 10 vials with each vial providing enough cells for a monolayer on a 24-well plate (*see* **Note 4**).

3.5. Collection of Murine Blastocyst Embryos

1. Set up natural matings of the *oct/neo* mice, cull females on d 3.5 post coitum. (The time of mating is presumed to be midnight.)
2. Remove the uterine horns and individually flush with approx 0.5 mL of isolation medium supplemented with HEPES buffer (20 m*M* final concentration) using a 25-gauge hypodermic needle clamped to the uterine horn with a pair of forceps (*see* **Note 5**).
3. Pool the contents of the flushed horns into a 6-cm Petri dish and examine at 25× magnification under a dissecting microscope.
4. With the aid of a drawn Pasteur pipet collect the embryos and transfer to a 50–100 µL microdrop culture under mineral oil.

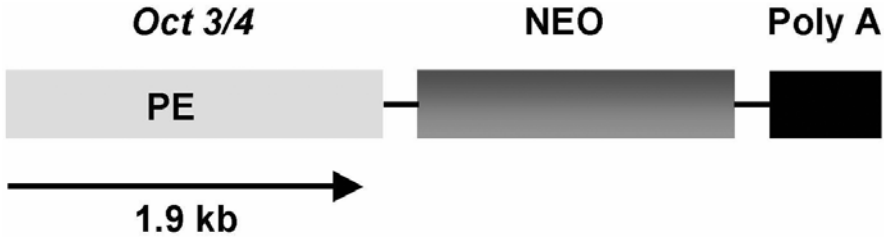


Fig. 2. The *oct/neo* transgene. A neomycin cassette driven by a 1.9 kb proximal portion of the *Oct3/4* promoter. NEO, *neomycin phosphotransferase* gene; PE, proximal element; Poly A, polyadenylation signal sequence.

5. Pass the embryos through two fresh drops of medium to wash them.

3.6. Selective Ablation of Mouse Explants

1. Transfer embryos individually into wells of a 24-well plate, preseeded with mitotically inactivated STO feeder cells.
2. Culture the explanted blastocysts in ES isolation medium with the appropriate concentration of G418. For our *oct/neo* transgenic line, G418 at a concentration of 50 µg/mL with hemizygous blastocysts and 100 µg/mL with homozygous blastocysts was most efficient (*see* **Note 6**).
3. The explants are cultured in selective media. Change the media every 3–4 d, until a primary ES explant forms, (*see* **Fig. 3**). *See* **Note 7** about additional feeders.
4. Pick primary ES explants after 14–21 d of selection. First, wash the well with 1X PBS, then add 0.5 mL of fresh 1X PBS. Pick the colony using a pulled Pasteur pipet and place in a microdrop of TEG solution. Gently break the colony into clumps and single cells, then transfer onto STO feeder layers in single wells of a 24-well plate.
5. Approximately 1 wk later, trypsinize the cells by treating with TEG solution and transfer the contents into one-well of a 6-well plate with ES isolation medium supplemented with G418, *see* **Note 7**.
6. The primary ES cell lines are expanded until about passage 5 or 6 and stored in liquid nitrogen. Remove newly derived ES lines from the isolation and selection medium to ES maintenance medium after passage 4, *see* **Note 8**.

3.7. Freezing and Storage of ES Cells

1. Cells are harvested by trypsinization with TEG for 2–3 min, ES medium added to stop digestion and centrifuged at 200*g* for 5 min.
2. The pelleted cells are resuspended in 0.5 mL aliquots of freezing medium and placed in a polysterene box that is incubated overnight at –80°C. On the following day, the cells are transferred to liquid nitrogen or –150°C refrigeration storage. From a near confluent 175 cm^2 flask of cells, make 10 0.5-mL vial freezes.

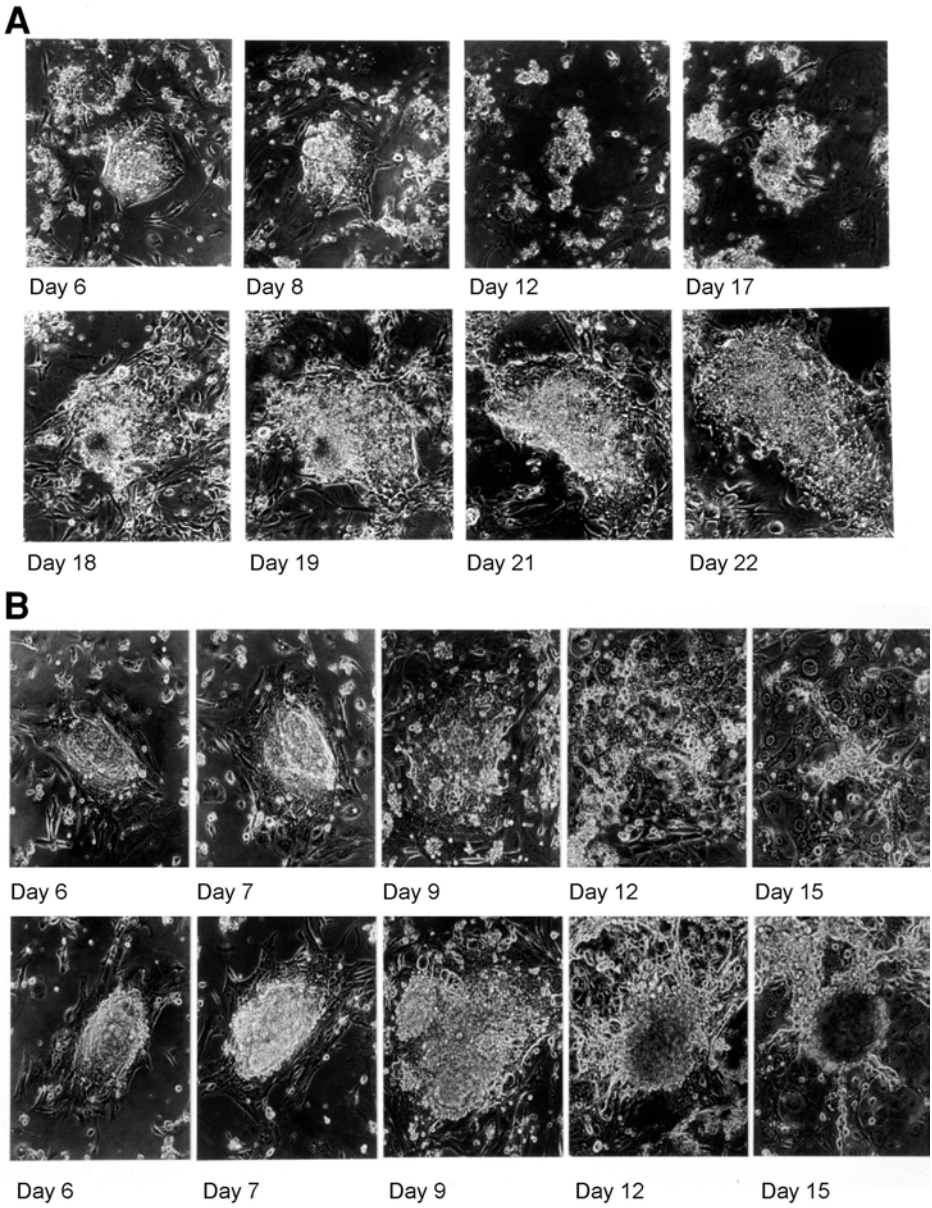


Fig. 3. The derivation of a primary ES cell colony from an explanted 94% CBA embryo expressing the *oct/neo* transgene. Panel **A** depicts the morphological changes of a single embryo explant under selective ablation conditions. There was initial growth, particularly from the ICM, up to d 6, after which the explants shrank in size as nonresistant cells were ablated by G418 action. At d 12, there appeared to be a small

3.8. Isolation of EG Cells by Selective Ablation

This EG cell isolation protocol is based on the procedures of Dr. B. Hogan and colleagues ***(20)***. The Sl^4-m220 feeder layer provided the requirement for exogenous Steel Factor, and G418 was added when isolating cells from CBA transgenic *oct/neo* embryos. The overall procedure is similar to that of ES isolation by selective ablation.

3.9. Preparation of m220 Feeder Cell Layers, General Comments

The procedure is identical to that outlined in the section preparation of STO feeders (*see* **Subheading 3.4.**).

3.10. Collection of Primordial Germ Cells (PGCs) for EG Isolation

1. Cull naturally mated females of the 94% CBA *oct/neo* line, at d 8.5, 9.5, or 12.5 postcoitum for embryo collection.
2. For d 8.5 and 9.5 embryos, dissect the posterior one-third, including the allantois *see* **Fig. 4**. Transfer the dissected tissue into a solution of 0.0025% trypsin and 0.02% EDTA in 1X PBS and incubate at 37°C for approx 10 min. Using a pulled Pasteur mouth pipet, disaggregate to a single cell suspension. Approximately 100 PGCs are present in a d 8.5 embryo.
3. For d 12.5 embryos, dissect the genital ridges and disaggregate as **step 2** above, *see* **Fig. 5**.
4. Approximately 26,000 PGCs are present in a d 12.5 embryo).

3.11. EG Cell Isolation by Selective Ablation of PGCs

1. Transfer the single cell suspension (*see* **Subheading 3.10.**) into a single-well of a 24-well plate preseeded with inactivated Sl^4-m220 cell feeder layer (each embryo was treated separately). For d 8.5, the whole embryo cell suspension was transferred to one well of a 24-well plate. For a 12.5 d embryo, split cells across four wells of a 24-well plate. Culture the cells with ES isolation medium supplemented with bFGF to final concentration 20 ng/mL *see* **Note 7**.
2. Add G418 selection to the isolation media at the required concentration *see* **Note 6**.
3. Pick primary EG colonies after 7–10 d of culture. First, wash the cells with 1X PBS, pick colony using a pulled Pasteur pipet and transfer to a microdrop of TEG

Fig. 3. (*continued*) group of ES precursor cells that were resistant to G418. Thereafter, the primary ES colony expanded until d 22 when it was picked and further expanded. It must be noted that fresh feeder cells were added between d 17 and 18. Panel **B** depicts two control embryos of the same genotype that have been cultured in the absence of G418. At d 12 and onward it was evident that terminally differentiated cells, such as giant trophoblast cells (top row) or endoderm-like cells (bottom row), were the major cell types produced.

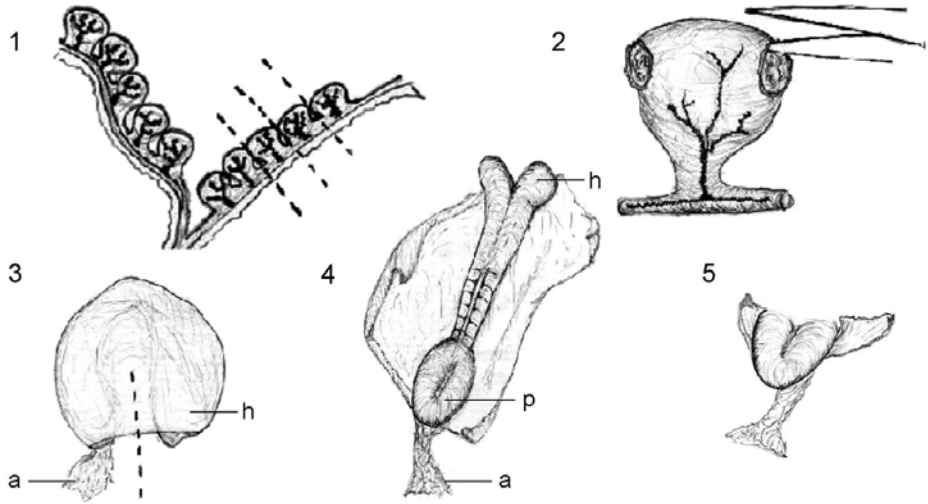
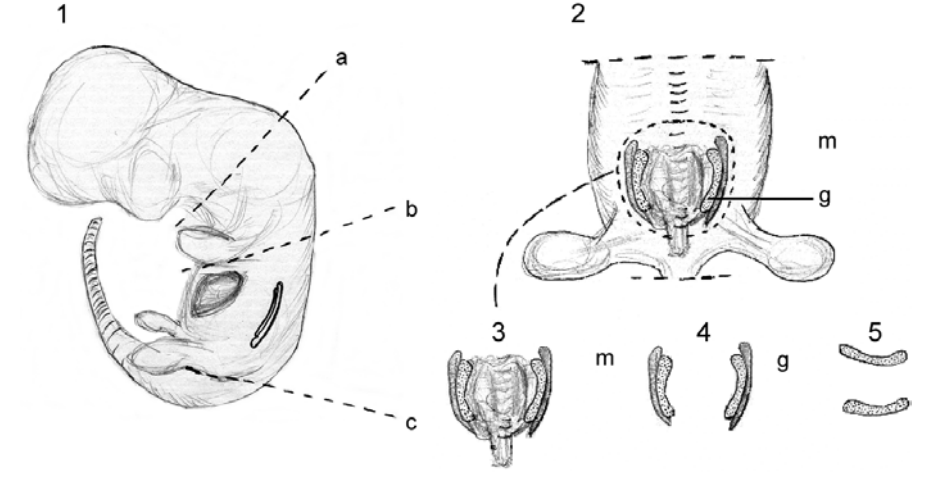


Fig. 4. The dissection of PGCs from d 8.5 embryos. Step 1: remove the intact uterus at d 8.5 of embryonic development and place in a dish of 1X PBS. Cut through the uterine wall as indicated by the dashed line to release the individual embryos. Step 2: cut through the uterine muscle layer using fine scissors and forceps to reveal the deciduum containing the embryo. Gently pull the deciduum apart with forceps and tease out the embryo. Step 3: under a dissecting microscope, orientate the embryo as shown, and cut through the amniotic membrane, taking care not to damage the embryo. Step 4: further cut the membrane to separate both ends of the embryo. Cut across the primitive streak and remove the posterior one-third of the embryo with the allantois intact. Step 5 collect the PGC containing portion of the embryo and proceed with **step 2** of **Subheading 3.10.** al, Allantois; h, head fold; p, primitive streak.



under mineral oil in a Petri dish. Disaggregate by pipetting the colony until it is reduced to small clumps and single cells; be careful not to introduce bubbles into the drop of TEG. Transfer the cell suspension onto Sl4-m220 feeders in a well of a 24-well plate.

4. Expand cell lines and store in liquid nitrogen.

4. Notes

1. The FCS used for the ES medium should be pretested on an established ES line, owing to serum batch variability. Samples of serum-batches can be obtained from most companies. The serum is tested alongside a proven serum sample for comparison. A 10% final volume of the FCS is added to the media and used to check its effect on plating density. Plate cells at low density and grow until colonies form, approx 10–12 d. Giemsa stain plates and count colonies. Only those serum batches with colony counts comparable or higher than the control are retained. It is also important to examine the morphology of the colonies; serum batches producing colonies with excessive differentiation are discarded as are those producing smaller colonies. Test the serum batches for toxicity by growing the cells in media containing 30% serum; any toxicity will produce cell death. Alternatively, some companies will supply recommended serum for ES cell medium (Life Technologies)
2. It is important not to over treat the cells with TEG therefore, monitor their progress under the microscope. Shaking and gentle tapping of the flask will help.
3. Do not grow the STO or Sl^4-m220 fibroblasts to confluence. Confluent or overconfluent cultures of cells can lead to inefficient inactivation of the cells with mitomycin C. This can cause the loss of primary ES cell lines as the "escapee" feeder cells can overgrow the ES cells.
4. Different frozen batches of feeder cells have different plating efficiencies. In our laboratory, we empirically determine the plating density of the cells by testing a vial of a freeze prior to use. We routinely seed cells to create a monolayer that is 80% or more confluent.
5. Use a blunted 24-gauge needle to prevent piercing of the uterine wall when flushing. Flushing from the wider cervix end of the uterine horn is less demanding

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Fig. 5. (*Opposite page*) The dissection of genital ridges from d 12.5 embryos. Step 1: remove day-12.5 embryos to a Petri dish containing 1X PBS. Decapitate embryos with an incision at the neck region (a), cut of forelimbs (b), and cut off the tail (c). Step 2: place the remainder of the embryo on its back under a dissection microscope, cut through the abdominal cavity, and remove the intestines and other organs to expose the genital ridges. Remove the genital ridges that are closely associated with the mesonepheros and developing kidneys. Step 3: detach the genital ridges and mesonepheros from the associated tissues. Step 4: carefully remove the mesonepheros, leaving the intact genital ridges. Step 5: proceed with disaggregation of the genital ridges as outlined in **step 2** of **Subheading 3.10.** g, genital ridge; m, mesonepheros.

than using a smaller bore needle to flush from the oviduct end of the uterine horn.
6. The required and maximum concentration of G418 will depend on the copy number and integration site(s) of the transgene. For this reason, it will be necessary to try a range of G418 concentrations. We have found that that low copy number transgenic lines with a range of G418 from 50 to 150 μg/mL work best ***(15,19)***.
7. Feeder cell layers die after 4–5 d of culture. We routinely add extra feeder cells along with fresh medium when required.
8. CBA-derived ES lines are more prone to spontaneous differentiation than their 129 strain counterparts. To rescue lines with a high degree of differentiating cells, we employ a high-plating density strategy. For several passages, we will subculture the cells at 1:2 or 1:1 splitting density to limit the expansion of the differentiating cells. We believe this strategy works for two reasons: as ES cells can grow in a three-dimensional fashion, the differentiating cells usually do not, and they are of a limited proliferated capacity, leading to their eventual loss and, hence, enrichment of ES cells.

Acknowledgments

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7

Using Multiphoton Excitation to Explore the Murky Depths of Developing Embryos

Jayne M. Squirrell and John G. White

1. Introduction

1.1. Multiphoton Microscopy

Fluorescence microscopy has become the method of choice for imaging living specimens, as it offers high signal-to-background and the ability to discriminate between multiple fluorophores. Recently developed techniques, such as confocal ***(1)*** or multiphoton imaging ***(2)***, permit optical sectioning of intact specimens. These may be collected as stacks of images at different focal depths to obtain three-dimensional (3D) structural data. Stacks of images may be collected at regular time intervals in order to reveal the dynamics of 3D structures in living tissue ***(3)***. However, living tissue is generally of poor optical quality; microinhomogeneities in refractive index caused by cytosol/membrane interfaces scatter light, thereby limiting the depth from which optical sections may be obtained. Multiphoton laser-scanning microscopy (MPLSM) is becoming increasingly favored for in vivo imaging because of its superior ability to obtain images from deep within specimens ***(4)*** and the improved viability that can be obtained ***(5)***.

The underlying principle of this MPLSM is as follows. At a very high-photon density, two or more photons may be simultaneously absorbed by a fluorescent molecule, producing a transition to a metastable-excited state. Fluorescence emission occurs conventionally following a transition to the ground state. The sum of the individual photon energies is equivalent to the energy of a single photon that would normally induce fluorescence ***(2)***. In the case of two-photon imaging, the excitation wavelength is set to about twice that of the absorption peak of the fluorophore being observed. Normally, this

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wavelength would not produce any appreciable fluorophore excitation. However, if a high-power ultrashort pulse laser is used, it is possible to achieve instantaneous photon densities that will give rise to a significant yield of two-photon events at the focal volume of an objective lens, while maintaining a mean power level that will not damage the specimen. In this manner, fluorophore excitation is confined to the focal volume because the photon density is insufficient to generate appreciable multiphoton events outside of this region. Optical sectioning is achieved because there is no appreciable fluorophore excitation above or below the focal volume (i.e., the plane of focus), thereby elegantly avoiding the problem of out-of-focus interference by not generating it to begin with.

Because scattering is lower at the longer wavelengths of excitation used in MPLSM, and also because there is no fluorescence excitation above the plane of focus, there is significantly reduced extinction of the excitation light from regions above the plane of sectioning when compared to confocal microscopy. Additionally, the emission signal does not have to be imaged and is therefore relatively insensitive to scatter. These characteristics provide the deep-sectioning capabilities of MPLSM. The depth of imaging that can be achieved with MPLSM is specimen-specific. In embryological studies, the reported depths range from >30 μm in frog gastrula ***(6)*** to 100 μm in bovine embryos (***4***; **Fig. 1**). However, imaging depths of <400 μm have been achieved in studies of neurons in rat brains ***(7)***.

1.2. Application to the Study of Embryonic Development

Because MPLSM has only recently become readily available to the developmental biologist, the number of embryological studies utilizing MPLSM is limited. However, the studies that have been performed cover several species and a variety of biological questions. These studies not only advance our knowledge of dynamic processes, but also exemplify the advantages of MPLSM, namely, imaging thick light-scattering living specimens over long periods of time.

1.2.1. Subcellular Localization

Cytoplasmic organization plays a significant role in the embryonic development of a variety of organisms. To gain a greater appreciation of these changes in subcellular organization, the application of live-cell imaging techniques, such as MPLSM, is critical. For example, MPLSM has been used to analyze changes in vesicle distribution in the early *Caenorhabditis elegans* embryo (**Fig. 2**; *see* **Note 1**). This work suggests that vesicle trafficking may play a role in the terminal phase of cytokinesis ***(8)***.

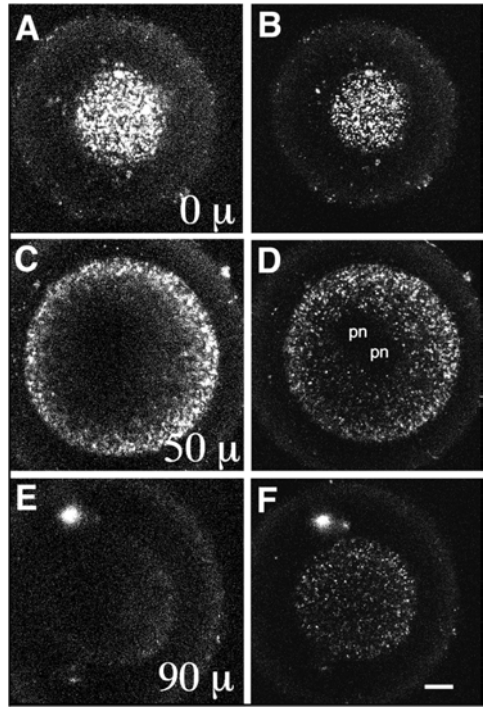


Fig. 1. Demonstration of increased imaging penetration depth with multiphoton microscopy in a living embryo. A bovine embryo stained with Mitotracker rosamine was imaged by confocal microscopy (*left*; 3.5 μW of 532-nm light) and by multiphoton microscopy (direct detection, *right*; 10 mW of 1047-nm light). Confocal microscopy gives the erroneous impression that mitochondria are localized to the region adjacent to the cell cortex. However, the multiphoton image indicates that the mitochondrion distribution is fairly uniform throughout the cytoplasm. Images were collected at a pixel resolution of 0.27 μm with a Kalman three-collection filter. Scale bar, 20 μm. Laser: YLF, 1047 nm. Copyright 1998, Biophysical Society, reproduced with permission ***(4)***.

MPLSM has also been used to examine changes in mitochondria distribution under various culture conditions in the oocytes and embryos of the hamster ***(9,10)***, bovine ***(11)***, and primate ***(12)***. Two-cell hamster embryos, labeled with the mitochondria-specific dye Mitotracker Rosamine, did not develop to the morula/blastocyst stage imaged with confocal microscopy. However, similarly prepared embryos imaged with MPLSM were capable of morula/blastocyst development at levels equivalent to nonimaged controls. Embryos imaged with MPLSM were also capable of fetal development and live birth (*see* **Fig. 3**; ***5***).

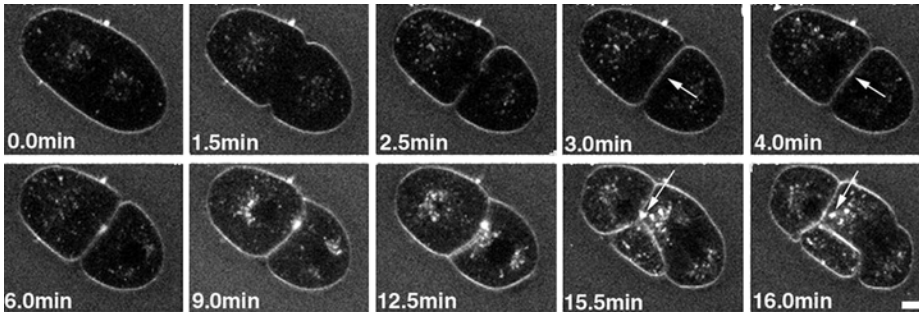
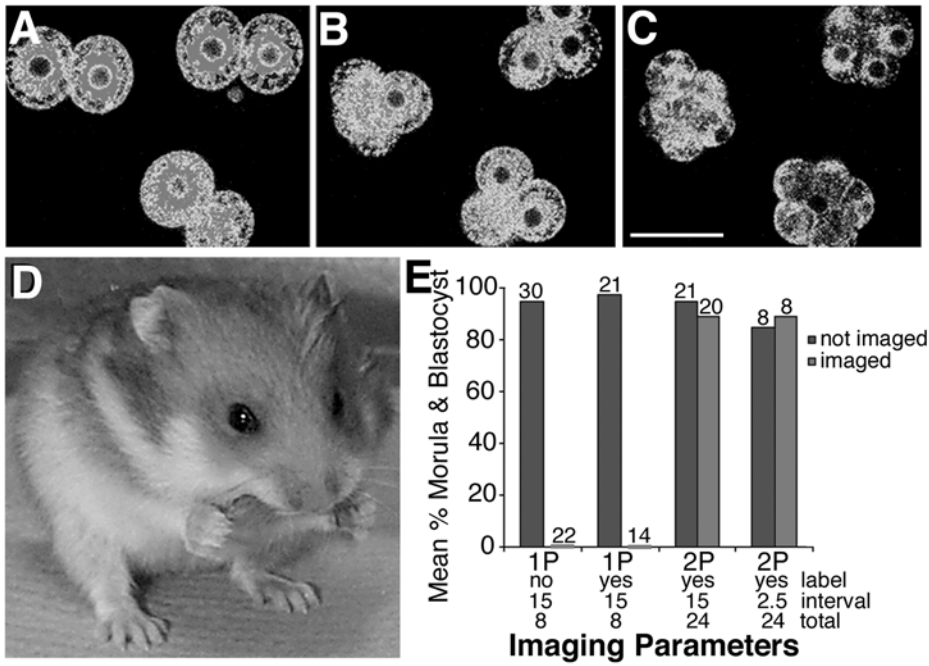


Fig. 2. Plasma membrane dynamics in the early *c. elegans* embryo. A multiphoton time course of a single focal plane taken from an embryo labeled with FM1-43 is shown (2D images). Furrow invagination began at 1.5 min and appears to have been completed at 3.0 min. Membrane accumulation at the intercellular canal occurred after furrow completion from approx 4.0 to 12.5 min. Note how the intensity of the staining in the spindle midzone region increases over time. At 16 min, the accumulated membrane appears to become dissociated from the plasma membrane and internalized. Scale bar, 5 μm. Laser: Ti:Sapph, 900 nm. Copyright 2001, Elsevier Science Ltd., reproduced with permission ***(8)***



In a very different type of study, the transmission from mother to offspring of a symbiotic bacteria has been examined in the parasitic wasp, *Aphytis*, using a combination of electron microscopy (EM), confocal, and MPLSM ***(13)***. The EM was used for the precise detection of the bacteria, whereas MPLSM were used to analyze the 3D distribution of DAPI label in fixed ovary tissue (*see* **Note 2**). The bacteria were observed in nurse cells and in both young and mature oocytes imaged with MPLSM. In contrast, when EM was used, the bacteria were only observed in the nurse cells and occasionally in young oocytes, but not at all in mature oocytes.

1.2.2. Cell Division and Cytoskeletal Dynamics

Because of their large size, the rapidity of the cell cycle, and their genetic and physical malleability, early embryos serve as extremely useful models for studying the mechanics and regulation of cell cycle progression. MPLSM has been used to image changes in cytoskeletal dynamics, chromatin morphology, and calcium levels in a number of organisms.

In *C. elegans*, green fluorescent fusion proteins (GFP; ***14***) have been used to analyze the effect of mutations on the early cell division. GFP::tubulin has been used to understand spindle dynamics during the early cell divisions ***(15)***. Similarly, a GFP::histone construct ***(16)*** has been used to examine chromosome segregation in wild-type (**Fig. 4**) and mutant embryos ***(17)***. Embryos expressing this construct were observed using a titanium:sapphire laser tuned

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Fig. 3. (*opposite page*) Embryo viability is maintained after long-term observation of mitochondrial dynamics in hamster embryos using multiphoton excitation. A multiphoton time series **(A–C)** of hamster embryos labeled with Mitotracker showing the localization of mitochondria in these embryos that undergo two cell divisions during the imaging period. Scale bar,50 μm. **(D)** A 18-d-old hamster, which developed from one of the embryos shown in A–C following transfer to a pseudopregnant recipient female. **(E)** Embryos imaged with CLSM do not develop to the morula and blastocyst stage when images are collected every 15 min for 8 h, in the presence or absence of a fluorophore. Embryos imaged for 24 h with MPLSM develop to the morula and blastocyst stage at levels similar to that of nonimaged controls, even when the frequencies of imaging collection is increased from 15 min to 2.5 min. Label indicates the presence or absence of Mitotracker, interval is time between image collection, in minutes. The total indicates the number of hours of imaging. Copyright 1999, Squirrell and Bavister, reproduced with permission. The graph is a summary of data published in **ref. *5***. Laser: Nd:YLF, 1047 nm

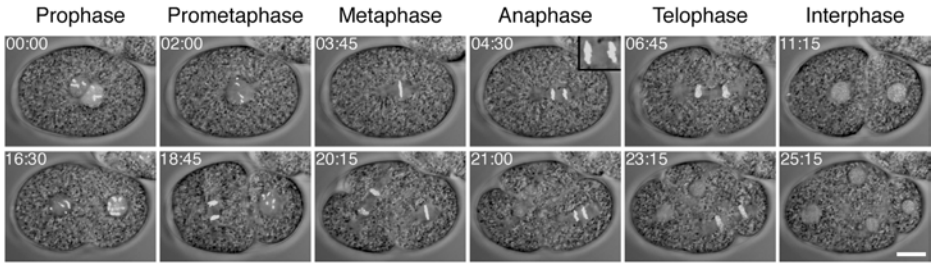

Fig. 4. Time-lapse analysis of the early mitotic divisions in the *C. elegans* embryo. Nomarski (blue) and GFP::H2B (green) images overlaid from the early mitotic divisions of wild-type embryos mounted on 2% agarose pads. The time at which each image was acquired is indicated in the top left-hand corner. Inlay shows anaphase figure at higher magnification. Scale bar, 10 μm. Copyright 2001, Elsevier Science Ltd., reproduced with permission ***(17)***. Laser: Ti:Sapph, 900 nm.

to 900 nm as an excitation source. Whereas two-photon excitation was used to visualize the histone::GFP signal, the single-photon infrared light was used to simultaneously generate bright-field images.

The early *Drosophila* embryo has a rapid (8–10 min at 25°C) cell cycle and, for the first 13 cycles, develops as a syncytium ***(18,19)***. The major challenge of observing the earliest cell cycles of these embryos is that the nuclei begin dividing deep within the center of light-scattering yolk. To examine the possibility of microenvironmental differences in cell cycle regulation, Ji et al. ***(20)*** utilized a MPLSM with 900 nm excitation to image cell cycle progression in fly embryos expressing a GFP::histone construct (**Fig. 5**).

To determine whether there is a relationship between the orientation of first-cleavage furrow and the subsequent bilateral axis of the sea urchin embryo, Summers et al. ***(21)*** used lineage-tracing techniques. In contrast to the previously described studies, these researchers used MPLSM to photolyze caged fluorescein in a single blastomere, which could then be used as a lineage marker (*see* **Note 3**). The probe was photolyzed in one blastomere of the two-cell embryo by scanning a portion of the blastomere with 700-nm light from a femtosecond titanium:sapphire laser. Subsequent observation and analysis of the embryos after development to the gastrula or pluteus stage was performed with confocal microscopy, which showed that the first-cleavage plane does not necessarily define the bilateral axis of the embryo. The advantages of this technique over conventional lineage-tracing techniques in which a single blastomere is injected with a fluorescent dye are: ***(1)*** because of its larger size, the entire zygote is easier to inject than a single blastomere; and ***(2)*** the irradiation with the MPLSM appears less damaging than the physical or electrical perturbation required by other lineaging techniques***(21)***.

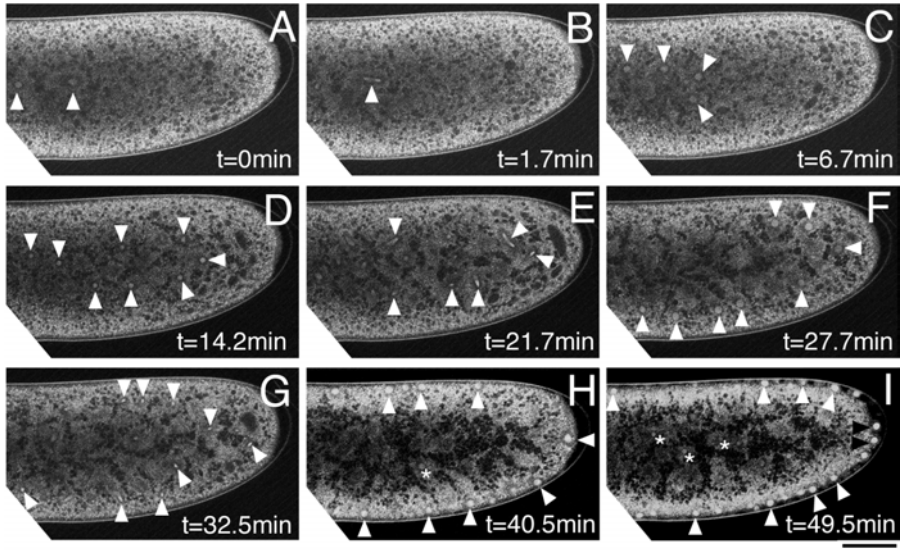

Fig. 5. Nuclear dynamics in the early *Drosophila* embryo. This multiphoton time series of single optical sections of a living *Drosphila* embryo transformed with a histone::GFP construct illustrates that changes in nuclear morphology and location can be assessed even in the yolk-rich interior of the syncytium. Arrowheads indicate some, but not all, nuclei in a given optical section. The nuclei are initially found in the interior and slightly anterior **(A)**. During the successive nuclear divisions, the nuclei spread along the anterior–posterior axis (**C–E**) and then out toward the cortex (**F–I**). Asterisks indicate those nuclei ("yolk nuclei") that remain in the interior of the embryo. **(A)** prophase cycle 5; **(B)** anaphase cycle 5; **(C)** interphase cycle 6; **(D)** early interphase cycle 7; **(E)** late anaphase cycle 7; **(F)** late interphase cycle 8; **(G)** telophase cycle 8; **(H)** interphase cycle 9; and **(I)** interphase cycle 10. Note pole bud formation at posterior end. Time (t) is time from the initiation of image collection. Anterior is toward the left, dorsal is up. Scale bar, 50 μm. Laser: Ti:Sapph, 900 nm. ***(20)***

1.2.3. Morphogenesis

One of the most intriguing and, for the microscopist, one of the most challenging, aspects of embryogenesis is the development of a complex, 3D organism from a single cell through a precisely orchestrated series of cell divisions, cell movements, and tissue reorganizations. The difficulty in trying to visualize such dynamic events lies in the conflict between needing high resolution to follow individual cells while gathering information in 3D space over extended periods of time. To date, the most common compromise is to generate 4D data sets, namely, the collection of optical sections in space (z-series) at a set of discrete time points. Although any optical sectioning microscopy technique can be used for 4D data collection, the advantages of MPLSM—in particularly

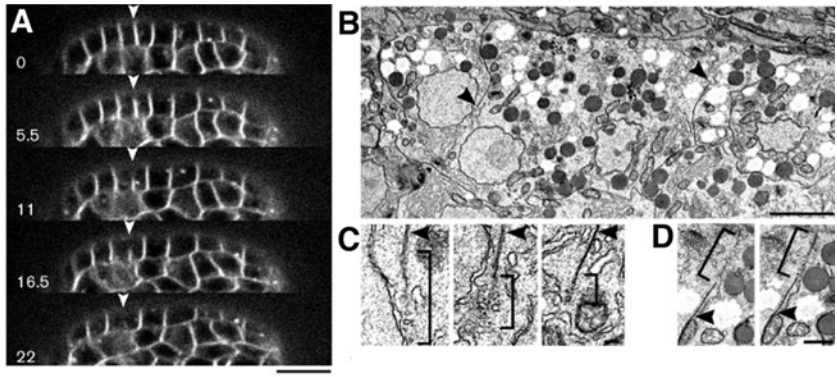

Fig. 6. Integration of MPLSM and EM for the study of morphogenesis in *C. elegans*. **(A)** Live MPLSM imaging of hypodermal cell fusions. Cell fusion proceeds in an apical-to-basal direction. Time sequence shows disappearance of lateral membranes (arrowhead) in an optical cross-section through a pair of fusing cells. Times displayed are in minutes. Scale bar, 10 μm. **(B)** TEM of an embryo fixed while two-cell fusion events were in progress (arrowheads). Scale bar, 2 μm. **(C)** Serial cross-sections through the leftmost fusing cell border in (A) shown at higher magnification. The arrowhead marks the region of intact membrane. The bracket indicates the zone of vesiculation. The spacing between sections is approx 300 nm. Scale bar, 100 nm. **(D)** Tangential sections through the vesiculating zone of the rightmost fusing border in (A). The spacing between sections is approx 70 nm. Scale bar, 500 nm. Laser: Nd:YLF, 1047 nm. Copyright 1998 Elsevier Science Ltd., reproduced with permission ***(42)***.

deeper optical sectioning and improved specimen viability—make it an obvious technological candidate morphogenetic analysis.

MPLSM has been used to study morphogenesis in *C. elegans*. To seal the interior of the organism from the external environment, the epithelia from each side of the embryo meet on the ventral side,and the free edges of these epithelia must join together. This sealing event has been examined using 4D MPLSM to observe GFP versions of junctionally localized proteins in wild-type and in embryos in which the expression of specific genes was suppressed by RNA interference ***(22)***. These experiments suggested that cadherin–catenin attachments play an important role in generating and stabilizing initial fusion events between the epithelial cells during the sealing process.

To further characterize morphogenesis in the *C. elegans* embryo, Mohler et al. ***(23)*** applied an integrated microscopy approach. Taking advantage of the strengths of different microscopy techniques, the authors used 1047 nm MPLSM of living embryos labeled with the membrane dye FM 4-64 (*see* **Note 1**) to maximize spatiotemporal resolution and viability and minimize photobleaching and EM to assess ultrastructure (**Fig. 6**). This study illustrates

the use of a combination of microscopy techniques to address different aspects of a biological question.

The large dense embryos of frog *Xenopus lavis* provide a considerable challenge for generating high quality images during morphogenesis. Periamsamy et al. ***(6)*** compared three microscopy techniques—two-photon laser scanning microscopy (TPLSM), confocal laser-scanning microscopy (CLSM), and digital deconvolution microscopy (DDM)—for imaging gastrulation in the frog embryo. The authors found that when optimized, TPLSM alone was the better imaging system, particularly for deep-tissue imaging (**Fig. 7**). However, they felt that the TPLSM images from deep within the tissue could be further improved by the application of digital deconvolution to remove some of the background noise.

1.4. Overview of Methods

The applications and methodologies available for the study of gametes and embryos using MPLSM are limited only by the skill and imagination of the researcher. For this reason, there is no "one-size-fits-all" protocol for using the instrument. Therefore, we have provided a list of factors that must be considered when imaging living cells and tissues with MPLSM in the Materials section and the methods that we have employed to address these various issues in embryos from three different organisms: the nematode worm *C. elegans*, the fruit fly *Drosophila melanogaster*, and a mammal, the golden hamster *Mesocricetus auratus*. These methods should provide a starting point to which adaptations could be made for visualizing other embryos or tissues of interest.

2. Materials

2.1. Microscope and Lasers

Currently, multiphoton microscope systems are commercially available. All of these systems function on the same basic multiphoton principle described in the **Introduction**, although each system differs in the specific components. The most commonly available laser for multiphoton systems is a tunable titanium::sapphire (Ti::Sapph) These lasers can vary in wavelength range, and the specific laser requirements depend on the application. For imaging endogenous fluorescence, shorter wavelengths (700–800 nm) are necessary; for fluorescein-type or GFP imaging, mid-range wavelengths are suitable (900 nm); and the more red-emitting dyes, such as rhodamine, yellow fluorescent protein (YFP) or DsRed, require longer wavelengths (>1000 nm). These lasers can also be either femto- or picosecond pulsing. Although currently there is no clear evidence for the preference of one type over another, anecdotal information suggests that the femtosecond pulses are better for maintaining specimen viability.

Fig. 7. Comparison of multiphoton, confocal, and deconvolution microscopy for imaging morphogenesis in the *Xenopus* embryo. Localization of GFP-GAP-43 fusion protein images in developing *Xenopus* embryos were acquired with same objective lens using Nikon epifluorescence-based TPEM, LSCM, and DDM microscope system. The details of the images are superior in TPEM mode. Generally, deterioration and less details of information of GFP-GAP-43 proteins were observed in all sections of the tissue in LSCM mode. The DDM system is not good for tissue imaging as demonstrated in the figure (images were deconvolved [15 iterations] using Deltavision software). Laser: Ti:Sapph, 870 nm. Copyright 1999, Wiley-Liss, Inc., Reproduced with permission. (Adapted from (**ref. *6***).

2.2. Labels and Probes

In order to maximize the utility of MPLSM for imaging living embryos, more than the instrumentation must be considered. Because MPLSM is a fluorescence excitation imaging system, the components of interest must be

fluorescently tagged. Currently there are three basic ways of accomplishing this: fluorescent vital dyes, fluorescently tagged transgenic proteins, and inherent autofluorescence. As their name implies, fluorescent vital dyes are relatively non-toxic and can be used in living cells. Generally, these dyes have properties that cause them to preferentially accumulate in particular cell compartments; for example, Mitotracker (Molecular Probes), a mitochondrial specific probe that enters and is subsequently trapped inside, respiring mitochondria due to their membrane potential ***(24)***. These types of dyes are frequently lipophilic, preferring to associate with membrane of particular compositions. For example, FM 4-64 labels plasma membranes and can be used to study endocytosis ***(25)***, whereas $DiOC_6$ ***(3)*** is incorporated into the endoplasmic reticulum (ER) ***(26)***. Although there are a number of such dyes now available for a multitude of labeling applications, caution must be exercised in their use. It is important to establish in a given tissue that the concentrations necessary for to get sufficient labeling for imaging are not detrimental to viability. This is particularly important when working with embryos hypersensitive to environmental conditions. Often, these dye are not specific and may label a variety of components, particularly at different dye concentrations ***(27)***. The ease of application of these labels varies with the dye as well as with organism. Many of the dyes easily cross cell membranes, being straightforward for labeling mammalian embryos. Unfortunately, this property can also make them difficult to use in an aqueous solution. The dye can be solublized in vegetable oil and a drop of the oil injected into the specimen to label internal membranes ***(28)***. Embryos, such as those from *Drosophila* and *C. elegans*, present the additional challenge that they are encased in a non- or only minimally permeable egg shell. Therefore, applying any dye, aqueous or otherwise, must be performed either by injection or laser ablation.

To eliminate both the problems of specificity as well as label permeability, much attention has recently been placed on the use of fluorescently tagged proteins ***(29)***, which can be transgenically expressed in the embryos of several species. GFP imaging has melded well with MPLSM use. The phototoxicity reduction with MPLSM verifies that changes in protein distribution in the embryo can be examined for long time periods, allowing the generation of developmental profiles of protein localization within a single embryo. Not only are these transgenic fluorescent tags useful for assessing protein expression and localization, it is now possible to transfect an organism with genetically engineered calcium indicators ***(30,31)***. With a suitable choice of promoter, these calcium indicators could be expressed in specific subsets of cells within the organism.

It is possible to avoid the use of fluorescent probes altogether by taking advantage of cellular autofluorescence. MPLSM has been used to assess

changes in metabolic status of a specimen by imaging changes in autofluorescence, specifically the fluorescence of NADPH. Although this type of imaging has successfully been performed in other tissues ***(32–34)***, its efficacy for use in the study of embryonic development remains to be determined. The disadvantage of relying on autofluorescence is that the researcher has no flexibility as to which cellular components are labeled. Furthermore, it is important that careful controls are performed to ascertain the origin of the autofluorescence. This may be particularly critical and potentially difficult when assessing changes in metabolic status, rather than structural components of the cell.

2.3. Maintaining Living Cells on the Microscope Stage

The most important first step in live-cell imaging is to determine the conditions necessary to maintain the viability of the embryo or cells before, during and following the observation period. Be rigorous. Make sure to start with the highest-quality tissue and components available for your biological system. To maximize the quality of the specimen, the culture medium must be the best available for the species of interest. A specimen preparation area should be located near the MPLSM system to minimize transport time. An additional challenge is how to culture imaging embryos on the stage. Some embryos, such as those from *C. elegans* or *Drosophila*, do quite well developing on a microscope slide with appropriate precautions taken to prevent dehydration. However, embryos that require more meticulous conditions must be grown in some form of culture dish that does not impair the imaging quality. One of the most critical aspects of culturing embryos on the stage is temperature. However, it is insufficient to just measure the temperature of the chamber because temperature gradients are common. There are stage and lens heaters available, but these too should be tested for accuracy and stability. Having confidence in one's ability to culture embryos on the stage not only saves time and reduces frustration, but also improves the reliability of the data collected.

2.4 Data Acquisition, Storage, Viewing and Analysis

Because of the additional temporal component, live-cell imaging data that are acquired must be more sophisticated than the "snapshots," whether analog or digital, collected with fixed-tissue imaging. Thus, live cell microscopy requires the use of software that permits the user to gather 4D data sets—stacks of images in the *z* direction collected over time. This type of software is generally included with commercial imaging systems or can be obtained separately. Because MPLSM provides improved specimen viability over other types of fluorescent imaging, and because developmental events frequently occur on the order of hours or even days (which translates into hundreds of megabytes

and even gigabytes), the embryologist using MPLSM faces the problem of what to do with all of the data. For data storage, the most efficient method is to store the data initially on a hard drive, necessitating the use of a computer with substantial hard drive space. This is especially critical for multiuser imaging facilities in which many people may need to collect large data sets. Writeable compact discs provide a relatively cheap and manageable archival medium for these large data sets after they have been collected.

How the data are collected depends on the specimen and type of biological questions of interest. There are trade-offs between image and data quality (spatial and temporal resolution) and specimen viability. The second issue is how to visualize the data after they have been collected. The 4D data sets from live-cell imaging generate two types of data: spatial and temporal. The individual time points can be reconstructed to generate 3D renditions of the embryo at different developmental stages. Alternatively, optical sections can viewed as a sequence of time points to make a four dimensional movie ***(3)***. Furthermore, the 3D reconstructions can be joined together into a temporal sequence to make a movie of the 3D data ***(23)***. All of these steps are obviously computer-intensive.

The final challenge in managing the huge data sets generated by MPLSM imaging of living embryos is how to analyze and quantify that data. The most straightforward approach is to collect measurements (e.g., size, distance, or intensity) from single optical sections at different time points. Accessible software programs with the capability for easy manipulation and analysis of a 4D data set as a complete entity are not yet readily available, but hopefully in the near future such programs will be available, permitting the researcher to take full advantage of the vast amount of information inherent in these 4D data sets.

3. Methods

3.1. Lasers and Microscopes

1. *C. elegans*: A titanium:sapphire laser (775–910-nm range, Spectra Physics, Santa Clara, CA) was generally set at 900 nm. Microscope was an inverted Nikcon Eclipse (Melville, NY) using a ×100 super fluor lens. A steerable nitrogen-pumped Coumarin dye laser (Laser Sciences,) at 450 nm was used for laser ablation (*see* **Note 4**).
2. *Drosophila*: A titanium:sapphire laser (775–910-nm range was set at 900 nm. Microscope was an inverted Nikcon Eclipse using a ×20 Plan Fluor multiimmersion lens with oil (*Drosophila*; *see* **Note 4**).
3. *M. auratus*: Neodymium-doped:yttrium lithium fluoride-based (Nd:YLF) laser with a 1047-nm fixed wavelength (Coherent/Microlase, Glasgow, Scotland).

Microscope used was an inverted Nikon Optiphot with a ×40 plan fluor oil immersion lens (*see* **Note 4**).

3.2. Specimen Prep Area

1. *C. elegans*: A dissecting microscope, heat block, flame, Pasteur pipets, and micropipettors were available on a bench in the same room as the microscope.
2. *Drosophila*: A similar set-up as for *C. elegans* was provided, but with the addition of a 25°C incubator for maintaining the flies and embryos prior to imaging.
3. *M. auratus*: A separate dark room with red lights (hamster embryos are light-sensitive), a water-jacketed, gas-controlled incubator, and a dissecting microscope are needed.

3.3. Culture Medium

1. *C. elegans*: Egg buffer is made up as follows ***(35)***, with all components purchased from Sigma (St. Louis, MO; *see* **Note 5**).

Component	Concentration	Amount in 1 L
NaCl	118 m*M*	6.9 g
KCl	48 m*M*	3.58 g
$CaCl_2$ $2H_2O$	3 m*M*	0.44 g
$MgCl_2$ $MgCl_2$ $6H_2O$	3 m*M*	0.61 g
HEPES, pH 7.2	5 m*M*	5 mL 1 *M* HEPES, pH 7.2, added after autoclaving

2. *Drosophila*: Not necessary.
3. *M. auratus*: We used hamster embryo culture medium 9 (HECM-9; ***36***). All components were purchased from Sigma, culture-tested quality when available. Make up in embryo culture-grade Milli-Q (Millipore, Bedford, MA) water. The 100X amino acid and vitamin solution can be made up, sterilized using a Millex-GV (Millipore, Bedford, MA) syringe filter, then stored at –80°C in 1-mL aliquots for up to 3 mo. Thaw an aliquot when ready to use, discarding the unused portion when done. Add PVA first by sprinkling over approx 75 mL of water. The polyvinyl alcohol(PVA) will dissolve within 2–4 min. Then add the other components, using gentle stirring with a stir bar to prevent precipitation. When all components (except vitamins) are added, bring up to 100 mL. Sterilize with a Millex-GV syringe filter, and store in a siliconized borosilicate glass bottle. If using plasticware for storage, use only polycarbonate or polystyrene. Store solution at 4°C for no more than 3 d. On the day of the experiment, add 10µL/mL of the amino acid solution to the desired amount of culture media:

Culture Medium

Component	Concentration	Amount in 100 mL
Poly vinyl alcohol (PVA)	0.1 mg/mL	0.01 g
NaCl	113.8 m*M*	0.6649 g
KCl	3 m*M*	0.0225 g

$CaCl_2 \cdot 2H_2O$	1.9 m*M*	0.0272 g
$MgCl_2 \cdot 2H_2O$	0.46 m*M*	0.0094 g
$NaHCO_3$	25 m*M*	0.2100 g
(DL) Lactic acid (60% syrup)	4.5 m*M*	0.0648 g
1 *M* HCl (to prevent ppt)		0.140 mL
Amino acid/vitamins (100X)	10 µL/mL	1 mL

Amino Acids and Vitamins (100X stock) Made in 100 mL of 157 m*M* NaCl

Taurine	0.50 m*M*	0.6250 g
L-Asparagine	0.01 m*M*	0.0132 g
L-Cysteine	0.01 m*M*	0.0176 g
L-Histidine	0.01 m*M*	0.0210 g
L-Lysine	0.01 m*M*	0.0182 g
L-Proline	0.01 m*M*	0.0115 g
L-Serine	0.01 m*M*	0.0105 g
L-Aspartic acid	0.01 m*M*	0.0133 g
L-Glycine	0.01 m*M*	0.0075 g
L-Glutamic acid	0.01 m*M*	0.0169 g
L-Glutamine	0.20 m*M*	0.2920 g
Pantothenate	0.003 m*M*	0.0071 g

3.4. Labels and Probes

See **Notes 1–3** for information on dyes used by other researchers as described in the **Introduction**.

1. *C. elegans*: Plasma membrane label FM 2-10 (Molecular Probes, Eugene, OR) is made up as a stock solution in double-distilled water at a 10-m*M* concentration. This stock solution was aliquoted and stored at –20C. For each experiment, an aliquot was removed to make up the dilution, which was made fresh each day before use. Any unused stock solution from that aliquot was discarded. To make the working concentration, 1 µL stock was added to 99 µL egg buffer. Embryos were placed into a 3-µL drop of egg buffer on a 22 × 22 mm coverslip and allowed to settle. The majority of the media was removed and replaced with 3 µL of 1 mg/mL trypan blue (Sigma) in egg buffer for 30 s. This facilitates the laser permeablization of the egg shell. The Trypan Blue solution was removed and replaced with 3 µL of the FM 2-10 solution.
2. *Drosophila*: Animals were expressing histone::GFP. No additional label was added.
3. *M. auratus*: A mitochondrial specific probe, MitoTracker-X-Rosamine (Molecular Probes, Eugene, OR), was made up as a 1 m*M* stock solution in culture-grade dimethyl sulfoxide (DMSO) (Sigma), aliquoted and stored at –20°C. For use, the

label was diluted to a working concentration by placing 1 µL of the stock solution into 1 mL of equilibrated (37°C; 10% CO_2, 5% O_2, 85% N_2) HECM-9. The embryos were placed into a 50-µL culture drop under mineral oil (Sigma), and 25 µL of the working solution was added to the embryos to make a final 330 nm concentration. The embryos remained in the label for 15 min at 37°C and were then rinsed in fresh HECM-9 before being placed for imaging into 36 ul culture drops, which had been temperature- and gas-equilibrated under 10 mL mineral oil.

3.5. Specimen Holder

1. *C. elegans*: Petroleum jelly squeezed out through an 22-gauge syringe needle was used to create a 1-cm circle on a 1 × 3-in. microscope slide. The coverslip with the drop containing the embryos was inverted over this circle and pressed down gently, resulting in an enclosed chamber ready for laser ablation and imaging (*see* **Note 6**).
2. *Drosophila*: We designed a re-useable open chamber by cutting a 3/4 X 1-in. square opening in a 1 × 3 × 1/8-in. piece of plastic (so it fits into a slide holder). Imaging coverslips were made by using a Pasteur pipet to put a stripe (about 3mm wide) of "heptane glue" down the center of a 22 × 40 mm-coverslip. The heptane glue was made by putting several strips of double-stick tape into a screw-capped 15-mL glass tube with approx 3-mL of heptane (Sigma). The tube was placed on a rocker for several hours and the glue dissolved into the heptane ***(37)***. The heptane stripe on the coverslip will evaporate, leaving a track of glue. Dechorionated embryos are lined up on this stripe of glue, then covered with halocarbon oil (Series 700, Halocarbon Products Corp., River Edge, NJ). The coverslip is then taped to the bottom of the plastic holder for the strip of embryos to be centered within the opening. This can be placed into the slide holder on stage and is ready for imaging (*see* **Note 7**).
3. *M. auratus*: A 60 mm culture dish (Becton Dickinson Biosciences, Palo Alto, CA) with a 1/2-in. hole drilled in the center of the bottom half of the dish, in which the hole is covered with a siliconized cover glass, works quite well ***(38)***. The cover glass (round #1; Bellco, Vineland, NJ) is glued to the bottom of the dish with epoxy (Devcon 5 Min Epoxy). The dishes were made up several days in advance. The epoxy was allowed to dry overnight, then the dishes were rinsed with culture-quality water and allowed to dry in a 40°C incubator overnight to permit the release of any volatiles from the epoxy. Similarly designed imaging culture dishes are now commercially available (*see* **Note 8**).

3.6. Maintaining Cells on the Stage

1. *C. elegans*: The *C. elegans* embryo is enclosed in an egg shell, which must be permeated to permit access by the label ***(23,39)***. The slide with the embryos bathed in labeling solution was placed on the microscope stage. Minute holes were made in the egg shell using the nitrogen-pumped dye laser. Single pulses controlled by a foot pedal were used to ablate specific sites on the egg shell, allowing the label to reach the membrane before imaging commenced ***(40)***.

2. *Drosophila*: No special modifications necessary.
3. *M. auratus*: In order to mimic incubator culture conditions, a large Plexiglas chamber surrounding the entire microscope containing a heater and thermostat served as an "incubator" (**Fig 8**). The imaging culture dish was placed into a 9.5 cm × 9.5 cm Plexiglass minichamber on the stage ***(41)***. Gas cylinders (air, nitrogen, and carbon dioxide) were connected to an adjustable gas mixing device (Cole-Palmer, Vernon Hills, IL). The mix was tested with a Fyrite indicator (Bacharach, Pittsburgh, PA), and adjustments were made until the gases reached proper proportions. Once these settings were determined, they remained relatively consistent from day to day. However, the gas concentrations were still checked prior to each experiment. The gas mixture was warmed and humidified by passing through a flask (*see* **Note 9**) of culture-quality water located within the large Plexiglass chamber. Temperature regulation is critical for mammalian embryo development and can be particularly difficult to control on a microscope stage. We found it necessary to adjust the temperature settings based on readings from a drop on the coverslip in contact with the immersion lens such that, on our system, it was necessary to warm the incoming gases to nearly 3 degrees above the desired temperature to maintain the culture drop at the correct temperature.

3.7. Acquiring Data (see Note 10)

1. *C. elegans*: Single optical sections collected as quickly as possible (4.37 s for a 512 × 512 slow scan) with manual control of the focus to follow items of interest over a period of about 30 min.
2. *Drosophila*: Images were collected from two focal planes using software to control *z*-axis stage motor movement every 10 s for over 1 h.
3. *M. auratus*: Five optical sections, 5 μm apart were collected either every 2.5 or every 15 min (depending on the experiment) for 24–48 h.

3.8. Viewing Data

1. *C. elegans*: Because the data set from these embryos contain images from one optical plane over time, they can be opened in ImageJ (Rasband, W.S., ImageJ, National Institutes of Health, Bethesda, MD; *see* **Note 11**) using the Bio-Rad plugin.
2. *Drosophila* and *M. auratus*: Because 4D data sets were generated for the experiments with these embryos, the program 4D Turnaround (LOCI 4D Suite, University of Wisconsin, Madison, WI) to convert Bio-Rad stacks into a more accessible form and the program 4D Viewer (LOCI 4D Suite, University of Wisconsin, Madison, WI) was used to view the data sets (*see* **Note 11**).

3.9. Analysis of Data

1. *C. elegans*: In addition to a descriptive analysis that compares wild-type and mutant embryos, including the timing of a specific event, measurements of specific membrane structures have been performed using the analysis tools of ImageJ (Rasband, W.S., ImageJ.

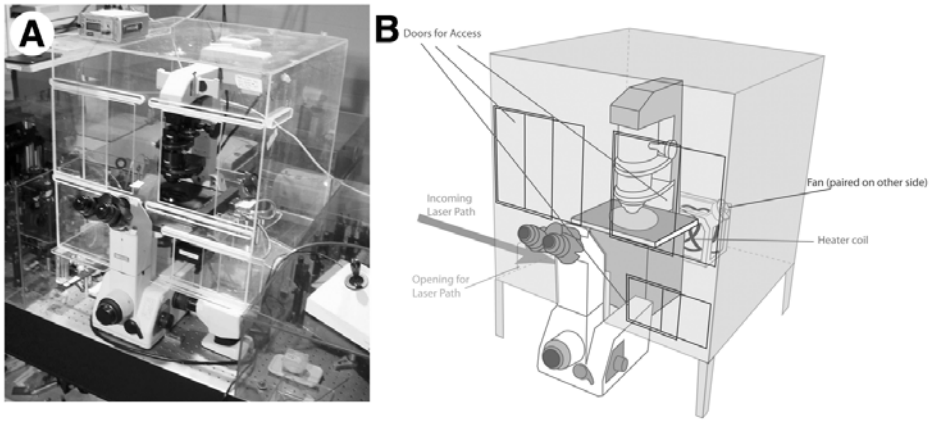

Fig. 8. Crystal Palace for maintaining living cells on the microscope stage. **(A)** A photograph of a Plexiglass chamber surrounding most of the microscope to provide a temperature-controlled environment for maintaining living cells on the microscope stage. **(B)** A diagram of the chamber shown in **(A)** with features labeled. Sliding doors permit access to the microscope as well as the specimen. The size of any openings in the chamber necessary for the laser path(s) should be kept to a minimum. Temperature is maintained via a thermostatically controlled heating coil and a pair of small fans. Appropriate gas mixtures can be established and maintained by using a minichamber on the microscope stage ***(41)***.

2. *Drosophila*: Viewing and defining timing of changes in mitotic status of nuclei in different regions of the embryos was done using 4D Viewer (LOCI 4D Suite, University of Wisconsin, Madison, WI).
3. *M. auratus*: Quantitive analysis of changes in mitochondria distribution in different culture mediums was performed using the analysis tools in NIH Image. Regional changes in pixel intensity were converted to ratios for each embryo, then these ratios were compared for different treatment groups as described in **Fig. 9**.

Fig. 9. Quantifying the change in the distribution of mitochondria over time in living embryos with altered intracellular pH (pHi). **(A)** These single optical sections of live 2-cell embryos labeled with Mitotracker and imaged with TPLSM illustrate how mitochondria move away from their perinuclear localization shortly after the addition of the pHi -altering compounds while embryos cultured in HECM-10 retain the perinuclear configuration. The HECM-10 embryo shown in the top row was cultured in the same dish as the HTMA-cultured embryo shown in the time series in the second row. The HECM-10 embryo shown in the bottom row was cultured in the same dish as the HDMO-cultured embryo shown in the time series in the third row. The $t = 0$ h images (left column) were collected prior to the addition of the pH_i-altering compounds. Scale bar, 50 μ. **(B)** To quantify the pattern of mitochondria distribution

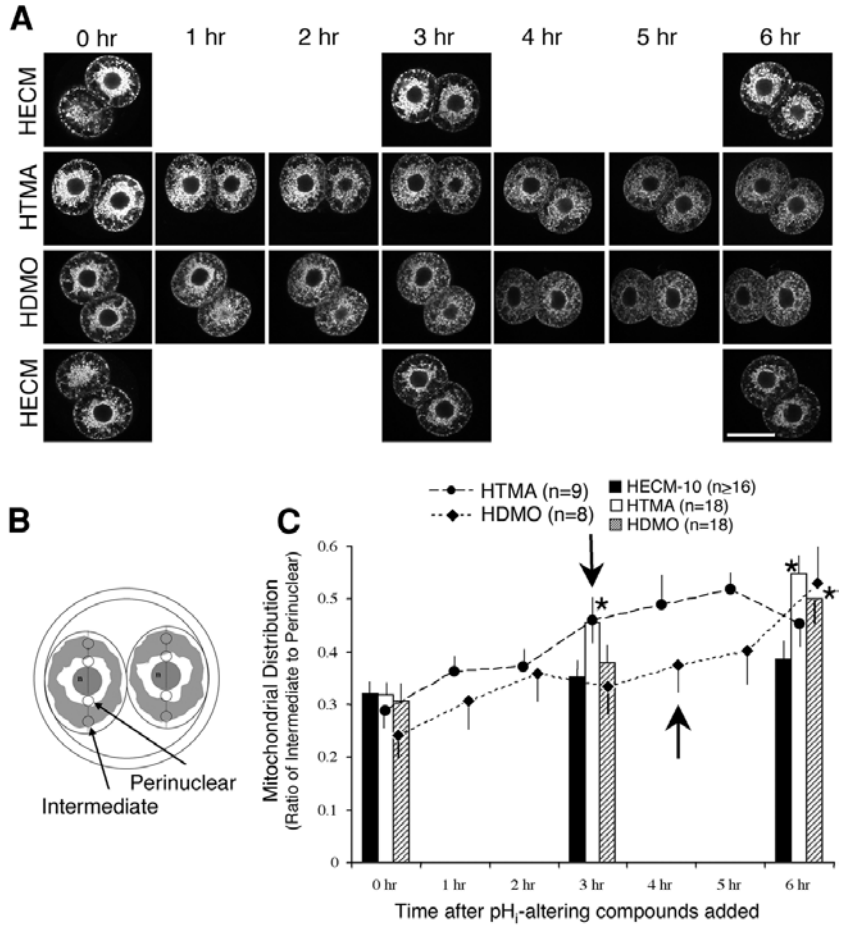

Fig. 9. (*continued from opposite page*) (white regions) in two-cell embryos, the average pixel intensity of the region within each of the four circles was determined. The ratio of the average pixel intensity of the intermediate region to the perinuclear region from the same side of the blastomere was identified. A ratio closer to one indicates a more homogeneous distribution of mitochondria, whereas a ratio closer to zero indicates a heterogeneous pattern, with a higher accumulation of mitochondria around the nucleus than in the intermediate region ***(46)***. The graph **(C)** illustrates the change of mitochondria distribution over time in living embryos. The bar graph shows data of individual images from each of the three treatments collected prior to the time lapse imaging (0 h) as well as at 3 and 6 h following the addition of the pH_i altering compounds. The data shown represent six replicates. *indicates statistical difference ($p < 0.05$) from control within a time point. Bars indicate standard error. The line graph shows the quantification of the mitochondrial pattern of individual images from a time-lapse sequence on a single culture. Each line represents three replicates. Bars indicate standard error. Arrows show first the time point that is statistically different ($p < 0.01$) from time 0 within a treatment. Laser: Nd:YLF, 1047 nm. Copyright 2001, Society for the Study of Reproduction, reproduced with permission ***(10)***.

4. Notes

1. For membrane labeling in *C. elegans* embryos, FM1-43 (Molecular Probes) was used at a 0.01 mg/mL concentration while internal cytoplasmic vesicles were visualized using 15 μg/mL Bodipy Brefeldin A (Molecular Probes ***8***). FM 4-64 (Molecular Probes) was used at a 10 μg/mL concentration ***(42)***.
2. For the wasp ovary study, DAPI was used on fixed tissue at a 0.1 μg/mL concentration in phosphate-buffered saline (PBS) and incubated at 37°C for 15–30 min ***(13)***.
3. To accomplish this, the authors injected the zygotes prior to the first division with a 100 mg/mL solution of caged fluorescein-dextran (10,000 MW; Molecular Probes; ***21***).
4. Both multiphoton systems used for these experiments are noncommercial systems that were designed and built at the University of Wisconsin–Madison. Additional details and specifications for these systems can be found in (**ref. *40*** for the *C. elegans* and *Drosophila* experiments or in **ref. *5*** or ***43***, *M. auratus* experiments.
5. A second recipe for *C. elegans* embryo culture can be used and may be particularly important for embryos undergoing morphogenesis. This recipe is: EGM: 65% Schneider's insect cell medium, 35% embryonic stem cell certified fetal bovine serum, 1X lipid concentrate, 1X BME vitamin concentrate (all components from Gibco-BRL; ***38***,***44***).
6. *C. elegans* embryos that express GFP fusion proteins do not require laser ablation and can be prepared on agar pads on microscope slides ***(45)***.
7. This design could be adjusted to accommodate a variety of cells or tissues. We are currently modifying this design using a 2-mm-thick piece of plastic and a 1-cm diameter smaller hole, to which a coverslip can be attached to both sides, creating a closed chamber for imaging tissue samples.
8. For example, one such dish, the Biocoat Poly-D-Lysine-coated cellware 35-mm coverslip bottom dish (Becton Dickinson Biosciences), has been used for imaging tissue culture cells on our system.
9. Flask with water for humidification should have a Teflon stopper (not black rubber, which can be toxic to mammalian embryos).
10. The specifics (scan rate, frequency of image collection, number of *z* axis sections, and so on) depend on the biological phenomenon of interest and the software controlling the system. Our system was controlled by the Bio-Rad Laser Sharp 1024 software (Bio-Rad, Hercules, CA).
11. The list of websites that follows for image acquisition/viewing (software referred to in **Subheading 3.**) are listed in bold are provided for information only. No endorsement of any particular product is expressed or implied.

    Commercial
    http://www.mediacy.com (Media Cybernetics—makers of ImagePro, ScopePro)
    http://www.api.com/DeltaVision/(Applied Precision Deltavision)
    http://www.image1.com (Universal Imaging-makers of Metamorph)
    http://microscopy.biorad.com/(BioRad Analysis Software)

http://www.zeiss.com/micro (Zeiss Software)
http://www.intelligent-imaging.com/(Analysis Software)
http://www.vaytek.com/vox.htm (Voxblast)
http://www.vitalimages.com (Vitrea 2)
http://www.scanalytics.com/(IPLab)
http://www.improvision.com (Volocity and Openlab)
http://www.bitplane.com (Imaris)
http://www.aqi.com (Autoquant)

Shareware/FreeWare

http://rsb.info.nih.gov/ij/(ImageJ)
http://rsb.info.nih.gov/nih-image/index.html (NIH Image)
http://www.scioncorp.com/(Scion Image)
http://www.loci.wisc.edu/4D (LOCI 4D suite 4D Turnaround and 4D Viewer)
http://www.loci.wisc.edu/visbio (LOCI VisBio program)
http://www.irfanview.com/(IrfranView)
ftp://ftp.genetics.bio-rad.com/Public/confocal/cas (Confocal Assistant)
http://ddsdx.uthscsa.edu/dig/itdesc.html (ImageTool)
http://www.openmicroscopy.org (Open Microscopy Environment)
http://www.gimp.org (GNU Image Manipulation Program)
http://www.imagemagick.org/(ImageMagick)

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8

Dual-Channel Confocal Ratioing of Calcium Dynamics in Living Eggs and Oocytes

Stephen A. Stricker

1. Introduction

Changes in intracellular calcium concentrations ($[Ca^{2+}]_i$) have been shown to regulate a wide variety of developmental processes. In particular, eggs that have completed meiosis or oocytes that are still in the process of undergoing meiotic maturation invariably display some form of a $[Ca^{2+}]_i$ elevation during fertilization ***(1–3)***. In order to image fertilization-induced calcium responses, unfertilized eggs and oocytes can be loaded with photoproteins, (e.g., aequorin) for subsequent detection of the luminescence that is generated when free calcium ions bind to the photoprotein ***(4)***. Alternatively, eggs may either be injected with or simply incubated in various calcium-sensitive fluorophores ***(5)***. Such calcium indicators can then be used in conjunction with fluorescence microscopy to track calcium dynamics during fertilization. The relative benefits and disadvantages of aequorin vs calcium-sensitive probes have been discussed previously ***(6)***, and both types of calcium indicators remain widely employed in imaging analyses that involves conventional microscopy.

As a complement to these methodologies, confocal laser-scanning microscopy (CLSM) provides marked improvements in spatial resolution, particularly along the *z* axis, in imaging studies utilizing calcium-sensitive fluorophores ***(7–9)***. A few confocal microscopes are equipped with a high-power "UV" laser that can excite dual-wavelength fluorophores and thereby provide true quantitative measurements of $[Ca^{2+}]_i$ ***(10)***. Alternatively, excitation at ultraviolet (UV) wavelengths is also possible to attain using femtosecond lasers and two-photon microscopy ***(11–13)***. However, the majority of confocal

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systems in operation today possess a low-power argon-ion laser, a krypton-argon laser, or a combination of lasers that are best suited for qualitative or semiquantitative analyses that employ single-wavelength, rather than dual-wavelength, calcium indicators.

This chapter outlines methods for imaging fertilization-induced calcium dynamics by means of confocal microscopy and single-wavelength fluorophores. Topics covered include fluorophore selection and application, data acquisition and processing, as well as image output and practical considerations. The particular protocols that are delineated have been used in our laboratory for investigations of marine invertebrate eggs. For alternative methodologies and more comprehensive theoretical treatments of confocal calcium imaging analyses, the reader is referred to various reviews ***(6,14–16)***.

2. Materials

2.1. Eggs and Oocytes

Fully grown eggs or oocytes obtained from ripe females (e.g., sea urchins, starfish, and nemertean worms) are typically maintained in filtered natural seawater (NSW) or artificial seawater (ASW) after having matured to a fertilizable state either spontaneously or in response to a maturation-inducing hormone (*see* **Note 1**). In most cases, optimal results are achieved when the mature gametes are kept at 12–15°C and imaged within approx 4–8 h after removal from the ovary (*see* **Note 2**).

2.2. Calcium-Sensitive Fluorophores

Of the single-wavelength fluorophores that have been commonly used in confocal calcium imaging studies, several (e.g., Fluo-3, Calcium Green, and Fura Red; Molecular Probes, Inc., Eugene, OR) are available as membrane-permeant acetoxymethyl (AM) esters that do not require microinjection protocols for successful dye loading ***(5,17)***. Such probes are excited at visible wavelengths and can therefore be used with a CLSM that is not provided with either UV- or two-photon capabilities.

In addition to analyses that utilize only one of these calcium indicators, Fluo-3 and Fura Red have also been used together in ratioing applications that employ a dual-dichroic filter and two separate detectors. As intracellular free calcium concentrations elevate, the Fluo-3 signal rises in the fluorescein channel, whereas the Fura Red emission decreases in the Rhodamine channel ***(18)***. Unfortunately, however, Fura Red is susceptible to certain artifacts ***(19,20)***, and both dyes are unavailable in dextran-conjugated forms, thus making them less suitable for long-term investigations (*see* discussion below).

For the eggs and oocytes we have imaged, dextran-conjugated Calcium Green (10,000 MW Molecular Probes, Eugene, OR) is routinely used as the calcium ion indicator. In addition, eggs are co-injected with a calcium-insensitive fluorophore, such as dextran-conjugated Rhodamine ***(21)***. The rhodamine, which can be either a tetramethylrhodamine or rhodamine B (Molecular Probes), typically shows similar photobleaching rates to the calcium green dextran ***(22)***. Moreover, the Rhodamine and Calcium Green signals are usually comparable to each other in different parts of the cell, although rhodamine B may not partition into the nucleus as well as tetramethylrhodamine, thus generating an artifactually higher resting ratio in the nucleus ***(22)***.

The combination of a calcium indicator plus a calcium-insensitive probe allows dual-channel ratioing of two fluorescent signals, which, in turn, helps to minimize the effects of artifacts associated with uneven dye-loading and differential pathlengths ***(6)***. In addition, dextran-conjugated forms of the calcium-sensitive probe tend to reduce dye compartmentalization, which is the process by which fluorophores are sequestered into membrane-bound compartments and become unavailable to register cytosolic calcium fluxes ***(17)***. Methods for performing such dual-channel confocal imaging of calcium dynamics are described below.

2.3. Solutions

1. Injection buffer: 10 m*M* HEPES, 100 m*M* potassium aspartate, pH 7.2 ***(23)***, made directly before mixture with fluorophores.
2. Protamine sulfate solution for affixing live eggs or oocytes: 5–10 mg/mL of protamine sulfate (Type X; Sigma Chemical Co.) in distilled water made within 12 h before use (*see* **Note 3**).
3. Calcium-free seawater: 376 m*M* NaCl, 49 m*M* $MgCl_2$, 26 m*M* Na_2SO_4, 10 m*M* Tris-HCl (from a 1 *M*, pH 8.0 stock solution), 8.5 m*M* KCl, 2.5 m*M* EGTA (from a 0.25 *M*, pH 8.0 stock solution), 2.25 m*M* $NaHCO_3$ in distilled water, pH 8.0. The solution should be filtered through coarse paper (e.g., 5-µm effective pore size; VWR Grade 413, VWR Scientific Co.) prior to use ***(24)***.
4. MBL ASW: 423 m*M* NaCl, 25 m*M* $MgSO_4$–$7H_2O$, 23 m*M* $MgCl_2$–$6H_2O$, 9.25 m*M* $CaCl_2$–$2H_2O$, 9 m*M* KCl, 2.1 m*M* $NaHCO_3$ in distilled water, pH 8.0. The solution should be filtered through coarse paper (e.g., 5-µm effective pore size; VWR Grade 413, VWR Scientific Co.) prior to use ***(25)***.
5. Stock solutions of calcium green dextran (10,000 MW) and rhodamine B dextran (10,000 MW) made in injection buffer (*see* **Note 4**).

2.4. Instruments

1. Confocal laser-scanning microscope with dual-channel capabilities, e.g., Bio-Rad MRC-600 system with a 15 mW krypton-argon laser (Bio-Rad, Hercules, CA).

2. Microinjection system: Eppendorf 5170/5242 (Brinkmann, Westbury, NY; *see* **Note 5**).
3. Thermoelectric cooling stage: KT-5000 (Micro Devices, Whitehouse Station, NJ).
4. Image processing system: MetaMorph image processing software version 2.0–5.0 (Molecular Devices, Sunnyvale, CA) running on a Windows-based PC (*see* **Note 6**).

3. Methods

3.1. Microinjection

Attach dejellied oocytes or eggs to a specimen dish that has been coated with protamine sulfate (*see* **Notes 7**and **8**). With a high-pressure microinjection system, load each cell to be imaged with the calcium green and rhodamine-B- Dextran stock solution to approx 1–5% of cell volume (*see* **Note 9**). Dye-injected specimens should be allowed to recover from the injection for at least 15 min prior to imaging. However, prolonged delays can lead to reduced fertilizability and/or increased dye compartmentalization.

3.2. Confocal Calcium Imaging

After microinjection, the cells are transferred to the Bio-Rad MRC-600 confocal microscope that is equipped with a thermoelectric cooling stage operating at 12–15°C. The microscope is then configured to provide simultaneous collection of the fluorescein and rhodamine signals (*see* **Note 10**), and one to several dye-injected specimens are placed in a single field of view for time-lapse confocal imaging. To maintain viability of the injected cells, the laser excitation should be reduced to an absolute minimum by means of a 1% or 3% transmission neutral density (ND) filter. In addition, imaging runs can include a time delay of one to several seconds between image acquisitions in order to reduce the phototoxicity that can build up under more constant illumination. For routine time-lapse runs, the slow-scan mode is chosen for optimal spatial resolution, and when using a ×20, NA 0.75 lens, the confocal aperture is typically set at "10" on the 1 (closed) to 15 (open) manually adjustable scale.

Paired calcium green and rhodamine images that are collected for each datapoint are routinely written to the hard drive. Thus, disk space of up to several hundred megabytes should be made available prior to each time-lapse run. Following the completion of an experiment, the data files can then be transferred via an ethernet connection or removable media (e.g., Zip disks or CD-ROMs) to an off-line system to allow processing without tying up the confocal apparatus.

3.2. Image Processing and Data Analysis

The monochrome 8-bit image files generated by the Bio-Rad MRC-600 system are 768 × 512 pixels in size and are written in a proprietary file format

(.pic) that typically needs to be converted to an alternative form to be processed by other software packages. Bio-Rad supplies a program (PIC2TIFF) that accomplishes such conversions. On the other hand, the MetaMorph software package can directly read Bio-Rad . pic files and convert them into various formats (e.g., .tiff files). In this way, MetaMorph is capable of handling full stacks of .pic files that are generated by the Bio-Rad system, provided that the host computer has sufficient RAM to load such stacks. Alternatively, in order to facilitate the processing of large data sets, it is often desirable to subdivide a long time-lapse run into several individual .pic files of smaller size while collecting data during image acquisitions.

Once opened in the MetaMorph processing system, each data set with dual-channel images is split into separate Calcium Green and Rhodamine stacks by placing a 384 × 512 region of interest first on the left half, then on the right half of each data set, followed by the "Edit-Duplicate Stack" command (**Fig. 1A**). Subsequently, an offset correction is performed for both the calcium green and rhodamine stacks (**Fig. 1B**). Such corrections compensate for any differences in the Black Level/Gain settings used for the two different channels of the confocal apparatus. To determine the offset that is to be subtracted from each image of the calcium green and rhodamine stacks, a modification of the procedure outlined by Finkbeiner ***(26)*** is used (*see* **Note 11**). Once the offset for each stack is determined, the offset value can be subtracted from every image in the stack by using the "Process-Arithmetic" command with the operation set to "Subtract" for positive offests or "Add" for negative offsets, and "Source 1–All Planes" set to the stack to be offset corrected. The "Source 2" is then set to "Constant," the "Result Depth" set to "8," and the Value set to the absolute value of the calculated offset. Typically, offsets range from –10 to +10 when using commonly employed settings of the photomultiplier tubes.

Following such procedures each offset-corrected stack of images is subjected to a thresholding procedure, in order to minimize background noise in the ratioed images (**Fig. 1C,D**). Such thresholding is accomplished using the "Process-Threshold" command, with the mode set to "Clip to Gray Level" and the gray level set to "0." With such parameters, the "High" slider is moved to the left from its initial 255 position until a thresholding range is found that ideally captures all of the background pixels, but none of those in the dye-injected cells. In most cases, however, a few pixels within the fluorescent cells also occur within the threshold range and/or some of the background pixels are not within range. But, for the most part, the threshold range that is selected should separate the background from the foreground (i.e., the fluorescent cells in each stack).

After thresholding, the calcium green stack is divided by the rhodamine stack by using the Process-Arithmetic command, with the operation set to "Divide,"

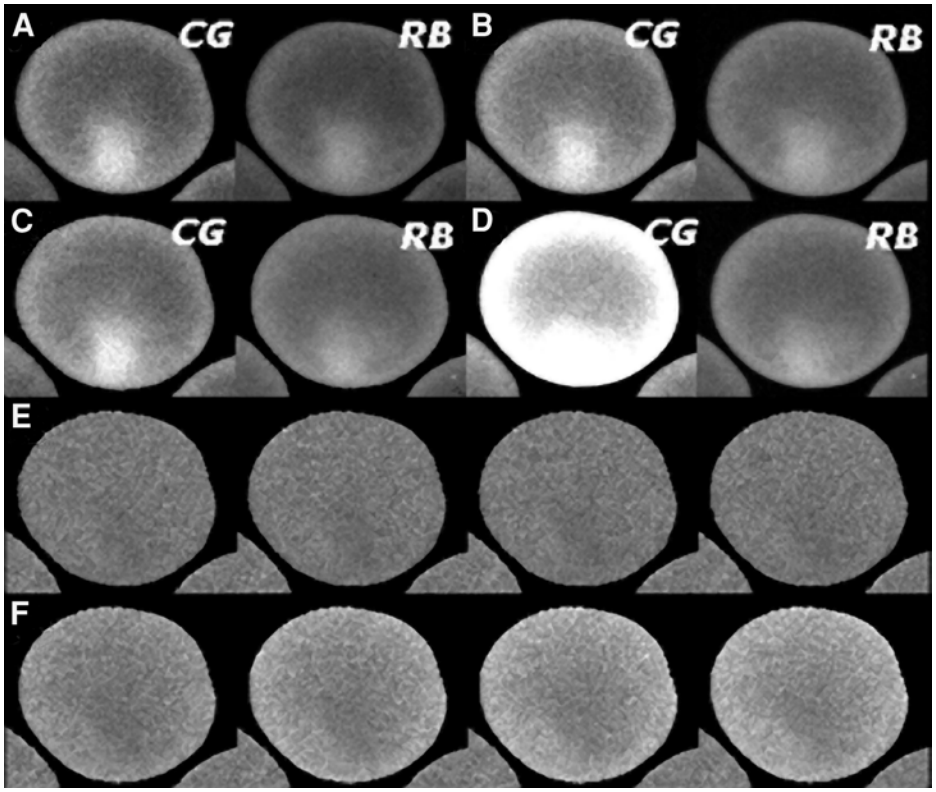

Fig. 1. Dual-channel confocal ratioing of fertilization-induced calcium dynamics in the nemertean worm *Cerebratulus* sp. **(A)** Separated Calcium Green (CG) and Rhodamine B (RB) images, showing a single optical section of an unfertilized oocyte approx 90 μm in diameter). Parts of two other injected oocytes are seen in the lower left and right corner of each image. **(B)** Images after offset correction wherein an offset of –8 and –10 was subtracted from the CG and RB images, respectively. **(C,D)** Offset-corrected and thresholded images before fertilization (C) and after a fertilization-induced calcium wave had spread through the oocyte (D). Note how the CG fluorescence has increased in D, whereas the RB signal remains relatively steady. **(E)** A montage of four CG/RB divided images taken 10 s apart just before the first calcium elevation of fertilization is elicited. Note how the ratioing procedure provides a relatively uniform signal throughout the unfertilized oocyte as compared to the artifactually elevated fluorescence in the cortex (=periphery) of nonratioed images, as well as the artifactual bright spot at 6 o'clock corresponding to the meiotic apparatus. (For more details on such artifacts, *see* **ref. *6***). **(F)** A montage of four CG/RB divided images taken 10 s apart as the first relatively low-amplitude calcium elevation of fertilization (=cortical flash) is elicited.

the Second Source set to "Image," the Result Depth set to "8," and the Source 1 and Source 2 set to the offset-corrected and thresholded calcium green and rhodamine stacks, respectively. In addition, prior to constructing the divided images, the "Numerator" is set to "64," whereas the Denominator is set to "1," to stretch the resultant images optimally across the 0–255 gray level scale.

The divided stack thus represents dual-channel calcium green/rhodamine ratioed images that help to correct for differential path length and dye-loading artifacts. Although such stacks do not readily lend themselves to quantitative measurements of calibrated calcium levels, they do provide valuable information regarding relative changes in calcium concentrations as compared to the baseline values at the onset of imaging. In this way, data from these stacks can be outputted in the form of a graph by placing one or more regions of interest in the divided images and subsequently constructing an Excel file using the Measure Brightness command of the MetaMorph software package. Alternatively, ratioed images can be configured in the form of a multiframe sequence by means of the Montage-Stack command and simply rendered as black-white images (**Fig. 1E,F**). In addition, a pseudocolor lookup table (e.g., the "geog" lut of Bio-Rad Corp.) can be applied to the ratioed images in order to yield better gray level discrimination than is available in monochrome images.

4. Notes

1. Sea urchin eggs complete meiosis within the ovary. Thus, no maturation-inducing hormone is required after eggs are spawned from fully ripe females ***(27)***. To elicit the resumption of meiosis and hence the progression to a fertilizable state in starfish and nemertean oocytes, ASWs can be treated with 1–5 μ*M* doses of 1-methyladenine or 5-hydroxytryptamine (Sigma Chemical Co.), respectively ***(28,29)***.
2. Aged eggs of sea urchins and starfish lose their capacity to undergo normal fertilization, presumably owing to apoptotic degradation ***(30,31)***. Similarly, nemertean oocytes that have matured to a metaphase I arrest point show suboptimal fertilization rates within a few hours after maturing, whereas immature oocytes arrested at prophase-I of meiosis can still undergo normal fertilization when subsequently matured and inseminated 24 h after removal from the ovary ***(32)***. Thus, to prolong the useful life of marine invertebrate oocytes, it may be possible to store oocytes in an immature state and/or with blockers of mitogen-activated kinases to retard degradation ***(33)***.
3. Protamine sulfate solutions are best mixed in glass containers (e.g., scintillation vials). In order to avoid incomplete solubilization of the protamine sulfate, the powder should be placed in a clean container, and water should be added without significant agitation for 30 min. Subsequently, the vial should be vortexed for at least 1 min. Such solutions can be used for up to 12 h, but become less effective

thereafter. In our experience, poly-L-lysine solutions tend to be more toxic than protamine sulfate.

4. Stock solutions of 2 m*M* calcium green dextran plus 0.5 m*M* rhodamine B dextran are mixed in the injection buffer. After 5 min centrifugation at 14,000*g* to sediment particulate material, the mixture is partitioned into 5-µL aliquots and frozen at –20°C. Freeze–thaw cycles and unnecessary exposures to bright room lights should be avoided. Under such conditions, the solutions remain capable of recording intracellular calcium fluxes for at least 1 yr.
5. As covered in more detail by Summers et al. ***(34)***, eggs and oocytes can be injected with dextran-conjugated dyes via a high-pressure picospritzer system corresponding to the kind described here, or they can be loaded by means of a low-pressure system, such as outlined by Kishimoto ***(35)***. In either case, it is possible to inject cells using commercially available micropipets (e.g., Femtotips, Brinkmann, Westbury, NY) or with borosilicate micropipets produced using a micropipet puller (e.g., Narashige PB-7; Narishige International USA, East Meadow, NY). For a detailed discussion of pipet pulling techniques and high-pressure microinjections, the reader is referred to DePamphilis et al. ***(36)*** and Summers et al. ***(34)***.
6. At least a Pentium II grade processor running at 300 MHz or above is required for handling the large data sets generated by this protocol. Certain routines (e.g., ratioing of confocal stacks) are optimized when there is at least twice as many megabytes of RAM as the size of the file to be processed.
7. To strip eggs/oocytes of their surrounding jelly coats, specimens can be washed several times in pH 5.0 seawater (2 µL of 0.5 *M* citric acid/mL of seawater to be titrated ***[34]***). Alternatively, mechanical removal can be achieved by passing the oocytes through an appropriate mesh Nitex filter (i.e. approx 20–30 µm larger than the diameter of the fully grown specimens to be dejellied). The filter can be made from a 1 × 1-cm patch of Nitex-brand mesh (Tetko, Inc., Briarcliff Manor, NY) that is glued with a hot-melt glue gun over the cut end of a 10- or 50-cc plastic syringe. The eggs/oocytes are then manually pulled with the plunger of the syringe several times back and forth across the Nitex mesh to dejelly them.
8. An inexpensive and easily manufactured specimen dish can be made from a 60-mm plastic Petri dish in which a 20-mm hole is made using a heated cork borer. The dish is then completed by gluing a no. 1 coverslip over the hole ***(37)***. For such dishes, epoxy glue should be used rather than "Super/Krazy glues" (cyanoacrylates), because the latter can be more toxic even after extensive curing. To attach eggs, coat the coverslip with 0.5 mL of the protamine sulfate solution for 5–10 min. Most of the solution is then discarded, and the remaining film is rubbed onto the coverslip with a clean powder-free latex glove in order to coat the entire surface more evenly. Approximately 10 mL of ASW or filtered NSW is then added to the coated dish, and a concentrated drop of eggs/oocytes is slowly added by means of a drawn out microcapillary pipet (e.g., 50 µL) so that the cells fall directly onto the coverslip in a high-density monolayer. Such additions are best achieved by mouth-pipetting, in order to obtain a steady stream from the tip

that is positioned as close to the coverslip as possible. Following addition of the eggs/oocytes, the dish should remain undisturbed for several minutes so that the specimens bind tightly to the coverslip. The dish is then transferred to the confocal microscope with the least amount of agitation possible. If the cells loosen, the following steps can be attempted to maximize attachment: (1) mix fresh protamine sulfate; (2) dejelly the eggs/oocytes again (because the jelly coat can reform wiyh long sittings); or (3) if possible, inject the specimens on the confocal microscope set-up itself, to avoid any dislodgings that occur during transfer from the microinjection set-up. Alternatively, if the samples will not stick properly to the protamine sulfate-coated dish, oocytes and eggs can be held securely in a modified Kiehart-type chamber ***(35)***.

9. To estimate the overall amount of dye delivered, the size of the initial injection bolus can be visually monitored so that approx 1–5% of the total cell volume is introduced. Using such qualitative methods, relatively uniform injections can be delivered so that neighboring cells in the field of view have similar baseline fluorescence signals (*see* **Fig. 1A** in **ref**. ***22***). Alternatively, for more quantitative methods of injection, the reader is referred to ***(35)***.
10. Numerous confocal microscopes that are more sophisticated than the Bio-Rad MRC-600 model are currently available. Such newer systems not only can be substituted in the protocol that is outlined here, but they can often provide a significant upgrade in capabilities. In preparing the MRC-600 confocal microscope for imaging, the confocal scan head and laser should be warmed up for at least 30 min in order to minimize baseline drift during imaging. Moreover, each photomultiplier tube of the two channels should be configured with the Black Level and Gain adjustments so that the background is dark, but not completely black, to avoid the clipping of relevant signals.
11. At the beginning and end of each imaging run, collect a calibration file consisting of two images as follows and make sure not to change any of the PMT settings or the confocal pinhole apertures used during imaging. Collect the first image of calibration file at the same ND setting used in imaging run (e.g., ND 3 = 1% transmittance). Collect the second image after changing the neutral density filter to ND 2 setting (= 3% transmittance). To determine offset, open up the calibration file in MetaMorph and set a region of interest (ROI) in different locations in the sample on the left half of the dual-channel image. Similarly, set three ROIs set in the right half of the image (note: the left and right sides do not have to match.) Then, calculate the average fluorescence of the six ROIs, by using: (a) "Log"–"Open Data Log"; (b) "Measure Brightness"; and (c) "Log"–"View Current Data Log" to display data as follows:

Plane	ROI 1	ROI 2	ROI 3	ROI 4	ROI 5	ROI 6
1	50	45	55	72	66	68
2	150	144	174	225	210	213

where ROI1–3 = average fluorescence in the three ROIs in Channel 2 (calcium green signal in left half of calibration images); ROI 4–6 = average. fluorescence

in the 3 ROIs in Channel 1 (Rhodamine B signal in right half of calibration image); plane 1 = ND 1%; plane 2 = ND 3%. To calculate the offset for Channel 2 in this particular example: (1) divide each of the three ND 3% readings by 3 (i.e. 150/3; 144/3; 174/3 = 50; 48; 58); (2) subtract each (ND 3%/3) value from the corresponding ND 1% (i.e., 50 – 50 = 0; 45 – 48 = –3; 55 – 58 = –3); (3) note: make sure to subtract the (ND 3%/3) from the ND 1%, and find the average of the three values (0 + –3 + –3 = –6/3 = –2); (4) multiply this average by 3/2 (–2 × 3/2 = –3). This –3 value is your offset for channel 2. Repeat the process for the three pairs of numbers obtained for Channel 1 (i.e., calculate the values for ROI 4–6 as described previously to obtain an offset of –5). These offsets must be subtracted from all of the pixel intensities obtained in your imaging run (i.e., add 3 to each pixel in the Channel 2 images, and add 5 to each pixel in the Channel 1 images in your time-lapse run).

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9

A Method for Producing Cloned Pigs by Using Somatic Cells as Donors

Liangxue Lai and Randall S. Prather

Introduction

Nuclear transfer (NT) is a procedure by which genetically identical individuals can be created. The applications of these NT techniques will be in agriculture, biomedicine, and basic research. Based on the source of donor cells, NT can be classified into embryonic cell NT and somatic cell NT. Somatic cell NT was first reported in 1996 ***(1)*** and includes more practical applications. Most importantly, it provides a promising method for producing transgenic animals. This concept is exemplified by the generation of transgenic sheep ***(2)***, pigs ***(3,4)*** and calves ***(5)***, along with gene-targeted sheep ***(6)*** and pigs ***(7)***, derived from NT approaches by using transfected somatic cells. For pigs, somatic cell NT has another specific significance, as it allows the use of genetic modification procedures to produce tissues and organs from cloned pigs with reduced immunogenicity for use in xenotransplantation ***(7)***. However, when measured as development to term as a proportion of oocytes used, the efficiency of somatic cell NT, has been very low (1–2%). Several variables influence the ability to reproduce a specific genotype by cloning. These include species, source of recipient ova, cell type of nuclei donor, treatment of donor cells prior to NT, the method of artificial oocyte activation, embryo culture, possible loss of somatic imprinting in the nuclei of reconstructed embryos, failure of adequate reprogramming of the transplanted nucleus, and the techniques employed for NT. In some species (e.g., pigs) there is an additional difficulty in that at least four good embryos are required to induce and maintain pregnancy.

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Procedures for NT include the following steps: acquisition of recipient oocytes and donor cells, enucleation (removal of the chromosome from recipient oocytes), insertion of donor nuclei into enucleated oocytes, artificial activation of reconstructed oocytes, and embryo transfer (transfer of the reconstructed embryos into a surrogate).

2. Materials

2.1. Solutions and Media

1. Cell culture medium: Dulbecco's modified Eagles' medium (DMEM), 15% fetal bovine serum (FBS), and antibiotics (100 IU/mL penicillin, 100 µg/mL streptomycin). Store at 4°C.
2. Digestion solution: Collagenase stock: 2000 U/mL in Dulbecco's phosphate-buffered saline (DPBS). Store at 4°C. Trypsin-EDTA solution: 0.05% Trypsin, 0.53 m*M* ethylenediaminetetraacetic acid (EDTA) in DPBS. Store at 4°C.
3. Wash solution: DPBS without calcium and magnesium.
4. Freezing medium: DMEM, 15% FBS, and 10% dimethyl sulfoxide (DMSO).
5. Saline plus antibiotics: 9 g NaCl, 0.012 g penicillin, 0.020 g streptomycin, 0.025 g gentamicin, 1000 mL double-distilled (dd)H_2O. Filter saline using a 0.22-µm filter unit. Store at 4°C.
6. 1X PVA-TL HEPES stock: 6.663 g NaCl, 0.237 g KCl, 0.168 g $NaHCO_3$, 0.041 g NaH_2PO_4, 1.868 mL Na lactate, 0.102 g, $MgCl_2 \cdot 6H_2O$, 2.383 g HEPES, 0.065 g penicillin G, 0.0l0 g phenol red, 0.294 g $CaC1_2 \cdot 2H_2O$, 0.l00 g polyvinylalcohol (PVA), 2.186 g sorbitol, 0.025 g gentamicin, 0.022 g pyruvate, and 1000 mL ddH_2O. Mix all chemicals together in the order given. After all reagents are added, adjust the pH to 7.40 using 1 *M* HCl and 1 *M* NaOH stocks. Next, check the osmolarity (275–280). Filter medium with 0.22-µm filter unit. Before mixing medium, add PVA to approx 100 mL of ddH_2O, and use mild heat and stirring to dissolve, then add to medium. Store at 4°C. Discard medium after 2–2.5 wk.
7. Maturation medium: basic medium: TCM-199, 0.1% PVA, 3.05 m*M* D-glucose, 0.91 m*M*, sodium pyruvate, 75 µg/mL penicillin, and 50 µg/mL streptomycin. The following are added fresh each time before use: 0.57 m*M* cysteine, 0.5 µg/mL Luteinizing hormone (LH), 0.5 µg/mL follicle-stimulating hormone (FSH), and 10 ng/mL epidermal growth factor (EGF) (*see* **Notes 1** and **2**).
8. Denuding medium: 5.460 g mannitol, 0.001 g bovine serum albumin (BSA), 5 mL TL-HEPES stock, 0.030 g hyaluronidase, and 95 mL ddH_2O. In a 100-mL graduated cylinder, place the TL-HEPES stock and approx 80 mL of ddH_2O. Add the other 3 chemicals. Fill to 100 mL final volume with ddH_2O. Adjust the pH to the 7.0–7.4 with 1 *M* HC1 and 1 *M* NaOH stocks. Filter with 0.22-µm filter unit. Place 1-mL aliquots in centrifuge tubes and store in the –20°C freezer.
9. Embryo manipulation medium: 9.500 g TCM-199, 0.050 g $NaHCO_3$, 0.750 g HEPES, 0.050 g penicillin, 0.060 g streptomycin, 1.755 g NaCl, 3.00 g BSA, and 1000 mL ddH_2O. After all the reagents are added, adjust the pH to7.2–7.4 with 1 *M*

HCl and 1 *M* NaOH stocks. Next check the osmolarity (295–310). Filter medium with a 0.22-μm filter unit. Store at 4°C. Discard after 2 wk.

10. Cytochalasin B stock: 1 mg cytochalasin B and 200 μL 100% ethanol. Store in the dessicator at -20°C for up to 2 wk.
11. Enucleation medium: 4 μL cytochalasin B stock and 3 mL embryo manipulation medium. Place 100-μL drops in a 100-mm Petri dish and cover with light mineral oil. Once mixed together, this medium is to be kept in the incubator and used within 48 hr discarded.
12. NCSU-23 (North Carolina State University) medium: 3.178 g NaCl, 1.053 g $NaHCO_3$, 0.178 g KC1, 0.081 g KH_2PO_4, 0.147 g $MgSO_4 \cdot 7H_2O$, 0.125 g $CaC1_2 \cdot 2H_2O$, 0.500 g glucose, 0.073 g glutamine, 0.438 g taurine, 0.273 g hypotaurine, 0.033 g penicillin G, 0.025 g streptomycin, and 500 mL ddH_2O. Mix all chemicals together in the order given. After all the reagents are added, adjust the pH to 7.4 with 1 *M* HCl and 1 *M* NaOH stocks. Check the osmolarity (295–310). Filter medium with a 0.22-μm filter unit. Store at 4°C until needed. Discard after 2–2.5 wk.
13. Embryo culture medium (NCSU-23 with BSA): 100 mL NCSU-23 and 400 mg BSA. After the BSA dissolves completely, filter medium with a 0.22-μm filter unit. This medium should be made fresh weekly. For embryo culture, add 500 μL this medium into a well (four-well multi-well dish) and cover with 100–200 μL light mineral oil. Place the prepared dishes in incubator (39°C, 5% CO_2 in air) for at least 3 h before adding reconstructed oocytes.
14. Fusion medium ***(4)***: 0.3 *M* mannitol, 1.0 m*M* $CaCl_2$, 0.1 m*M* $MgCl_2$, and 0.5 m*M* HEPES. Filter medium with a 0.22-μm filter unit. Store at 4°C. Warm to 39°C before use.

2.2. Instruments

1. CO_2 incubator: For cell and embryo culture.
2. Micromanipulator: For enucleation and microinjection.
3. Inverted microscope with epifluorescence and warm stage: For enucleation and microinjection.
4. Stereo microscope with warm stage: For embryo and oocyte manipulation.
5. Sachs-flaming micropipet puller: For making enucleation and injection pipet.
6. Microforge: For making enucleation and injection pipet.
7. Grinding wheel: For making injection pipet.
8. BTX Electro-Cell Manipulator: For activation and fusion.
9. Coagulation tubes: For enucleation and injection pipet

3. Methods

3.1. Preparation of Donor Cells

Ten different somatic cell types have proven successful for complete cloning competence thus far in mouse ***(8)*** and cattle ***(9)***. In pigs, fibroblast and

cumulus cells have been clonable ***(3,4,7,10)***. Fetal fibroblasts are excellent for use as the nucleus donors to produce cloned pigs because fetal fibroblasts are dispersed in connective tissues throughout the fetal body, and they are capable of extensive proliferation, which is an advantage for genetic modification of donor nuclei before NT. Therefore isolation of the fetal fibroblast is described in this chapter.

3.1.1. Recovery of Fetuses

1. Euthanize the mated pig approx 35–40 d after fertilization.
2. Open the abdominal cavity and remove the entire uterus.
3. Exteriorize the conceptuses from the uterus and transfer them into a plate, then cut the membrane and expose the fetuses in a clean bench.
4. Thoroughly rinse fetuses with DPBS three times.
5. Remove head, intestine, liver, and heart.
6. Finely mince the fetuses into 1-mm^3 pieces in DPBS by using scissors.
7. Collect the minced tissue and transfer into a 15-mL tube. When the tissues settle, remove the buffer.

3.1.2. Tissue Digestion

3.1.2.1. Collagenase Method

1. Transfer tissue pieces to a 125-mL flask, then add 45 mL DMEM + 15% FBS, 5 mL collagenase stock (2000 U/mL), then incubate at 39°C for 24 h without agitation.
2. Disperse tissue pieces by vigorously aspirating them in and out of pipet, then transfer the solution into a 50-mL tube. Let residual clusters of tissues settle for 5 min.
3. Carefully aspirate dispersed cell suspension without residual clusters of tissues into another 50-mL tube. After centrifugation at 500*g* for 5 min, remove supernatant.
4. Resuspend the pellet in 15-mL cell culture medium and seed the cells in a 250-mL culture flask for further culture.

3.1.2.2. Trypsinization Digestion Method

1. Transfer tissue pieces into a 50-mL tube, add 10 mL trypsin-EDTA solution, and incubate at 39°C for 30 min with shaking.
2. Add 40 mL cell culture medium. Disperse tissue pieces by vigorously aspirating them in and out of the pipet, and transfer the solution into a 50-mL tube. Let residual clusters of tissues settle for 5 min. Then carefully aspirate dispersed cell suspension without residual clusters of tissues into another 50-mL tube. After centrifugation at 500*g* for 5 min, remove supernatant.
3. Resuspend the pellet in 15-mL cell culture medium and seed the cells in a 250-mL culture flask for further culture.

3.1.2.3. Cluster Culture Method

1. Transfer the minced tissue pieces without digestion into a 250-mL flask, add 20 mL cell culture medium, and incubate at 39°C for 24 h–48 h without agitation.
2. After formation of monolayer cells, remove the medium and rinse the cells with DPBS.
3. Add 10 mL trypsin-EDTA solution to digest cells for 5–10 min at 39°C.
4. Add 40 mL cell culture medium. Disperse cells by vigorously aspirating them in and out of the pipet.
5. Transfer the solution into a 50-mL tube. Let residual clusters of tissues settle for 5 min.
6. Carefully aspirate dispersed cell suspension without residual clusters of tissues into another 50-mL tube. After centrifugation at 500*g* for 5 min, remove supernatant, resuspend the pellet in 15 mL cell culture medium, and seed the cells in two or three 250-mL culture flasks for further culture.

3.1.3. Passage and Subculture

1. When the cells are confluent, remove the medium and rinse the cells with DPBS.
2. Add 5 mL trypsin-EDTA solution. Incubate at 39°C for 5–10 min.
3. Add 10-mL culture medium. Pipet bottom of the flask and collect the cells into a 15-mL tube.
4. Centrifuge the cell suspension at 500*g* for 5 min, then remove the supernatant, and add 10 mL culture medium and resuspend cells.
5. Aliquot the cells into three 250-mL flasks. Put them into the incubator for further culture.

3.1.4. Freezing Early Passaged Cells

After cells grow confluently, they need to be frozen in liquid nitrogen for long-term storage.

1. Remove the medium and rinse the cells with DPBS.
2. Add 5 mL trypsin-EDTA solution. Incubate at 39°C for 5–10 min. Then add 10 mL culture medium. Pipet bottom of the flask and collect the cells into a 15-mL tube. Centrifuge the supernatant at 500*g* for 5 min. Remove the supernatant, then add 1mL freeing medium and resuspend cells.
3. Determine the cell concentration, dilute the cells to 10^7/mL, and aliquot the cells into freezing tubes (100 µL/vial). Transfer tubes with cells into a freezing box. Keep cells in –80°C overnight.
4. Transfer the tubes with cells from –80°C to liquid nitrogen.

3.1.5. Thawing the Cells Prior to NT

1. Cryopreserved donor cells are thawed at 39°C and 200 µL FBS is added. The suspension is kept at room temperature for 30 min. Subsequently, 800 µL cell culture medium is added and the cells are pelleted at 500*g* for 5 min.

2. The supernatant is removed and embryo manipulation medium is added to resuspend the cells (*see* **Note 3**).

3.2. Collection of Oocytes

3.2.1. Collection of In Vivo Matured Oocytes (10)

1. Oral administration of 18–20 mg Regumate (Altrenogest, Hoechst) mixed into the feed for 4–14 d by using a scheme dependent on the stage of estrous cycle.
2. Estrumate 250 μg (Bayer) is administered intramuscularly (im) on the last day of the Regumate treatment.
3. Superovulation is induced with single im injection of 1500 IU of pregnant mare serum gonadotropin (PMSG) 15–17 h after the last Regumate feeding, and 1000 U of human chorionic gonadotropin (hCG) are administered im 82 h after the PMSG injection.
4. Collect oocytes 46–54 h after the hCG injection by reverse flush of oviducts by using prewarmed TL-HEPES containing 0.3% BSA.

3.2.2. Collection of in vitro Matured Oocytes

For pig NT, matured oocytes are needed in large numbers and in vivo matured oocytes are very expensive to acquire. Thus, many laboratories have chosen to use in vitro matured oocytes. Immature oocytes are derived from ovaries obtained from the slaughterhouse and subsequently matured in vitro. Recently, progress of in vitro production of pig embryos in our laboratory has resulted in a routine chemically defined system of producing embryos that are developmentally competent ***(11)***. By using this defined system, a large number of oocytes can be synchronized in a specific development stage.

1. Prepubertal porcine ovaries are collected from an abattoir and transported to the laboratory in a thermos filled with saline maintained at 30–35°C within 4 h after collection.
2. Ovaries are washed 3–4 times with saline at 37°C to remove the blood. Follicular fluid from 3–6-mm antral follicles is aspirated by using an 18-gauge needle attached to a 10-mL disposable syringe.
3. Cumulus-oocyte complexes (COCs) with uniform cytoplasm and several layers of cumulus cells are selected and rinsed two times in TL-HEPES plus PVA and three times in basic maturation medium.
4. Approximately 50–70 COCs are transferred into each well of four-well multidishes containing 500-μL maturation medium covered with 100 μL-light mineral oil. The oocytes are matured for 42–44 h at 39°C, 5% CO_2 in air.

3.3. NT

3.3.1. Treatment of Matured Oocytes

1. After maturation, oocytes are transferred into denuding medium in a 1.5-mL centrifuge tube.

2. Vigorously vortex oocytes for 4–5 min, then transfer oocytes into embryo manipulation medium in a 35-mm dish.
3. Oocytes with an intact plasma membrane, round shape, and visible perivitelline space are selected and kept in embryo manipulation medium until use.

3.3.2. Treatment of Donor Cells

Considerable variation in development exists between individual cell populations, and, at present, no definitive method is available for identification of cell populations that are best suited for NT. Factors that seem to influence the suitability include the effects of oxidative damage associated with metabolism, genome instability, and chromosomal pathologies. All of these factors may be influenced by the method of isolation and culture and the number of population doublings in culture. Even different subclones of fibroblasts derived from the same fetus and cultured under the same conditions at the same generation lead to different in vitro developmental potential of reconstructed embryos ***(12)***. Another factor affecting the efficiency of NT is the cell cycle phase of donor cells, which is still a topic being debated in the NT field. Wilmut et al. ***(13)*** state that the donor cells for NT must be in G0 of the cell cycle (quiescent phase), but Cibelli et al. ***(14)*** later showed that cycling cells, which contained cells in different cycle stages, could successfully be used for NT in cattle. G2/M stage cells are another choice, as shown in some early studies on NT using G2/M stage blastomeres as nuclear donors to produce cloned mice ***(15)***, and sheep ***(16)***, and in recent successes using G2/M stage embryonic stem (ES) cells as donors to produce cloned mice ***(17–19)***. Also, fetal fibroblasts treated with colchicine, which synchronizes the cells at the G2/M stage of the cell cycle, produced a live cloned pig ***(4)***.

3.3.3.1. Creation of Cycling Cells

1. Thaw cells as described in **Subheading 3.1.5.** After centrifugation, remove the supernatant and add 500 µL culture medium to resuspend the cells, then transfer the cells into a well of a four-well dish.
2. After culturing 2–3 d, before cells are confluent, remove the medium, rinse the cells with DPBS, and add 100 µL trypsin-EDTA solution. Incubate at 39°C for 5 min.
3. Add 500 µL culture medium. Pipet bottom of the well and collect the cell suspension into a 1.5 mL centrifuge tube and centrifuge at 500*g* for 5 min. Remove the supernatant.
4. Centrifuged pellet is resuspened in 200 µL manipulation medium.

3.3.3.2. Creation of Serum-Starved Cells

1. Thaw cells as described in **Subheading 3.1.5.** After centrifugation, remove the supernatant and add 500 µL culture medium to resuspend the cells.

2. Transfer the cells into a four-well dish.
3. After culturing 12–24 h, before cells are confluent, remove the old medium completely, and add 500 µL DMEM with 0.5% FBS and culture for 5 d, or add 500 µL DMEM with 0.1% FBS for 3 d, and collect the cells by trypsinization treatment as described in **Subheading 3.3.3.1.**
4. Resuspend centrifuged pellet in 200 µL manipulation medium.

3.3.3.3. Creation of Colchicine Treated Cells

1. Thaw cells as described **Subheading 3.1.5.** After centrifugation, remove the supernatant and add 500 µL culture medium to resuspend the cells, then transfer the cells into a well of a four-well dish.
2. After culturing 12–24 h, before cells become confluent, remove the old medium completely, and add 500 µL cell culture medium with 1.0 µ*M* colchicine and culture for 24 h.
3. Collect the cells by trypsinization treatment as described in **Subheading 3.3.3.1.**
4. Resuspend centrifuged pellet in 200 µL of manipulation medium.

3.3.4. Enucleation of Matured Oocytes

Oocytes need to be enucleated before use in NT. One of the chemicals routinely used during enucleation to aid in visualizing the chromosomes is bisbenzimide.

1. The matured oocytes are stained with 5 µg/mL bisbenzimide in manipulation medium for at least 30 min.
2. Oocytes are transferred into the drops of enucleation medium in 100-mm dishes covered with light mineral oil. Five minutes later, enucleation is accomplished by aspirating the first polar body and the metaphase II plate in a small amount of surrounding cytoplasm by using a beveled glass pipet with 25–30 µm in diameter.
3. Withdraw the pipet from the oocytes. Confirmation of successful enucleation is observed by visualizing the karyoplast, while still inside the pipet, under violet light (*see* **Note 4**).

3.3.5. Construction of NT Embryos

There are two approaches to put the donor nuclei into the cytoplasts: one is direct injection of donor nuclei into enucleated oocytes and the other is to inject the intact donor cell into the perivitelline space and subsequently fuse the donor cell with the recipient oocyte by electrical pulses. With direct microinjection, the plasma membrane and much of the cytoplasm material of the donor cell is not transferred, and only remnants of cytoplasm are injected with the donor nucleus. In contrast, with cell fusion, all of the components of the donor cell (nuclear, cytoplasmic, and plasma membrane) merge with the enucleated oocyte.

3.3.5.1. Direct Injection of the Donor Nucleus into the Cytoplasm of Enucleated Oocytes

1. After enucleation, oocytes are transferred to NCSU-23 with BSA medium and kept at 39°C, 5% CO_2 in air.
2. Put the enucleated oocytes and donor cells into the drops of embryo manipulation medium in 100-mm dishes covered with light mineral oil.
3. Plasma membrane of fibroblast cell is broken and visible cytoplasmic material is removed by gently aspirating it in and out of the injection pipet that has a sharp beveled 10-μm (for G0/G1 stage donors) or 15-μm (for G2/M stage donors) diameter tip ***(4)***.
4. The nucleus and remaining cellular debris are then injected into the cytoplasm of enucleated oocytes by using the same slit in the zona pellucida as made during enucleation (*see* **Note 5**).

3.3.5.2. Injection of Donor Cells into the Perivitelline Space of Enucleated Oocytes

1. Put the donor cells and unenucleated oocytes into the same drop of enucleation medium in 100-mm dishes covered with light mineral oil.
2. Enucleate oocytes as described above.
3. Withdraw the pipet from the oocytes after enucleation is accomplished, and push the cytoplasm with chromosomes and polar body out of the pipet.
4. Take a single donor cell into the same pipet and inject the cell into the perivitelline space of enucleated oocytes using the same slit in zona pellucida as made during enucleation.
5. Injected oocytes are kept in embryo culture medium before fusion and activation.
6. Fusion is achieved with two 2 DC pulses (1 s interval) of 1.2 kV/cm for 30 μs on a BTX Electro-Cell Manipulator 200 (BTX, San Diego, CA) ***(3,4)*** in a chamber consisting of platinum wire electrodes 1 mm apart (**Fig. 1**).
7. Embryos are kept in NCSU-23 with BSA for another 40–60 min before the fusion rate is evaluated (*see* **Note 6**).
8. Fused embryos are cultured in 500 μL NCSU 23 with BSA overlayed with mineral oil.
9. The surviving embryos (intact plasma membrane) are selected for transfer into surrogates after culture for 18–22 h

3.3.6. Activation

After the donor cells are transferred into the enucleated oocytes, the reconstructed oocytes must be activated to initiate subsequent development. Activation of oocytes can be induced artificially by a variety of physical and chemical agents.

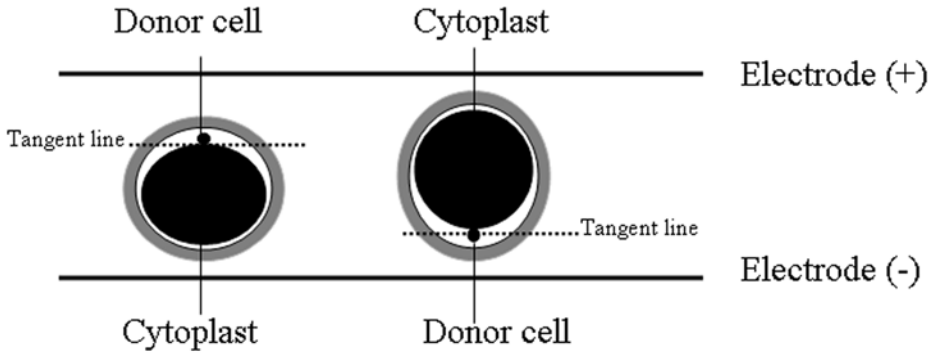

Fig. 1. Align the oocytes to make imaginary tangent lines across the oocyte and donor cell parallel to the two electrodes.

3.3.6.1. Electrical Activation

With the above fusion medium (with 1.0 m*M* Ca^{2+}), oocyte activation can be achieved during fusion. If the reconstructed oocytes are made by direct injection of donor nuclei into the cytoplasm, the oocytes can be activated electrically in this fusion medium with the same parameters used for fusion.

3.3.6.2. Chemical Activation

Combined thimerosal/dithiothreitol (DTT) treatment of the oocytes also can effectively activate porcine oocytes ***(20)***.

1. Treat the oocytes with 200 μ*M* thimerosal in embryo manipulation medium for 10 min, then wash the oocytes in embryo manipulation medium one time.
2. Treat the oocytes with 8 m*M* DTT for 30 min, wash oocytes in embryo manipulation medium one time, and then wash oocytes in NCSU-23 with BSA two times (*see* **Note 7**).

3.3.6.3. Electrical Fusion Combined with Chemical Activation ***(21)***

1. Fuse the donor cells into the cytoplasm of enucleated oocytes in a chamber consisting of platinum wire electrodes 500 μm apart and SOR2 medium (0.25 *M* sorbitol, 1.0 calcium acetate, 0.5 m*M* magnesium acetate, 0.1% BSA, pH 7.2, and 250 mOsm osmolarity) with an electrical pulse of 95 V for 45 μs.
2. Treat the reconstructed oocytes with 15 μ*M* ionomycin for 20 min, followed by 1.9 m*M* 6-dimethylaminopurine (DMAP) in CR2 medium for 3–4 h.
3. Wash oocytes in embryo manipulation medium one time, then wash oocytes in NCSU-23 with BSA two times.

3.3.7. In Vitro Culture of Embryos

After activation, embryos are transferred to 500 µL embryo culture medium in a four-well dish and covered with 100–200 µL light mineral oil. A pronucleus can be found 8–15 h after activation. Cleavage can occur 24–36 h after activation. Blastocysts can form on d 6–7 after activation.

3.4. Preparation of Surrogate Gilts

3.4.1. Naturally Cycling Surrogate Gilts

Potential surrogates are heat checked twice a day. Depending on the exact time of estrus, NT-derived embryo transfers can be performed 5– 36 h following the onset of estrus *(7)*.

3.4.2. Estrus-Synchronized Surrogate Gilts **(22)**

1. Administer 18–20 mg Regumate (Altrenogest, Hoechst) mixed into the feed for 14 d using scheme dependent on the stage of estrous cycle.
2. Inject 1000 U of hCG im 105 h after the last Regumate treatment.
3. Perform embryo transfer 22–26 h after the hCG.

3.5. Embryo Transfer

Pregnancy initiation in the pig requires a critical minimum signal from four embryos to the mother around d 12 of gestation *(23)*. To minimize any adverse effect from in vitro conditions on the NT development of embryos some researchers have reduced the culture time to a minimum before they were transferred into the surrogate oviduct. Because the NT embryos are generally of a low quality, a large number (>100) of one-cell stage embryos cultured 18–22 h with good shape (intact membrane) are surgically transferred into an oviduct of the surrogate.

1. Anesthesia is initially induced with 30 mL pentothal and maintained with 3% halothane.
2. The surrogate is treated with im injection of 2 mL banamine before the beginning of surgery.
3. While in a dorsal recumbent position, the surrogates are aseptically prepared for surgery and a 10-cm incision is made into the abdominal medline to expose the oviduct.
4. Transfer embryos from culture medium into the manipulation medium and load embryos into a Tomcat catheter attached to a 1-mL syringe.
5. Embryos are placed in the ampullar region of oviduct by inserting 5 cm of the catheter through the ovarian fimbria and into the ampulla (*see* **Note 8**).

4. Notes

1. When maturation medium is made, the basic medium should be stored at 4°C and used within 3 wk. 100X LH and FSH and 1000X EGF stocks can be made and stored long term at -20°C. 5X cysteine stock must be made daily. All stocks should be diluted in basic medium. Make oocyte washing drops in three 35-mm dishes with basic medium and oocyte maturation dishes (four-well multi-well dishes) with 500 µL of the maturation medium. Place the prepared dishes with media in incubator (100% humidity, 39°C, 5% CO_2 in air) for at least 3 h to balance pH before adding oocytes.
2. Although in vitro matured oocytes derived from the ovaries of prepubertal animals can result in development to the blastocyst stage and term ***(3,4)***, oocytes from sexually mature pigs may be more suitable ***(12,21)***. For the maturation of oocytes derived from sows, the procedure is similar to that for the prepubertal pigs. The following maturation medium is recommended ***(12)***: TCM199-HEPES supplemented with 5 µg/mL insulin, 10 ng/mL EGF, 0.6 m*M* cysteine, 0.2 m*M* Na-pyruvate, 3 µg/mL FSH, 25 µg/mL gentamicin, and 10% porcine follicular fluid, cultured at 39°C.
3. If the fetal fibroblast cells have been cultured for short time (<30 d) and passaged a few times (<7 passages) after primary culture, the thawed cells are recommended to be cultured further before being used for NT, because fresh cultured cells fuse at a higher rate. For cells cultured long term, the attachment and proliferative ability becomes very low; thus, the thawed cells should be used as donors for NT immediately.
4. The exposure of oocytes to violet light should be no more than 3 s. Exposure to bisbenzimide has deleterious effects on the development of pig oocytes to the blastocyst stage. Because the condensed chromosomes are always located in the cytoplasm underneath the first polar body, enucleation of in vitro matured metaphase II oocytes can be performed by simply aspirating the first polar body and adjacent cytoplasm without staining the chromosomes. By using this "blind enucleation" method, the enucleation rate varies between 85% and 90% ***(24,25)***.
5. When direct injection method is used to insert the donor nuclei into enucleated oocytes, care should be taken to inject a nucleus into the cytoplasm with as little medium as possible. Be certain that the oocyte membrane is penetrated and the donor nucleus is left inside the oocyte. When the pipet is withdrawn, the puncture in the membrane should be automatically seal immediately.
6. When the fusion method is used to insert the donor nuclei into enucleated oocytes, the round cells with a smooth surface should be selected as the donor cells. Be certain that the donor cell contacts the membrane of oocyte tightly by pushing the donor cell through the zona pellucida with the enucleation pipet.
7. Thimerosal is very sensitive to light. Prevent the medium from exposure to light. If the color of the medium becomes deep, discard it.
8. If enough NT embryos are unavailable for a transfer, the following three strategies have been attempted as an aid to inducing and maintaining pregnancy:

a. NT embryos are cotransferred with 30–40 parthenogenetic embryos of the same developmental stage, which are thought to enhance the signal for maternal recognition of pregnancy, but would degenerate later in gestation because of genomic imprinting ***(26)***.
b. Estradiol, which should be a signal to stimulate the uterus development for accepting attachment of the embryos, is injected on day 12 after embryo transfer ***(4)***.
c. Transfer reconstructed embryos into a naturally bred gilt ***(7)*** on the day the surrogate is fertilized.

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10

Pronuclear Microinjection of Mouse Zygotes

Thomas Rülicke

1. Introduction

The most important tools to investigate the genome of an organism are spontaneous or induced mutations. In mammals, with the exception of humans, the mouse is genetically the most thoroughly analyzed species. Hundreds of different mutant mouse strains are being bred in laboratories and stored as frozen embryos or germ cells in repositories around the world. Because spontaneous mutations are very rare events, different methods to increase the incidence of mutagenesis were developed several decades ago. One example is the alkylating agent *N*-ethyl-*N*-nitrosourea, a powerful tool for producing random point mutations in premeiotic spermatogonia. This "phenotype driven" approach is most helpful to identify unknown genes and their function. Complementary to this large-scale random mutagenesis technique are the so called "gene-driven" approaches, which involve selective manipulation of the mouse genome with the objective of creating transgenic mice. In contrast to chemical mutagenesis, where phenotypic changes are a prerequisite to identify the induced mutation (forward genetics), transgenic technology is based on the reintroduction of a previously isolated and in vitro recombined DNA sequence (reverse genetics).

The term "transgenic" was introduced by Gordon and Ruddle in 1981 ***(1)*** to describe mice whose germline had been genetically modified by the injection of transgenes into one of the pronuclei of a zygote. Today, this term is applied to characterize an organism whose genome (including its germline) has been altered by the stable integration of a recombinant DNA sequence, irrespective of the activity or phenotypic relevance of the foreign DNA and the method used.

Because the germline of mammals is well protected against the incorporation of foreign genetic material, early embryonic stages (i.e., before the cells

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differentiate into the precursors of body and germ cells) are best suited for genetic manipulation *(2)*. Three different methods are used to routinely generate transgenic mice: (a) transfection of germ cells *(3,4)* or early embryos *(5)* with recombinant viral vectors carrying a foreign gene; (b) microinjection of transgenes into one of the pronuclei of a zygote *(6)*; and (c) transfection of pluripotent embryonic stem cells (predominantly used for targeted mutagenesis by homologous recombination) *(7)*.

Most transgenic mice used in biomedical research are generated by pronuclear microinjection of cloned transgenes into one of the pronuclei of a zygote. The successful microinjection of embryonic cells of mammals has been developed with oocytes and zygotes of mice *(8)*. Mammalian geneticists who pioneered this technique recognized the great advantages of choosing the laboratory mouse as their model. In many respects, both biological and economical, the mouse was the ideal tool for this purpose: high fertility under laboratory conditions, short-generation span, relatively low costs for husbandry, and plenty of information on the mouse genome already available. Furthermore, the availability of appropriate and well-characterized inbred strains and mutants, bred and maintained since the beginning of the past century, was crucial in the rapid development of the transgenic technology in mice.

The microinjection of transgenes into one pronucleus involves the introduction of a small amount of defined genetic material directly into the location where a haploid set of the mammalian zygote's DNA is sequestered. This method of DNA transfection has proven to be very efficient and practical in obtaining stable integration and germline transmission of transgenes. Continuous refinement of the process, and yields of often more than 20% transgenic offspring, have led to a broad application of this method in both laboratory and farm animals. Although restricted to the addition and non-targeted integration of a foreign DNA sequence, the pronuclear injection route offers several applications in biomedical research. This chapter focuses predominantly on practical methodological aspects to increase the success of transgenesis in mice by pronuclear microinjection.

2. Materials

2.1. Mouse Strains, Treatments And Animal Husbandry

Several prerequisites are necessary to set up a unit for the successful generation of transgenic mice. First, a variety of different mouse strains are required. It is important to select the appropriate strain or stock for each particular purpose. Additionally, the animals need to be maintained and treated under suitable housing conditions. Finally, the staff needs to have great experience in genetics, cell biology, reproductive biology, and, last but not least - laboratory animal science.

2.1.1. Zygote Donors

1. To provide a high number of zygotes for pronuclear injection, females and males of hybrid strains or outbred stocks are most suitable because of their superior fitness (viability and fertility), as well as their good response to hormonally induced superovulation. However, for certain research projects, it may be important to analyze the transgene against a defined genetic background. In this case, in order to avoid long periods of backcrossing, we use zygotes created by mating the desired inbred strain. Yet, it must be taken into account that inbred strains are less efficient than hybrids in nearly all crucial aspects of pronuclear injection. An alternative approach to save at least one generation of backcrossing is to use the hybrid effect of F1 females as donors mated with males of one of the parental inbred strain. For example, the zygotes of B6D2F1 × C57BL/6 will already share, on average, 75% of the genome with C57BL/6.
2. Two other important aspects influencing the efficiency of transferring genes into mouse zygotes are the size and visibility of the pronuclei. The albino inbred strain FVB/N (FVB for Friend virus B-type susceptibility) is characterized by a high yield of zygotes and large, prominent pronuclei. Both characteristics of the pronuclei facilitate microinjection ***(9)***. In crossbreeds, it has been shown that the phenotype of large pronuclei is a dominant trait of the FVB/N oocyte and is independent of the genotype of the fertilizing sperm. Because this feature is genetically controlled, it can be capitalized on as a useful maternal characteristic in FVB hybrids or in a cross between other inbred strains. Unfortunately, FVB/N is a homozygous carrier of the retinal degeneration 1 mutation that causes blindness ***(10)***.
3. To increase the number of zygotes, donor females are hormonally stimulated to superovulate. The recommended dose of pregnant mare's serum (PMS), used to mimic follicle-stimulating hormone (FSH), and human chorionic gonadotropin (hCG), used to mimic luteinizing hormone (LH), is 5 IU each for most strains. The hormones are injected intraperitoneally with an interval of 46–48 h between the PMS injection and hCG injection (for time of injection, see below). Note that aqueous solutions of hormones are unstable and must be stored frozen.
4. The response to superovulation stimulation depends on the strain and age of females. In most strains, young adults of 6–12 wk of age give good results (i.e., high numbers of well- and equally developed zygotes). For some inbred strains with low response rates, hormone stimulation is recommended in immature females (approx 32 d old). This is because the endogenous estrus cycle, present in adult females, can interfere with hormone treatment.
5. A further improvement in the yield of ovulation, especially in inbred strains, is possible with the help of additional pheromonal stimulation of females (Whitten effect) ***(11)***: approx 24 h after PMS administration, the females are put into the cage of stud males, but are separated by a grid (**Fig. 1**). Immediately after the hCG injection, the grid is removed to allow mating. Another advantage of this procedure is that neither animal needs to change cages for mating. A new cage may induce exploration behavior, particularly in males, and therefore prolong

Fig. 1. Stimulation of females by male pheromones before the females are used as zygote donors or surrogate mothers. The cage of the stud male is divided into two halves by a grid. The female is put into the other half 24–48 h prior to mating.

the start of copulation. Using the combination of superovulation and pheromonal stimulation, nearly all females ovulate and mate at about the same time, resulting in synchronous-developed zygotes. Furthermore, it becomes unnecessary to check for vaginal plugs as verification of copulation. To ensure high-copulation rates, the super-ovulated females should always be separately mated with one male, and the stud males should not be used more than twice a week.

6. In most strains, the optimal time point to obtain zygotes with well-developed pronuclei is 24 h after hCG injection and mating. Without hormone administration, mice naturally ovulate around midnight, and fertilization occurs a few hours later. Therefore, in the absence of hormonal stimulation, pronuclei suitable for injection will be available during the late afternoon of the day following mating. To avoid night shifts for the scientist, ovulation can be induced by hormone application at the appropriate time point. For example, a hormone injection at 10 AM will result in high numbers of injectable pronuclei between 10 AM and 2 PM the next day. Consequently, the transfer of injected zygotes should be performed in the afternoon following microinjection.

2.1.2. Surrogate Mothers

1. Pseudopregnant females are used as surrogate mothers for the microinjected zygotes, and they are generated by mating females of a hybrid strain or outbred

stock with vasectomized males. Good foster mothers with a high fitness, for example, are adult females of the strains CD2F1 and NMRI. To avoid vasectomy of the males, it is also possible to use mutant mouse strains in which the males are sterile (e.g., homozygous males of the Dpl^{tm1} mutation) ***(12)***. The mating behavior and the ability to produce prominent vaginal plugs should not be influenced in the male mutants.

2. For surrogate mothers, we prefer females between 7 and 20 wk old. Older females are less suitable because of their reduced fertility and the increased adiposis that complicates the embryo transfer. The surrogate mothers are prepared in a similar fashion as the zygote donors (using the Whitten effect), but without the application of hormones. The introduction of group-housed females into the stud male's cage may help induce a synchronous short estrus cycle. More precisely, the pheromonal stimulation of the surrogate mothers takes place 24–48 h before mating, (simultaneously with the donors). Superovulation of the foster mothers is not applicable because of infrequent results in successful pregnancies.
3. As the ovulation cycle in mice is 4–5 d in length, one would expect on average 20–25% of females is expected to be mated per night, as evidenced by a vaginal plug (**Fig. 2**). Frequently, a strong deviation from the expected number of vaginal plugs is observed, which may be partly the result of a synchronization of the estrus cycle of females housed in groups. Moreover, not every female with a vaginal plug is really pseudopregnant. Therefore, ovulation has to be confirmed before embryo transfer. This is best done by checking for a noticeably swollen ampulla (**Fig. 3**). Superfluous pseudopregnant females cannot be used for approx 2 wk to allow them to return to a normal cycle.
4. In our experience, more than 90% of surrogate mothers become pregnant after transfer of microinjected zygotes. Delicate treatment during surgery and appropriate anesthesia are important prerequisites for high-pregnancy rates. In mice, anesthesia is usually performed by ip injection of anesthetic substances. However, many protocols are inadequate and do not produce surgical tolerance ***(13)***. Therefore, we prefer to use inhalation anesthesia with sevoflurane, because it is directly controllable and does not interfere with pregnancy (**Fig. 4**). To avoid hypothermia following surgery, it is important to keep the mouse warm until it has recovered from the anesthesia. We prefer to use a heated surface with a controlled temperature of 37°C instead of an infrared heater (**Fig. 5**). An embryo transfer without complications does not cause strong pain, and the mice soon recover from surgery. Indicators of a good recovery are normal movement, clean fur, and a newly built nest in the cage (if nesting material, such as paper towels or Kleenex® tissues, are provided) on the morning after surgery.
5. Another often neglected effect on pregnancy rates is the social housing conditions. After embryo transfer, foster mothers are not returned to the cage of the vasectomized stud male. If a recently mated female is removed from the stud male's cage and exposed to the odor of another (genetically different) male, pregnancy fails in more than 80% of cases, and the female soon returns to estrus (Bruce effect) ***(14)*** (*see* **Note 1**). Offering the mouse appropriate nesting material

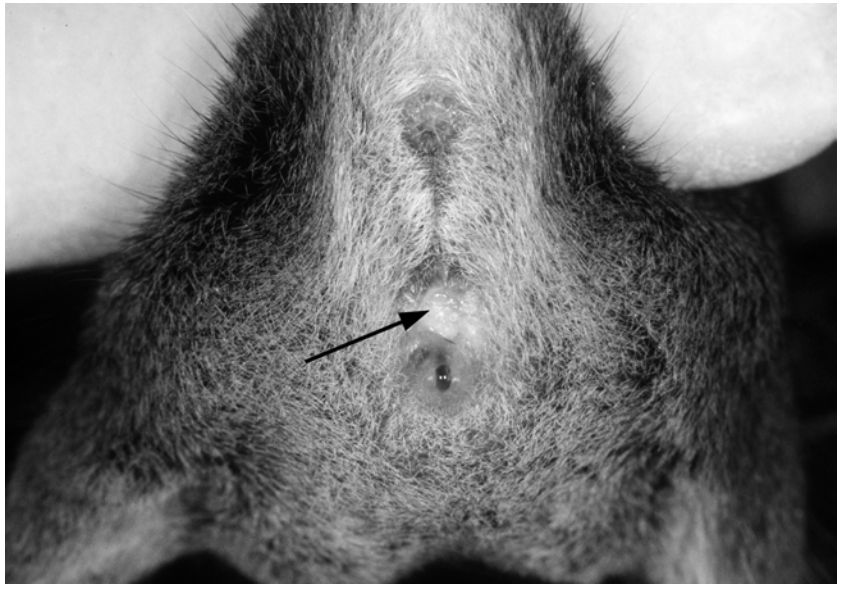

Fig. 2. Copulation plug in the vagina of a mouse. Plugs consist of coagulated proteins of the seminal fluid and can be seen in most strains up to 24 h after mating. In some strains (e.g., C57BL/6), the plug lies deep in the vagina and can only be detected with a probe.

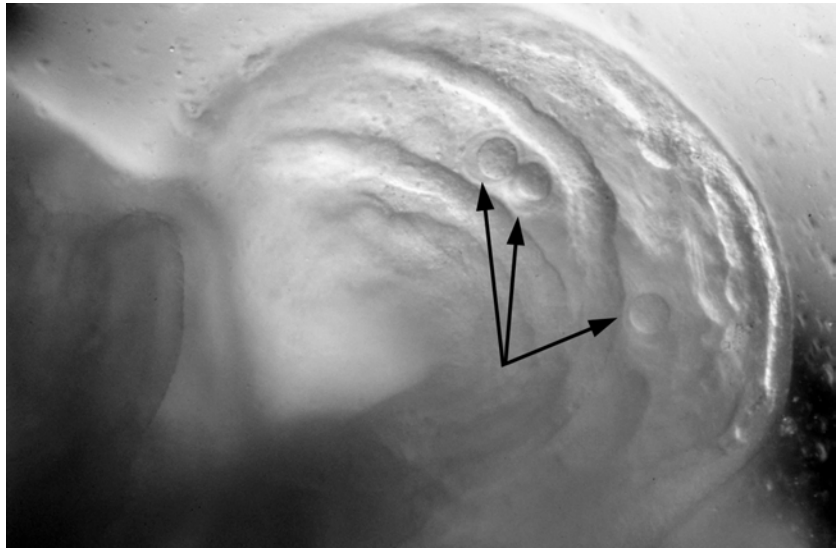

Fig. 3. Swollen ampulla of the mouse oviduct after ovulation. Unfertilized eggs or zygotes can be seen inside (arrows).

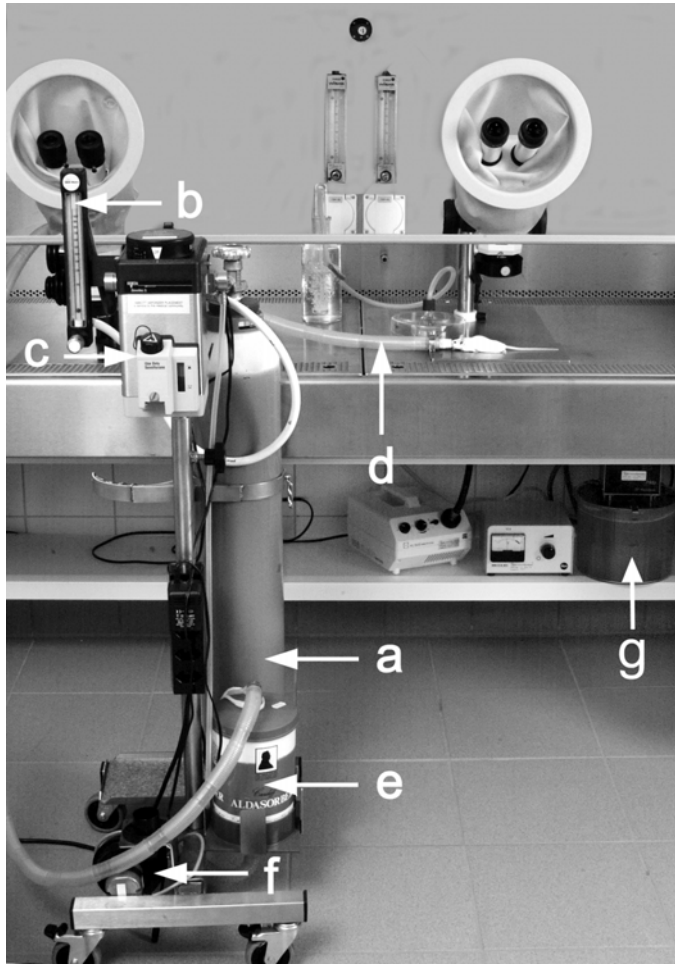

Fig. 4. Anesthesia equipment. Embryo transfers are performed in a laminar hood under sterile conditions: *(a)* air tank; *(b)* flow meter; *(c)* vaporizer; *(d)* flexible double tube ending in the anesthetic mask (the inside tube delivers the fresh anaesthetic mixture, the outside tube removes exhaust gas); *(e)* exhaust gas filter; *(f)* pump; *(g)* thermostat for heating parts of the table of the laminar hood.

or putting a non-transparent nest box into the cage will support the natural behavior of the females and may additionally improve the pregnancy rates and brood care.

2.2. Preparation and Culture of Zygotes

1. After the oviduct has been dissected, the zygotes can be collected and placed into M2 medium (Sigma) at two different time points. Early pronuclear stages (<22 h

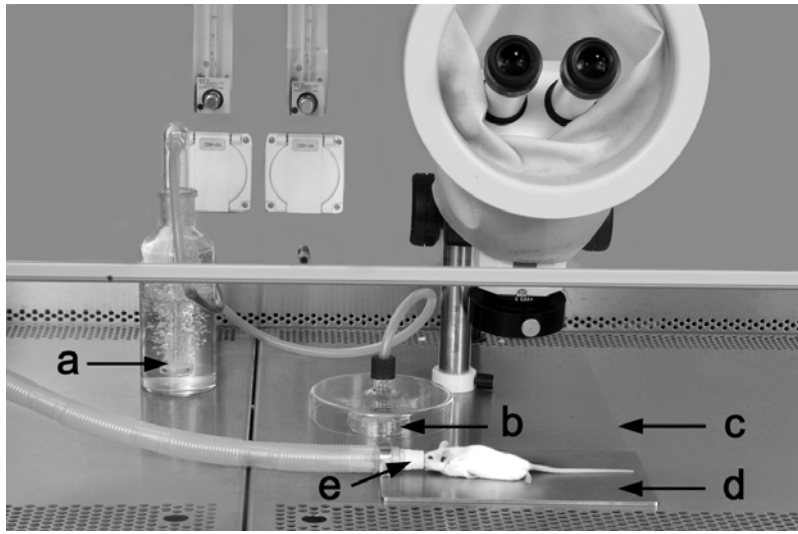

Fig. 5. Anesthesized surrogate mother for embryo transfer: *(a)* washing flask to moisturize air containing 5% CO_2 (for the cultured zygotes); *(b)* cultured zygotes under a glass cover; *(c)* warming plate in the table of the laminar hood; *(d)* metal plate upon which the sleeping mouse can be easily moved or rotated; *(e)* mask for the inhalation anesthesia.

after hCG injection), which are still packaged in a complex of cumulus cells, are best released from the swollen ampulla by simply tearing the ampulla with fine forceps or injection needles. Flushing at this stage may result in an occlusion of the oviduct by the egg–cumulus complex and an uncontrolled disruption of the oviduct. Whereas some hours later (>24 h after hCG injection), the zygotes are partially or completely released from the cumulus complex and therefore need to be flushed from the oviduct via the infundibulum with a blunt hypodermic 32- or 33-gauge flushing needle (Hamilton, pointstyle 3).

2. In order to separate the zygotes from sticky cumulus cells, we incubate the flushed complex with hyaluronidase (Sigma, Type IV-S: from bovine testes; approx 1 mg/mL) in M2 medium. The treatment with hyaluronidase may take a few minutes and should be continuously monitored. Because a longer exposure may be harmful, the zygotes have to be removed from the hyaluronidase solution as soon as possible and washed immediately.
3. Mainly used for collecting embryos and handling outside the incubator, M2 medium has a less intensive red color than M16 medium and is therefore preferred for the microinjection step (the pronuclei are more easily visible). However, mouse zygotes are cultured, in M16 medium (Sigma), which is previously equilibrated to incubator conditions. Prior to and following microinjection, the zygotes are maintained in an incubator at 37°C with 5% CO_2 in air (*see* **Note 3**).

4. All manipulations can be done at room temperature, as short phases of decreased temperature do not seem to be detrimental to the zygotes. However, to facilitate the various steps, we prefer to work at a laminar flow bench with a 37°C thermostatted surface. Between steps, the cultured zygotes are kept under a glass cover ventilated with 5% CO_2 in moisturized air (**Fig. 5**). Lowered temperatures or changes in osmolarity of the medium (due to evaporation) will cause shrinkage of the zygotes, thus complicating the microinjection step. Additionally, temperature equilibrated injection slides (preheated in the incubator) and a heated microscope stage are advantageous and support the injection procedure.

2.3. Preparation of Transgenes

Several factors and preparation steps contribute to the success in generating transgenic mice. In addition to the microinjection step itself, the quality of the DNA solution is a fundamental parameter. Depending on the size of the transgenic construct and the researcher's personal preference and experience, several protocols have been developed to prepare the DNA for microinjection. Therefore, only some general rules will be reviewed.

1. DNA samples used for microinjection need to be of the highest purity possible. It is very important to remove all contaminants from the different preparation steps. Highly concentrated DNA is advantageous, because the final dilution in the microinjection buffer will additionally reduce the effect of unknown toxic agents. No pregnancies or a low number of progeny may be due to a remaining contamination that has impaired embryo survival (*see* **Note 2**). A further dilution of the DNA sample by a factor of 2–3 for a second round of injections, can sometimes overcome this problem.
2. Because the outside diameter of an injection microcapillary is smaller at the tip than the diameter of the head of a mouse sperm (the inner orifice is approx 1 µm), it is indispensable that the DNA solution be free of all matter that could occlude the injection capillary. It is strongly recommended to filter all solutions used for DNA preparation through a prewashed 0.2-µm filter (Millipore, Ultrafree-MC). If using centrifugal filter units, it is important to avoid extensive centrifugation, because parts of the filter might peel off and pollute the solution. All pipet tips and tubes used to prepare and store the DNA sample should be rinsed with distilled water prior to use.
3. Transgenes are injected as linearized molecules. In contrast to circular molecules, the integration frequency of linearized fragments is independent of the cell cycle ***(15)***. Moreover, the free ends are thought to function as substrate to stimulate the recombinogenic machinery of the cell ***(16)***. Therefore, the integration of linear molecules is about five times more efficient than circular molecules ***(17)***. Instead of simple linearization of the cloning vector, the insert has to be isolated and separated completely from the prokaryotic sequence. Interestingly, the inactivation of transgenes by *de novo* methylation has frequently been observed for constructs containing viral sequences ***(18)***. To avoid contamination with prokaryotic

DNA, we refrain from loading size markers on preparative agarose gels. Cointegration of prokaryotic DNA with the transgenes may activate defense mechanisms of the mammalian cell against foreign (parasitic) DNA and therefore interfere with the later activity of the transgenes.

4. Removing the DNA construct by cleavage with appropriate restriction enzymes should generate an overhang of approx 100 dispensable base pairs, preferably not of prokaryotic origin (*see* **Note 4**).
5. For microinjection, the DNA sample is diluted to a 1–3 ng/µL concentration. We use an injection buffer: 5 m*M* NaCl, 5 m*M* Tris-HCl, pH 7.5, 0.1 m*M* EDTA, in high quality distilled water. This buffer has been used successfully for constructs up to 40 kb. Fragments longer than 15 kb require careful handling to prevent shearing of the DNA. Higher concentrations of DNA (>10 ng/µL) and EDTA (>1.0 m*M*) are toxic and result in a significant decrease of embryo survival ***(17)***. To prepare the aliquots for microinjection, a volume of approx 400 µL (containing the transgenes at the final DNA concentration) is centrifuged for 10 min in a precooled microcentrifuge at maximum speed (15,000–20,000*g*) at 4°C. Only the upper two-thirds of the entire volume are carefully withdrawn and subdivided into 30-µL aliquots, and the rest is used to determine the final DNA concentration. For this purpose, we run an agarose gel with the DNA solution and compare ethidium bromide (EtBr) intensities with that of DNA standards of known concentration. Although the DNA is stable in the buffer, it is recommended to store transgenes up to 15 kb frozen. Repeated thawing and freezing should be avoided.
6. Fragments of exogenous DNA that range in size up to several hundred kilobase pairs, cloned as bacterial or yeast artificial chromosomes (BACs or YACs), have been transferred by pronuclear microinjection. To apply this technique successfully, the large DNA molecules should be stabilized to prevent shearing during passage through the injection capillary. As recommended by Schedl and coworker ***(19)***, we use a buffer (100 m*M* NaCl, 10 m*M* Tris-HCl, pH 7.5, 0.1 m*M* EDTA) containing 100 µ*M* polyamine (30 µ*M* spermine and 70 µ*M* spermidine) which does not compromise the survival of the injected zygotes ***(20)***. Additionally, we use injection capillaries with an inner diameter of approx 2 µm and inject slowly at low pressure to reduce shearing forces. To make sure that some molecules are transmitted with each injection, the DNA concentration should be approx 5 ng/µL injection buffer. Larger fragments, such as BACs and YACs, can be maintained at 4°C for up to 2 wk.
7. To exclude a mistake during the preparation of the DNA solution, we always control the DNA sample, especially if it was not prepared in our laboratory. During the course of our work with transgenic animals, we have had samples from other research groups that have been completely lacking in DNA, and samples that contained only the vector backbone or degraded DNA although they were labeled as "transgene for microinjection"!

2.4. Microinjection Equipment

1. The basic equipment used to manipulate preimplantation embryos consists of an inverted microscope and two micromanipulators. To facilitate the microinjection

of zygotes, the microscope should be equipped with a special optical element that improves the visibility of the pronuclei. We prefer Nomarski differential interference contrast optics, but this must be used with glass object slides. Another option is the use of Hoffman modulation contrast optics, which can be used with disposable plastic tissue culture dishes. Unfortunately, the resolution of the Hoffman optics is lower than that of the Normarski product. While training the technique, microinjection of pronuclei is usually performed at a magnification of 400×, using a 40× objective. Experienced personnel tend to prefer a magnification of 200×.

2. Microinjection can be performed with mechanical, hydraulic, or electrical micromanipulators. The choice of the microinjection device depends on the range of micromanipulation techniques in which the laboratory intends to conduct. Particularly in combination with a piezo drill (used for nuclear transfer and intracytoplasmatic sperm injection [ICSI]), the highest precision is usually possible with mechanical manipulators.
3. When using Nomarski optics, 1-mm-thick glass depression slides are used as an injection chamber. The depression helps to cover the drop of medium placed in the center of the slide with oil. It is often recommended to treat the slides with silicone to make the surface water-repellent. A drop of medium will thus remain quite immobile once the injection instruments have been inserted; this aids in the manipulation of the zygotes. Nevertheless, it is unnecessary to siliconize the glass slides if they are boiled in distilled water without detergent before use.
4. The micromanipulators translate the movement of the operator's hand into the subtlest motions. These micromanipulators move only in the X-Y plane. Thus, ideally, the capillary should be inserted into the zygote from the horizontal axis. At this angle, the tip would enter cleanly, like an arrow, without tearing the cell membrane. However, because of the diameter of the pipet holder and the depression in the slide itself, the capillary inevitably enters at a slight angle (α, **Fig. 6**). To reduce the risk of an irreparable trauma to the zygote, this angle should be as small as possible.
5. Although we use a tempered microscope stage, we do not inject more than 20–30 zygotes per slide, to keep the period outside of optimal culture conditions as short as possible (**Fig. 7**).
6. The microinjection procedure is carried out with a finely drawn glass capillary. As the zygotes are nonadherent cells, they must be fixed with a holding capillary for injection. Injection and holding capillaries are commercially available, but both are expensive. If transgenic animals are generated frequently, capillaries can be made in the transgenic laboratory using a capillary puller and a microforge. Making good capillaries takes time and practice, but it is essential for a successful micromanipulation procedure.
7. We prefer to pull injection capillaries immediately before microinjection in order to avoid contamination during storage. It is critical to store and handle the crude pipets and drawn capillaries in such a way that avoids dust accumulation. For example, if capillaries are delivered in a plastic dust cover, open the package only at one corner with an opening not much larger than the capillary itself.

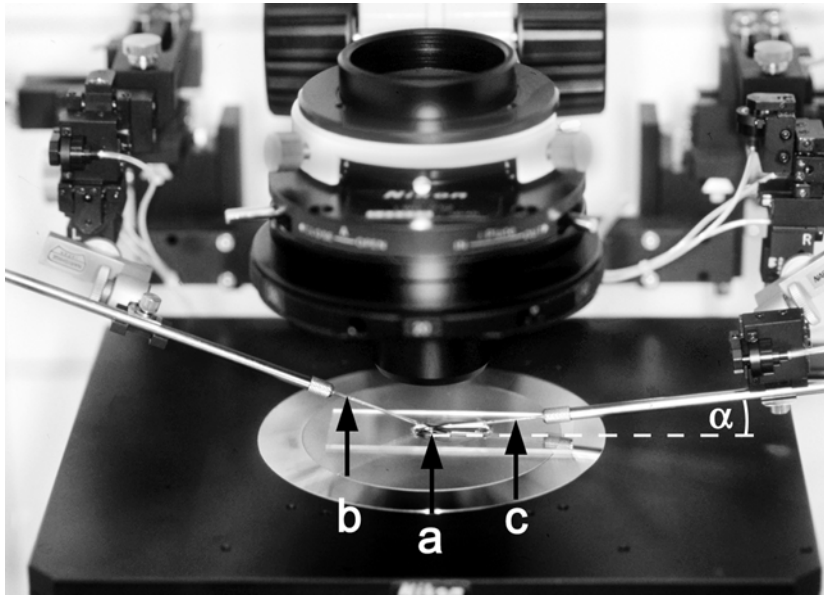

Fig. 6. Microscope stage with a depression glass slide *(a)* containing the oil-covered drop of medium with the zygotes to be injected. The angle (α) between the injection capillary *(c)* and the microscope stage should be as small as possible (*[b]*, holding capillary).

8. Usually, capillaries for injection can be used without further treatment. If the tip of the injection capillary is blocked, it can be opened by a slight tap on the holding capillary.
9. For easy handling of the zygotes, it is very important to use holding capillaries with a smooth and symmetrically shaped tip (**Fig. 8**). Furthermore, the outside diameter should be slightly smaller than that of a zygote (approx 70 µm). This allows for an additional fixation of the sucked zygote against the floor of the culture dish, a position which supports a stable optical focus of the pronucleus during microinjection.
10. We control the holding and injection capillaries with 500-µL Hamilton syringes, which are fixed in Narishige microinjectors (consisting of a pressure holder to fix the capillary, a teflon tube to connect the syringe with the holder, and the syringe attachment with a magnetic base). The small volume of the syringes has proved advantageous in providing gentle fixation and well-dosed pronuclear microinjection of zygotes. Reproducible injections are guaranteed by using automatic microinjectors (e.g., FemtoJet, Eppendorf) with programmable parameters (volume and flow).
11. The thawed DNA aliquot should be centrifuged for 10 min at 4°C to pellet any remaining particles. The transfer of the DNA solution into the tip of the microin-

Fig. 7. Mouse zygotes prepared for microinjection. To distinguish injected from uninjected zygotes, we place the latter in the 'northern area' of the drop. Injection is performed in the middle area with the holding capillary coming from the West and the injection pipet coming from the East. Injected zygotes are grouped in the southern area.

jection capillary is facilitated by the presence of an internal glass filament that supports capillary action. This allows the injection pipet to be filled passively by dipping the blunt end into the DNA solution. For active transfer, microloaders are commercially available. A cheaper method is to load the injection capillary with a self-made thin drawn-out, glass capillary connected to a mouth-controlled silicon tube (as used for embryo transfer). To transfer the DNA solution, the transfer pipet is inserted into the injection capillary to the place where it tapers. Facilitated by the inner glass filament, the solution will rapidly reach the tip of the capillary. Small air bubbles in the tip can be removed by carefully rotating the loaded capillary in a vertical position. Approximately 3 mm of the tip, filled with DNA solution is more than enough to inject approx 30 zygotes. The empty part of

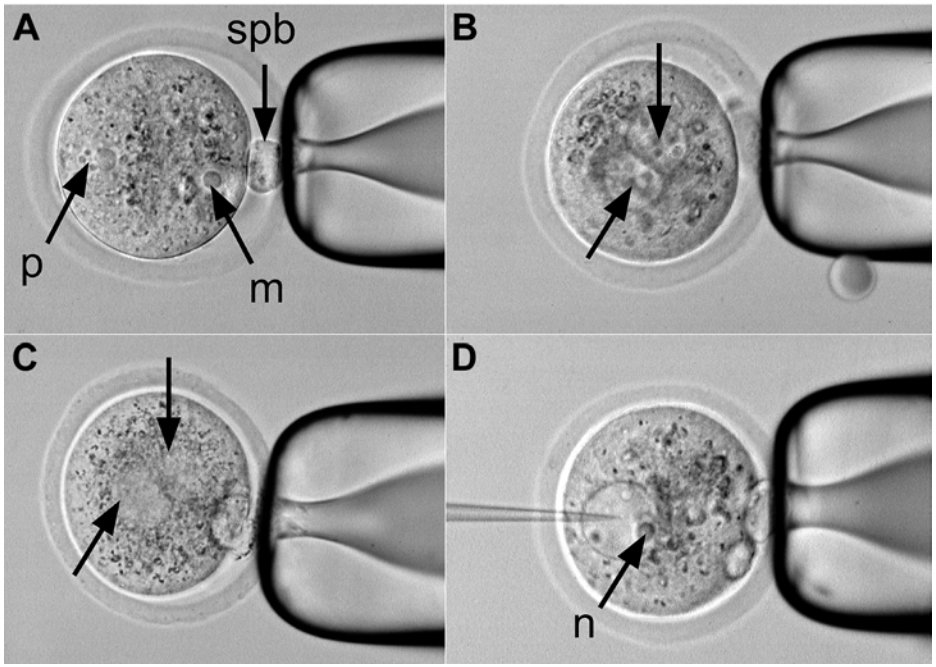

Fig. 8. Development of pronuclei and microinjection of transgenes into the mouse zygote. **(A–C)** development, migration, and disappearence of pronuclei (as indicated by arrows) (*p*, paternal pronucleus; *m*, maternal pronucleus; *spb*, and second polar body). **(D)** DNA microinjection visible as a clear swelling of the injected pronucleus *n*, nucleolus).

the injection capillary is used as an air cushion, regulated by the injection syringe, to induce a steady flow of the DNA solution. In contrast, all air bubbles should be removed from the holding pipet system; this is necessary for that the zygote can be directly controlled.

3. Methods

3.1. Pronuclear Microinjection

1. Initiation of the first cell cycle of the zygote is associated with the penetration of fertilizing sperm. Fertilization triggers the second meiotic division, visible as the extrusion of the prominent second polar body. Nuclear membranes then form around the decondensing maternal and paternal chromosomes, building two haploid pronuclei. Both pronuclei first become visible near the periphery of the zygote (**Fig. 8A**). The maternal pronucleus usually develops later and is initially located near the second polar body. Both pronuclei migrate toward the center of the zygote until they lie adjacent to each other (**Fig. 8B**). This migration takes

several hours, during which the pronuclei increase in size and the first DNA replication takes place. The optimal conditions for microinjection are during the last 3 h of migration. At that time, the pronuclei are well-developed, and the plasma membrane is easier to penetrate. Waiting too long after the pronuclei have reached the center of the zygote allows the nuclear envelopes and the nucleoli dissolve (making the pronuclei difficult to detect), and the first cell division is initiated. Therefore, it is no longer possible to guarantee a successful microinjection (**Fig. 8C**).

2. The two pronuclei differ in their DNA structure. The maternal DNA of the oocyte is packaged into chromatin, whereas the paternal DNA of the sperm is packaged into protamines, which are replaced by histones provided by the oocyte ***(21)***. However, there is apparently no difference in the frequency of transgenic offspring whether injection is carried out in the paternal or maternal pronucleus ***(17)***. Zygotes with three pronuclei, usually due to polyspermy, will not develop to term. Unfortunately, frequently we find this phenomenon in zygotes of the FVB/N strain, the strain which is recommended for pronuclear injection because of its large pronuclei.
3. Before injection, ensure that the injection capillary is open and adjust the steady flow of the DNA solution, which is controlled by the injection syringe. This can easily be done by targeting the stream of DNA solution tangential to the central axis of a zygote. The optimal flow rate will cause the zygote to rotate very slowly. A steady flow of DNA solution helps to prevent the capillary from becoming occluded and also prevents the unintentional aspiration of medium.
4. The zygotes can be gently immobilized with the holding pipet. If possible the zygote should be aspirated in the region of the second polar body (**Fig. 8D**). However, more critical for successful injection is an optimal position of the pronucleus (toward the injection capillary and near the horizontal axis of the zygote). Although it is possible to inject the maternal pronucleus, the paternal pronucleus is preferred, because of its larger size and better position within the cell. The pronucleus to be injected is optimally positioned, if, after refocusing the microscope on it, both the plasma membrane and the zona pellucida are also clearly in focus. After inserting the injection capillary into the zygote, make sure that the pronucleus remains in focus.
5. The cytoplasm of mouse zygotes is less viscous and its "wound healing" capacity is inferior to that of other species used for microinjection. Therefore, a crude injury to the plasma membrane will result in immediate cytolysis (**Fig. 9**). Moreover, the plasma membrane of mouse zygotes is far more elastic than that of other rodents. This is especially noticeable if the zygote is injected before the pronuclei are sufficiently developed. At this stage, it is possible to push the sharp injection capillary to the cortex of the opposite side of the zygote without penetrating the membrane.
6. An optimal microinjection is performed as follows. After focusing on the outline of the pronucleus, the capillary tip is refocused, and the capillary is slowly pushed toward the pronucleus to be injected. Although the zona pellucida is easily pen-

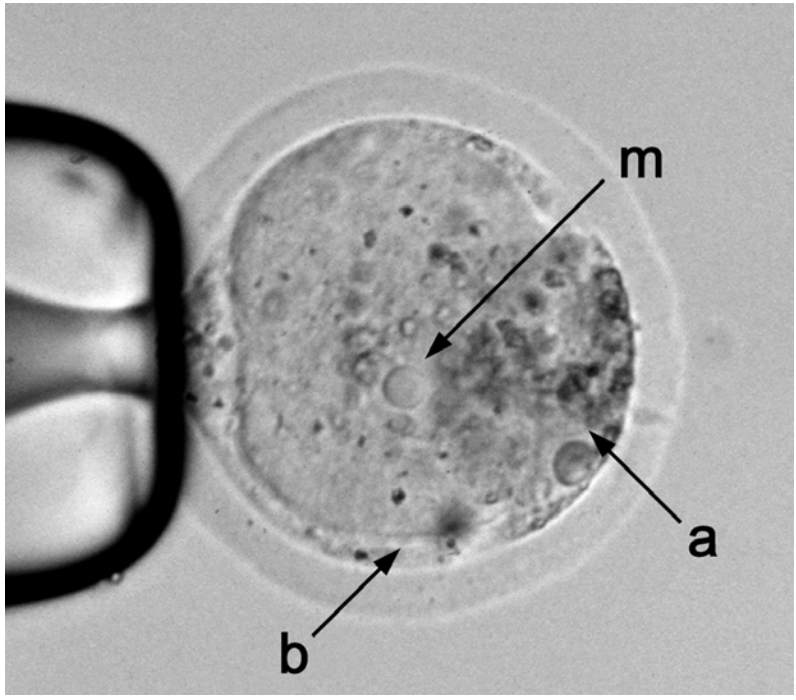

Fig. 9. Lysed zygote immediately after microinjection. The plasma membrane has been torn and cytoplasm with the male pronucleus *(a)* floats out to fill the perivitelin space *(b)*. *m*, maternal pronucleus.

etrated, the plasma membrane may not be ruptured until the capillary already appears to be inside the pronucleus. If the membrane resists injection, it must be pierced by a delicate jab with the capillary. Depending on the flow rate of the DNA solution, a successful piercing of the membrane is indicated by a slow, but visible, swelling of the pronucleus (the pronucleus diameter will increase from approx 15 µm to 20 µm, **Fig. 8D**). A DNA solution flow rate that is too high (causing a rapid swelling of the pronucleus and strong movements of the nucleoli) may disturb pronuclear organization and impair further embryo development. Additionally, it would be difficult to control the injection volume (*see* **Notes 5** and **6**).

7. Because the plasma membrane is usually penetrated inside the pronucleus, and the needle is withdrawn quickly after injection, only a very small amount of DNA and buffer is released into the cytoplasm. If the capillary is not pulled out fast enough, chromosomal DNA or other pronuclear components will frequently stick to it, resulting in a developmental stop or destruction of the zygote (**Fig. 10**). If DNA threads are pulled from the pronucleus even though the injection needle is quickly pulled out, the tip has likely become dirty (material from a previous

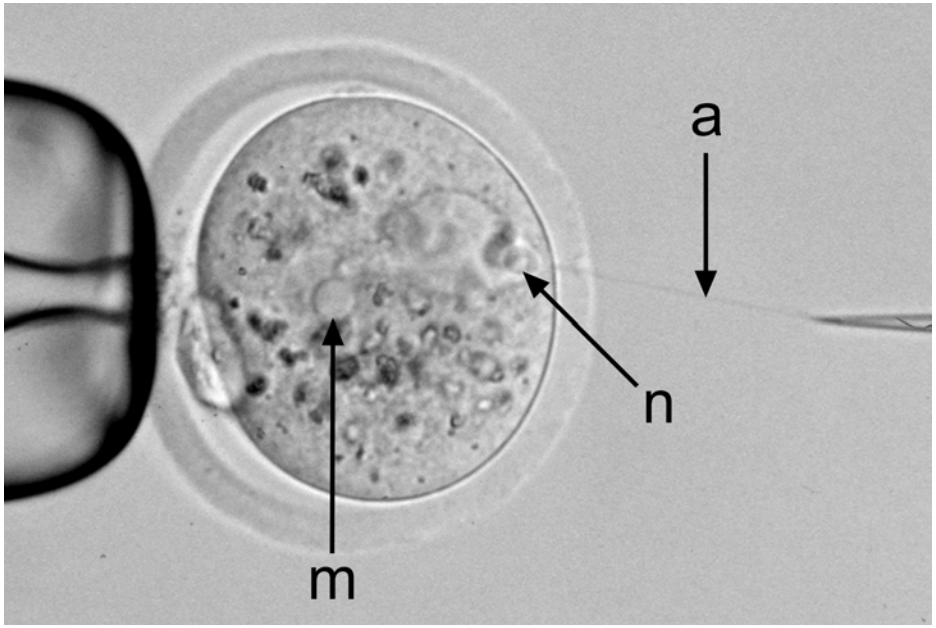

Fig. 10. A strand of nuclear DNA *(a)* sticking to the tip of the injection capillary after the latter's withdrawl. Note the elongated shape of the injected pronucleus caused by a nucleolus *n* attached to the DNA that has been pulled out (*m*, maternal pronucleus with one nucleolus).

zygote has become stuck to the capillary). In this case, the capillary should be replaced without delay. Never touch nucleoli because they will become glued to the capillary, resulting in the loss of both the capillary and zygote.

8. An injection capillary can be used provided that it performs satisfactorily. Although the sharpness of the tip decreases with each injection, it is sometimes possible to inject up to 60 zygotes with the same capillary. On the contrary, it is strongly recommended to change the injection capillary if it becomes difficult to penetrate the plasma membrane, if it cannot be pulled out without pronuclear components sticking to it, or after repeated lyses of the zygotes immediately following injection.
9. The volume of DNA solution introduced by microinjection into a pronucleus is approx 1 pL (for a 5 kb transgene this corresponds to approx 200 injected molecules). From the construct size and DNA concentration in the solution, we can estimate the number of copies transmitted, but the amount of transgenic DNA remaining in the pronucleus after injection is unknown.
10. The oocytes that survive the injection procedure (up to 90% yields are possible, depending on the skill of the operator and the strain origin of the zygotes) are sorted out and cultured for approx 2 h in the incubator. Healthy looking zygotes

are simple to distinguish by their distinct cell outline; in lysed zygotes, the perivitelline space is filled with cytoplasm that has leaked out (**Fig. 9**). Vital staining, easily performed with fluorescein-diacetate (FDA) ***(22)***, is usually unnecessary.

3.2. EmbryoTransfer

1. Healthy zygotes are transferred bilaterally into each of the two oviducts of a surrogate mother. Appropriate transfer capillaries with an internal and external diameter of approx 100 and 140 µm, respectively, facilitate the procedure. If there are insufficient pseudopregnant surrogate mothers available, the zygotes can be cultured overnight in M16 medium. For overnight culture, we use center-well organ culture dishes without an oil cover. The resulting two-cell stages can still be transferred into freshly mated females (d 1 of pseudopregnancy) (*see* **Note 7**).
2. After transfer, approx 70% of nonmanipulated mouse zygotes will normally develop to term. Depending on the genetic background, this frequency is reduced to 15–30% after DNA microinjection as a result of mechanical damage during the microinjection procedure and the non-targeted integration of foreign DNA. Even if it is a non-active sequence, it may cause developmental malfunctions and abortion of the growing embryo ***(23)***. In mice, small numbers of successful implantations can increase the risk of abortions, giant fetuses, or bad brood care. Consequently, the number of embryos transferred after microinjection should be nearly double that of natural ovulations (i.e., approx 12–15 into each oviduct) to ensure a stable pregnancy and the stimulation of lactation in the surrogate mother.

3.3. Screening the Progeny for Transgenic Founders

1. After weaning, the littermates of the same sex are maintained in groups per cage. To correlate the screening results with each mouse, an individual identification system is necessary. Depending on the number of animals identified, desired durability of the identification, age/size of the animals, and associated burden caused by the identification procedure, we suggest various identification methods (**Table 1**). For weaned animals (4–5 wk old), we prefer the ear punch, which is simple, permanent, and also provides material for the biopsy.
2. Due to non-targeted insertion and the unpredictable copy number, each founder generated by pronuclear DNA injection creates a unique transgenic line and must be named individually according to the Guidelines for Mouse Strain Nomenclature ***(24)***.
3. Among the offspring, the founder animals are first identified by polymerase chain reaction (PCR) analysis. As a positive control, the cloned transgene can be mixed with the wild-type mouse DNA (the wild-type mouse DNA should also be used as negative control). PCR analysis is a sensitive method, but it is susceptible to false-positive results caused by contamination of the specimens. Therefore, founders should always be confirmed by Southern analysis.
4. For these analyses, high-molecular-weight genomic DNA can be isolated from different tissues of the animals. Although Southern hybridization requires micrograms of DNA, only tiny amounts are necessary for PCR. Convenient sources of

Table 1
Identification Methods for Mice

Method	Age	Durability/burden
Body painting	Neonate to adult	Nonpermanent, noninvasive, more suitable for newborns and mice with white or pale fur
Ear notching/punching	> 3 wk	Permanent, invasive, useful as biopsy material
Ear tags	> 3 wk	Permanent, but can easily be lost, often too heavy for mice
Microchipping	> 3 wk	Permanent, invasive, expensive
Foot tattoo	Neonate	Permanent, invasive
Ear tattoo	> 3 wk	Permanent, invasive
Tail tattoo	> 3 wk	Permanent, invasive
Toe amputation	Neonate	Permanent, invasive, useful as biopsy material

genomic DNA for these analyses are listed in **Table 2**. Tail biopsies are widely used to prepare genomic DNA samples, but other sources, some obtainable while marking the animal (ear punch), are also suitable. Nevertheless, for extensive screening procedures, the tail biopsy is the appropriate and preferred tissue to isolate sufficient amounts of genomic DNA from live animals.

5. An extended Southern blot analysis with a variety of probes and enzymes can provide additional information on the integrity of the transgene sequence and the estimated number of copies integrated at the chromosomal locus. The number of copies integrated can be more precisely estimated by real time PCR. It is significant to note that a high percentage of founder animals are genetic mosaics or have multiple integration sites. Neither of these founders are appropriate for the genetic and phenotypic characterization of the new line.

3.4. Different Types of Founders

1. In our laboratory, approx 25% of the progeny produced have integrated the injected transgene. Occasionally, however, we do not find transgenic progeny following the injection of a construct. If the DNA solution and the injection procedure are known to be flawless, the prenatal expression of the transgene itself is possibly the reason for no transgenic offspring. Transgene products might interfere with the normal development and viability of the embryo, resulting in abortions or perinatal death. These consequences are hard to predict. Therefore, it is extremely important to inspect all aborted pregnancies and the litter immediately postpartum. All stillbirths, dead sucklings or remains thereof should be analyzed for transgene integration. If, indeed, it is the prenatal expression of the transgene that is responsible for the non-appearance of transgenic offspring,

Table 2
Methods of Tissue Biopsy as Source of Genomic DNA for the Analysis of Transgenic Mice

Tissue	Biopsy (age)	DNA quantity	Burden	Reference
Tail	> 3 wk	+++	Invasive, amputation	***(25)***
Tail	Neonate	++	Invasive, amputation	***(26)***
Ear	>3 wk	++	Invasive, obtained during identification procedure	***(27)***
Toe amputation	Neonate	++	Invasive, amputation, obtained during Identification procedure	***(28)***
Blood sample	Neonate to adult	+	Invasive	***(29)***
Stool specimen	>3 wk	+	Noninvasive	***(30)***
Rectal epithelial cells	>4 wk	+	Noninvasive, rectal swab	***(31)***
Oral epithelial cells	>3 wk	+	Noninvasive, oral swab	***(32)***
Saliva, oral epithelial cells	>3 wk	+	Noninvasive, flushing the oral cavity	***(33)***
Hair	>3 wk	+	Noninvasive	***(34)***

we recommend constructing the transgenes in inducible or conditional expression vectors to establish the required transgenic lines.

2. Among all founders, we observe, on average, 41% mosaics (i.e., founders who harbor the transgene in only a fraction of their somatic and germ cells). Mosaics are the result of transgene integration after the first round of DNA replication. Indeed, the injected DNA is frequently detectable as free molecules at the blastocyst stage ***(35)***. However, due to the rapid assembly of a few large multicopy tandem arrays, predominantly by homologous recombination between the injected DNA molecules ***(36)***, the transgenes are unequally distributed to the daughter cells during each cell division. The degree of mosaicism can vary from founders that produce approx 50% transgenic offspring (as would be expected for a completely hemizygous founder), extending down to animals that produce no transgenic progeny. The term "hemizygous" is used because there is no corresponding allele on the sister chromosome. To avoid mistakenly eliminating a weakly mosaic animal with poor germline transmission, we analyze (if necessary) up to 50 offspring per founder.

3. Nearly 13% of all founders generated in our laboratory have significantly more than 50% transgenic progeny. This is usually the result of multiple non-linked integration sites that segregate in the F1 progeny. These sites have to be separated by careful breeding and screening to establish stable independent transgenic lines.
4. Because of the unique and different genotypes among transgenic founders generated by pronuclear microinjection, the detailed analysis of the transgenic locus and determination of the in vivo expression of the transgene should be done using completely hemizygous and homozygous transgenic progeny of the F1 and later generations, rather than the founder itself. Transgenic animals, homozygous for the transgenic locus, can be identified by Southern analysis, by real-time PCR, or by the fact that they will produce 100% transgenic offspring after mating with a wild-type mouse. Moreover, homozygous individuals have been detected by semiquantitative PCR *(37)*. It is important to note that unexpected phenotypes caused by recessive insertional mutations may be detected in homozygous offspring.

3.5. Breeding Transgenic Animals and the Significance of the Genetic Background

1. More than 90% of the transgenic founders generated in our laboratory by pronuclear injection are able to transmit the mutation to their progeny. Usually, the founders are first crossed with animals of the zygote donor strain. In subsequent generations, brother–sister mating is often performed to obtain homozygosity for the transgenic locus. To breed transgenic lines with an undefined genetic background, it is essential to consider all sources of genetic changes in a population of restricted size, such as selection, inbreeding, or genetic drift. Also, the significance of genetic background must be taken into account to correctly interpret specific characteristics of a transgenic model. Two important factors are described below:
   a. Transgenic lines are usually bred as a small isolated population. In a mixed or undefined genetic background, random genetic changes may occur as a result of genetic drift. Furthermore, uncontrolled inbreeding will ensue if the population is too small to be able to adhere strictly to the rules of specific breeding systems for laboratory outbred stocks. The subsequent problems are changes of phenotypic characteristics and finally inbreeding depression, usually recognized by poor breeding performance.
   b. Transgenes integrated into an established genome have to develop their function in concert with many other genes of the organism. Pleiotropic genes and polygenic traits are examples of the complex network of interactions between genotype and phenotype. A stably integrated transgene is part of the genome and may be influenced by endogenous genes (genetic modifiers) via a number of different mechanisms. Therefore, the phenotype of a genetically modified organism varies depending on the genetic background, defined as the sum of all genes present in an organism that influences a trait *(38)*. Particularly against a mixed or an undefined genetic background, the polymorphism of

genetic modifiers could confound the interpretation of experimental results from a transgenic line ***(39,40,41)***.

2. Genetic modifiers are best identified by analyzing the mutation against different genetic backgrounds. This analysis can be systematically accomplished by backcrossing the transgene into appropriate inbred strains, an approach used to establish congenic mouse strains ***(42)***. Currently, marker assisted breeding (speed congenic or supersonic congenics) can reduce the time necessary to produce congenic strains ***(43,44)***.
3. The use of inbred strains with well-characterized uniform genetic backgrounds greatly facilitates the interpretation of experimental results with genetically modified mice. Nevertheless, each strain is a unique genetic combination and has its own set of characteristics. The possible interaction of modifiers with specific characteristics of a transgenic model should be taken into consideration when selecting the zygote donor strain, as well as when deciding the further breeding strategy of the transgenic line. Furthermore, the genetic background of a transgenic line should be known in detail, and appropriate control animals of this line should be available, so that control experiments can be carried out and results can be compared between laboratories ***(45)***.

3.6. Phenotypic Characterization of Transgenic Mice

1. New transgenic mouse lines must be thoroughly characterized early in order to judge their suitability for research, as well as to evaluate possible animal welfare problems caused by the mutation. Depending on the transgene function, in some strains no clear phenotypic changes arise, whereas in others, serious clinical symptoms are seen ***(46)***. Although there has been continuous and rapid development in transgenic strategies and the functional quality of transgenes, the phenotypic consequences of transgene integration cannot be completely predicted. Besides the phenotypic relevance of the transgene expression itself, we have to be aware of other effects due to the pecularities of each technique used for transgenesis. After pronuclear injection, the random integration of the transgenes may interfere with the expression of endogenous loci by a phenomena termed transcription interference ***(47)*** and cosuppression ***(48)***. Furthermore, insertional mutations induced by the random integration into an endogenous locus may lead to partial or complete inactivation of the affected gene. The resulting phenotypic changes, intended or accidental, may be crucial to the animals' welfare. Only a careful and comprehensive phenotypic assessment, carried out during the first-breeding generations of a newly generated line, can determine whether phenotypically relevant influences of the mutation occurred, and, if so, how to deal with them ***(49,50)***. Because the transgenes are inserted randomly after pronuclear injection, each founder animal will create a unique genotype. Therefore, each transgenic line must be characterized separately. It is insufficient to characterize one founder line and extrapolate the results to all others generated with the same transgene.
2. The documentation of each strain should include all information about the mutation, including its phenotypic relevance, genetic background, breeding

system, number of generations, relevant control wild-type strain, and microbiological status. Additionally, all information regarding the necessity of specific treatments, as well as humane endpoints for genetic burdens, are also indispensable.

3.7. Outlook

Genetically engineered mammals are a qualitatively important extension to the wide range of laboratory animals. The ability to alter the genome of mammals by the introduction of defined mutations provides a powerful approach in examining the molecular mechanisms of mammalian development and mimicking the pathogenesis of human diseases or aspects thereof. Nevertheless, to successfully investigate an isolated gene, the complexity of the developing organism and the modifying influences of the biotic and abiotic environment on phenotypic characteristics must be taken into account ***(51,52)***. Furthermore, these novel techniques can also lead to unknown difficulties for researchers in their work with laboratory animals. Foreign DNA sequences, artificially constructed and randomly integrated into an unknown chromosomal site, may interfere with the correct function of parts of an established genome. The resulting phenotype may mask or change the desired effect of the transgene with the risk that experimental results may be misinterpreted. To minimize these potentially confounding effects, it is indispensable to have a thorough understanding of the genetic peculiarities of transgenic animals ***(53)***.

Since the first generation of transgenic mice nearly two decades ago, the wide experience gained in transgenic technology today facilitates the efficient application of transgenesis by pronuclear injection in different mammalian species. Nevertheless, not all factors that influence the phenotype of a transgenic animal can be controlled and managed by the scientist. In the case of pronuclear injection, it is mainly the non-targeted integration site (position effects), including structural changes to the endogenous flanking regions and the varying number of tandem repeats of the integrated concatamer, that are considered responsible for the strong phenotypic variation between individual founder lines. Although the number of integrated DNA copies can be reduced and controlled with the help of prokaryotic recombination systems, such as Cre/loxP ***(54)***, feasible approaches to target DNA integration at specific appropriate loci in the genome after pronuclear injection still remain elusive. The expression pattern of the same transgene in two different mouse lines exemplifies the importance of these effects for the quality of the transgenic model (**Fig. 11**).

Another important factor, that can be influenced by the scientist is transgene construction. The majority of transgenes are chimeric constructs, i.e., fusions of regulating and coding sequences of different origin. Although the expression of a transgene may be influenced by posttranscriptional and posttransla-

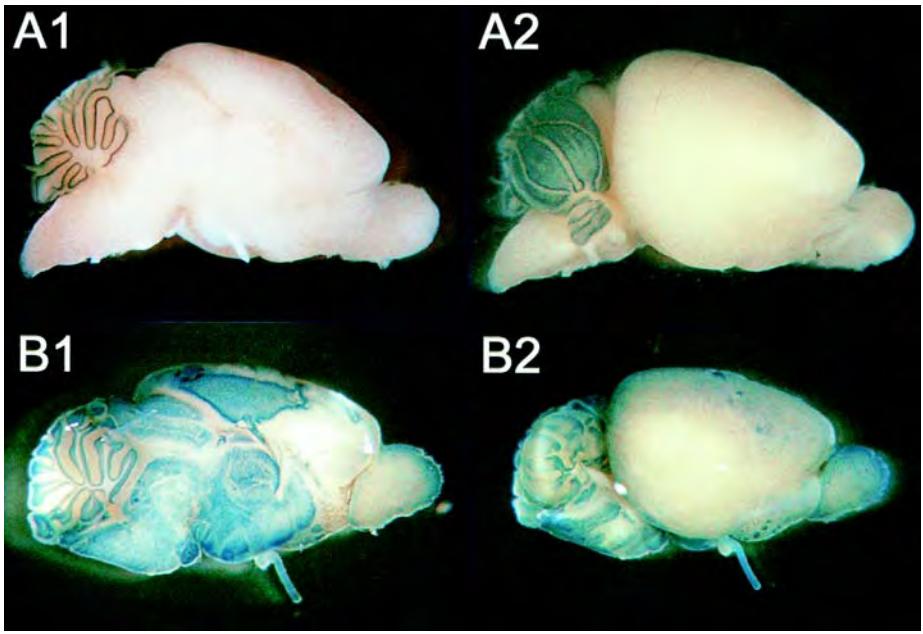

Fig. 11. Expression pattern of a lacZ reporter gene in the ROSA26 locus activated by Cre-mediated recombination (L7-Cre) in the brain of doubly transgenic mice. The tissue-specific activity of the L7 promoter should restrict the expression of Cre and lacZ exclusively to the Purkinje cells. In animal **A**, the X-Gal staining is specifically confined to the Purkinje cells (A1, midsaggital brain section, stained cells on the left; A2, lateral view of the brain). In animal **B**, the lacZ transgene is expressed ectopically in the entire brain (**B1** midsaggital section; **B2** lateral view). Pictures courtesy of S. Brandner.

tional processes, the regulation of transcription *per se* is the most stringent method of controlling gene expression. The transcription rate, as well as spatial and temporal activity of a transgene largely depends on the presence of specific control elements, absence of prokaryotic target sequences that trigger cellular defense mechanisms, and on the correct structure (exon–intron) of the transgene. However, the different control elements governing the gene of interest are rarely known in detail. If the entire genomic sequence was taken, then all these control elements would be present. However, genomic sequences are not always available (only the cDNA is available, i.e., the coded portion of the gene without the control elements), or the genomic sequence is far too large to be inserted into one of the standard vectors, which are usually used to clone transgenes. Unfortunately, artificial gene sequences are often extremely sensitive to effects of the non-targeted integration site (position effects), as well as cellular defense mechanisms. This is also true, but often forgotten, for

transgenes of prokaryotic origin injected to generate inducible or conditional mouse models. To anticipate these serious problems, it is essential to analyze several transgenic lines containing the same construct and to validate the results of a transgenic experiment in at least two different lines with comparable phenotypes. The methods described in this chapter will help in the success of pronuclear DNA injection of mouse zygotes, so that the adequate number of transgenic lines will be available.

4. Notes

1. In newly inseminated mice, a so-called pregnancy block can be induced by urinary pheromones, resulting in the reduction in release of hypophyseal prolactin. This reduction causes the regression of functional corpora lutea, which results in a decreased progesterone level and failure of embryo implantation. Therefore, foster mothers should be housed individually and without exposure to strong olfactory cues from other mice (neither male nor female).
2. Small litters or no progeny may be the result of toxic contamination of the culture system or DNA sample. Controlling the in vitro culture from the zygote to the blastocyst stage and/or monitoring the entire pregnancy may help to identify the reason for poor results. Sometimes a foster mother may still be pregnant on d 12 following embryo transfer, but no longer be pregnant 1 wk later. In such a case, a hysterectomy and an inspection for abortions and/or resorptions is recommended.
3. New batches of M16 medium should always be tested by overnight culture of spare zygotes before use. An adequate number of two-cell stages should be found the next morning (approx 90% for zygotes of hybrid donors), and their viability should be checked by embryo transfer. Attention should also be given to the oil used to cover the medium drop during manipulation. Light paraffin oil (BDH GPR™) or mineral oil (light white oil, Sigma) is commonly used for this purpose. Oil should be sterilized by passing through a 0.22-µm filter, rather than by autoclaving (the latter results in toxic degradation products). Surprisingly, we repeatedly found batches of oil (designated as embryo tested) that had adverse or harmful effects on the further developmental capability of cultured embryos. Normally, it is unnecessary to equilibrate the layering oil before use by mixing with culture medium, but this may also help to improve the culture conditions.
4. Naked, linearized constructs are sensitive to nucleases that degrade unprotected DNA. Prior to integration, partial digestion, can result in transgenes bearing deletions at both ends. Indeed, after sequence analysis of transgene–chromosome junctions in transgenic mice, we found degradations of up to 133 bp from the transgene end. In those cases, where important coding or regulatory sequences lie near the ends of the construct, nuclease degradation of the ends could be the cause for a lack of expression. Whenever feasible, we try to consider this fact throughout cloning and isolation of the insert.
5. DNA injection into the cytoplasm will not (or very rarely) result in transgenic progeny. The injection of a large volume of DNA solution close to the pronucleus

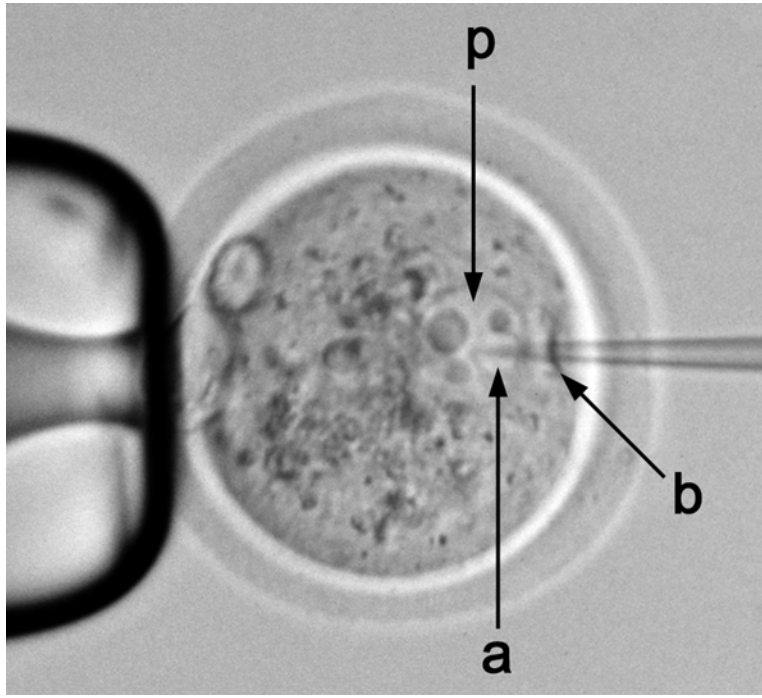

Fig. 12. Incomplete penetration of the egg plasma membrane. The expelled solution forms a visible bubble *(a)* within the paternal pronucleus *(p)*, surrounded by the plasma membrane of the zygote. Note also the indented membrane around the injection capillary *(b)*.

will dilute the surrounding cytoplasm, making it appear lighter in color. For an unpracticed injector, this lighter-colored area may appear to be like a pronucleus swollen by injection. Injection in the pronucleus itself will cause the nucleoli to swim around in reaction to the flow of buffer.

6. Another often observed mistake is the incomplete penetration of the plasma membrane. Instead of pronucleus swelling, only a bubble containing a drop of DNA solution surrounded by the plasma membrane is formed around the tip of the capillary (**Fig. 12**). When the capillary is withdrawn, the DNA will flow backward into the perivitellin space.
7. As a rule of thumb, no culture condition is better than nature. Therefore, we transfer the injected zygotes to a surrogate mother as soon as possible after microinjection. As the in vitro development of mouse embryos often tends to stop at the two cell stage (two cell block), we recommend performing the transfer no later than 24 h after flushing the zygotes.

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11

Parthenogenesis and Nuclear Transfer in Rabbit Oocytes

Yukio Tsunoda and Yoko Kato

1. Introduction

Rabbits are widely used as experimental animals in various fields of research. However, unlike mice and rats, rabbits with a homologous genetic background are difficult to obtain, as few inbred strains have been established because of the fact that inbreeding decreases reproductive ability. Moreover, an embryonic stem (ES) cell line with germline chimeras has not been established in rabbits; and thus, there are no reports on production of transgenic rabbits by gene targeting.

The most ideal, but unrealistic, method of producing genetically homologous animals is parthenogenetic activation of oocytes. A variety of methods have been used to stimulate mammalian oocytes, including those of rabbits, to develop to blastocysts at a high rate and to fetuses if transferred to recipients ***(1–4)***. But fetuses originating from parthenogenotes die near midgestation owing deficient expression of paternally derived imprinted genes ***(5)***.

One method of producing clones is the separation or bisection of preimplantation embryos into several groups following transfer to recipients. Identical offspring in a multitude of mammals, including rabbits, have been produced ***(6,7)***, but the number of identical offspring is usually limited to two.

Another method of clone production utilizes nuclear transfer (NT) technology. Cloned rabbits have been produced by NT of blastomeres from eight-cell to morula-stage embryos into oocytes whose maternal chromosomes were previously removed (***8–11***; **Table 1**). The procedures for parthenogenetic activation have an important role in the developmental potential of NT oocytes. Theoretically, many clones can be produced if NT oocytes that develop to the

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Table1
Successful Production of NT Rabbits

Origin of donor cells	No. of offspring/no. of embryos transferred	(%)	Reference
Preimplantion embryo	5/164	3%	***(8)***
	1/85	1%	***(9)***
	23/110	21%	***(10)***
	8/243	3%	***(11)***
Adult somatic cell	6/371	2%	***(21)***

eight-cell to morula stage are used for serial NT, ***(12,13)***. The maximum number of clones actually produced, however, is less than 10.

Since the first report, where a normal sheep was produced after NT of somatic cells obtained from an adult and cultured in vitro ***(14)***, several cloned sheep, mice, bovine, goats, and pigs have been produced ***(7)***. Although no studies have examined the maximum quantity of clones produced by somatic cell NT, there may be no limit. Somatic cell NT technology is also used for the production of gene targeting transgenic sheep ***(15)*** and pigs ***(16,17)***. The in vitro developmental potential of NT rabbit oocytes receiving somatic cells is high, but live cloned offspring were only recently produced ***(3,18–21)***. Cloned rabbits were recently produced after transfer of NT oocytes receiving adult somatic cells to asynchronous, but not to synchronous recipients ***(21)***. Yet, the success rate for the production of somatic cell clones, is still low, and further technical improvement for NT of rabbit somatic cells is required. In this chapter, parthenogenetic and NT procedures are discussed.

2. Materials

Although the materials used in our laboratory are listed, equivalents can also be used.

2.1. Superovulation

1. Mature Japanese white or New Zealand white rabbits.
2. Follicular-stimulating hormone (FSH).
3. Human chorionic gonadotrophin (hCG).

2.2. Recovery of Oocytes and Embryos

1. Medium for in vitro maturation and in vitro culture of oocytes (complete EBSS; ***18***): bicarbonate-buffered Earle's balanced salt solution (EBSS) supplemented with nonessential and essential amino acids (NEAA and EAA), 1 m*M* L-glutamine, 0.4 m*M* sodium pyruvate, and 10% fetal bovine serum (FBS).

2. TCM-199. (Gibco-BRL, cat. no. 31100-027, Eggenstein, Germany).
3. FBS: inactivate the FBS at 56°C for 30 min.
4. Bovine serum albumin (BSA; Sigma Chemical Co., cat. no. A-4378, St. Louis, MO).
5. Hyaluronidase, (Sigma Chemical Co., cat. no. H-3506) with a final concentration of 300 U/mL Dulbecco's Ca^{2+} and Mg^{2+}-free phosphate buffered saline (PBS), is stored at –20°C.

2.3. Donor Cell Preparation for Preimplantation Embryos

1. Medium for embryo recovery and manipulation: PBS supplemented with 20% FBS.
2. 1% Pronase with 1% polyvinylpyrrolidon (PVP; Sigma Chemical, Co.cat. no. PVP-40) in PBS: dialyze against a large volume of PBS for 1 d.

2.4. Donor Cell Preparation for Somatic Cells

1. Dulbecco's modified Eagle medium (DMEM).
2. Disruption for subculture: 0.05% trypsin and 0.05% EDTA in PBS.
3. Cell proliferation kit (Amersham, code RPN 20, Buckinghamshire, UK).
4. Chromosomal analysis: 0.075 *M* KCl, hypotonic solution, fixative solution (methanol 3 : acetic acid 1), 3% Giemsa solution (pH 6.4), 3 µg/mL nocodazole (1000X stock solution, stored at –20°C).
5. Gelatin-coated culture dish: 0.1% gelatin solution in PBS, four-well multidish (cat. no. 176740, Nunck, Denmark).
6. Freezing medium: Cell Culture Freezing Medium-dimethyl sulfoxide (DMSO; Gibco-BRL, cat. no. 1110-011).

2.5. Preparation of Microtools

1. Microforge, MF-9 with a 10× or 20× objective (Narishige, Tokyo, Japan).
2. Pipet puller, Narishige, PN-3 or Sutter Instruments, (cat. no. P-97/IVF, Novato)
3. Micromanipulator system (Narishige).
4. Capillary beveling device, EG-40 (Narishige).
5. Glass capillary: holding pipet (G-1, 1 × 90 mm, Narishige), enucleation/injection pipet (Sutter Instruments, cat. no. 120-90-10), needle (10 µL microdispenser, Drummond, cat. no. 510G-310G-210G).

2.6. NT

1. Inverted microscope (Nikon TE300, Tokyo, Japan or Olympus IX70, Tokyo, Japan).
2. Electrofusion system (Electro Cell fusion Model LF101, BEX, Tokyo, Japan).
3. Manipulation chamber: microdrops are made on this chamber with mineral oil (**Fig. 1**).
4. Fusion chamber (**Fig. 2**): two stainless wires 1.0 mm apart attached to polished borosilicate glass dish (93 mm inner diameter, 13 mm depth).
5. Medium for micromanipulation: EBSS supplemented with 10% FBS.
6. Warm/cool plate for inverted microscope (Thermoplate MATS 555 RT, Tokai Hit, Tokyo, Japan).

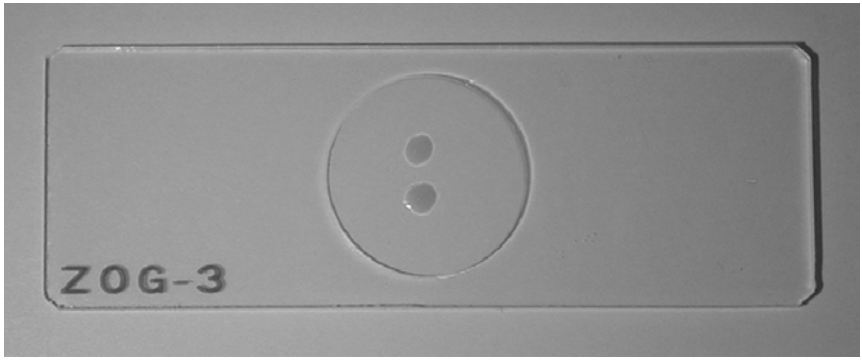

Fig. 1. Manipulation chamber with two microdrops under oil.

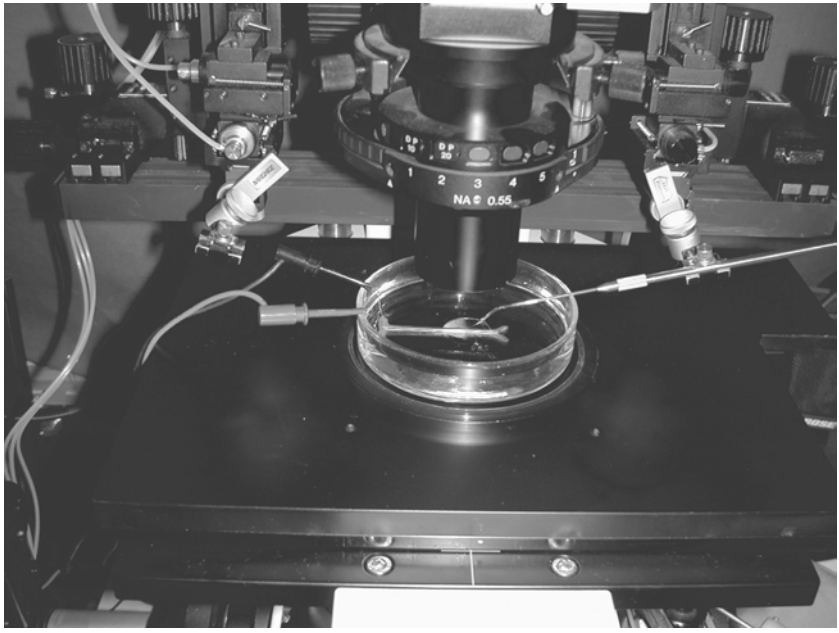

Fig. 2. Fusion chamber with two wires and glass needle under inverted microscope.

7. 7.5 µg/mL Cytochalasin B (CB): A 1 mg/mL stock solution in DMSO, stored at –20°C.
8. Demecolcine (colcemide): A 10 µg/mL stock solution in PBS, stored at 4°C.
9. 5 µg/mL Hoechst 3342 (Calbiochem-Behring, cat. no. 382065, San Diego, CA). A 1 mg/mL stock solution in ddH_2Oin an opaque vessel can be stored at 4°C for 1 mo.

Table 2
The Composition of Zimmerman Cell Fusion Medium

Compound	Molarity	g/L
Sucrose	0.28 *M*	95.84
$Mg(C_2H_3O_2)_2{\cdot}4H_2O$	0.5 m*M*	0.107
$Ca(C_2H_3O_2)_2$	0.1 m*M*	0.016
K_2HPO_4(anh)	1.0 m*M*	0.174
Glutathione	0.1 m*M*	0.031
BSA	0.01 mg/mL	0.01
PH 7.0		

2.7. Parthenogenetic Activation

1. Fusion medium: Zimmerman cell fusion medium (**Table 2**) or 0.3 *M* mannitol, Ca^{2+} and M^{2+}-free EBSS containing 3 μg/mL BSA.
2. Activation medium: complete EBSS with 2 m*M* 6-dimethylaminopurine (6-DMAP; Sigma Chemical Co.).

2.8. In Vitro Culture

1. Complete EBSS.
2. Light mineral oil.

2.9. Embryo Transfer

1. Anesthetic: Nembutal sodium solution (Abbott Laboratories, North Chicago, IL).

3. Methods

3.1. Superovulation and Recovery of Ovulated Oocytes (See Note 1)

1. Because female rabbits sometimes ovulate during transportation, it is essential to keep them individually housed for 15 d before the superovulation treatment.
2. Inject 0.5 IU FSH into mature females subcutaneously six times 12 h apart.
3. Inject 100 IU hCG intravenously 12 h after the final dose of FSH (**Fig. 3**).
4. Flush oviducts with EBSS containing 10% FBS 14–15 h after hCG administration (**Fig. 4**). Then, under a microscope, collect oocytes surrounded by cumulus cells. The cumulus cells are removed by treatment with hyaluronidase.

3.2. Superovulation and Recovery of Follicular Oocytes

1. Inject 0.5 IU FSH subcutaneously six times 12 h apart.
2. Remove ovaries 12 h after the final FSH dose.
3. Fully grown oocytes are collected by rupturing follicles 1–3 mm in diameter in TCM199 supplemented with 3 mg BSA/mL.
4. Fewer than 50 oocytes are cultured in 500 μL of complete EBSS in an atmosphere of 5% CO_2 at 39°C for 10–14 h.

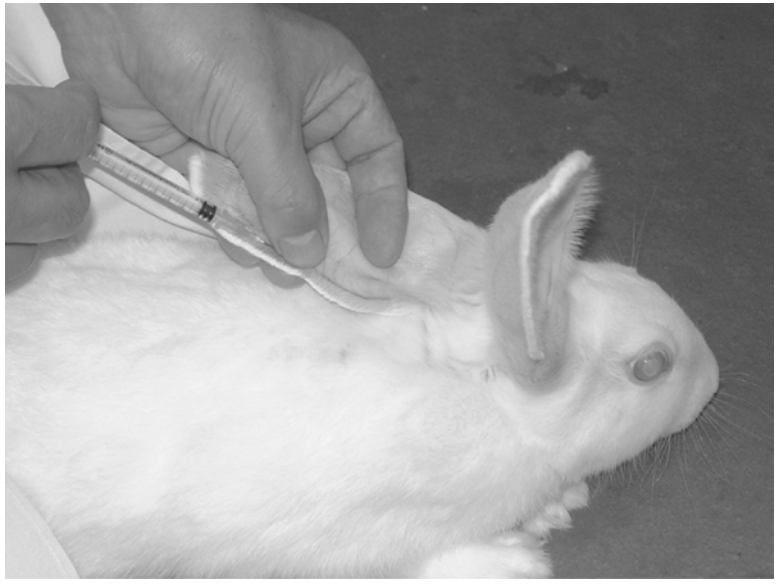

Fig. 3. Intravenous injection of hCG.

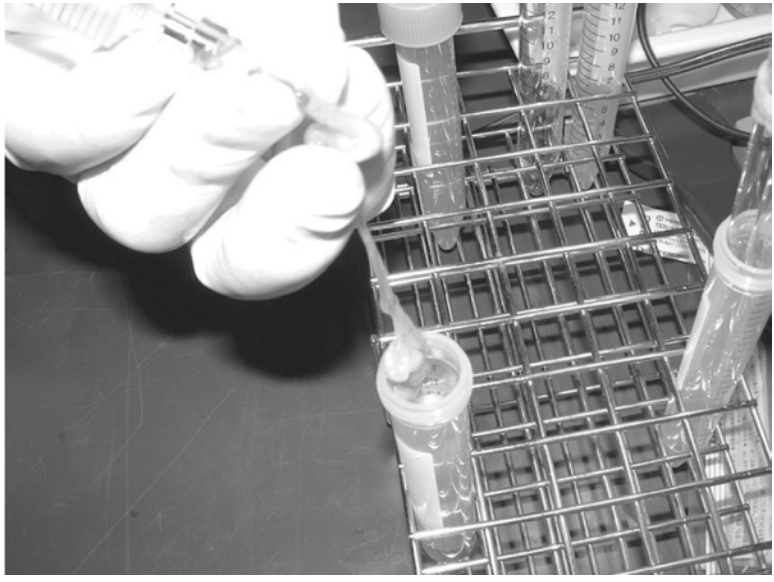

Fig. 4. Flushing oviduct with medium.

5. The cumulus cells are removed by hyaluronidase treatment. Oocytes with a first polar body are considered to be matured.

3.3. Donor Cell Preparation for Preimplantation Embryos (See Note 2)

1. The mucin coat and zona pellucida of eight-cell to morula-stage embryos are removed by 1% pronase treatment.
2. Zona-free embryos are separated into single blastomeres by pipetting in Ca^{2+} and Mg^{2+}-free PBS.
3. Separated blastomeres are maintained in EBSS supplemented with 10% FBS at room temperature until NT.

3.4. Donor Cell Preparation for Somatic Cells (See Note 3)

1. The various tissues obtained from fetus or adult are cut into 1–3-mm fragments and placed in 1 mL DMEM supplemented with 10% FBS in gelatin coated dishes.
2. After 7–14 d when cell growth extends to 70–90% of the bottom of the culture dishes, cells are dispersed with PBS containing trypsin and EDTA.
3. Cells are centrifuged at 300*g* for 5 min at 4°C; 4 mL DMEM supplemented with 10% FBS is added to the precipitation.
4. One milliliter of cell suspension is placed in a fresh gelatin-coated dish (passage 1), and cells are used for NT within seven passages, with each passage occurring every 3–5 d.
5. To induce a quiescent state, semiconfluent cells are cultured in DMEM with 0.5% FBS for 3–14 d (serum starvation) or by culture in 10% FBS for 5–21 d (contact inhibition).
6. To determine whether cells are in the mitotic phase, the BrdU incorporation is analyzed using a cell proliferation kit following the manufacturer's instructions. Cells are cultured in DMEM with BrdU substituted for thymidine. At the end of BrdU exposure, cells are washed and fixed. After immunocytochemical detection with reconstituted nuclease/anti-5-bromo-2'-deoxyuridine and peroxidase anti-mouse IgG2a, specimens are immersed in diaminobenzidine (DAB) solution containing substrate/intensifier. To delineate the cell borders, 0.5% eosin for 2–3 min is used as a counterstain. BrdU-positive cells in S-phase are stained blue-black. When serum-starved or contact-inhibited cells are cultured with BrdU overnight, the incorporation rate is low (0.5–1.0%).
7. The detailed cell cycle is analyzed using flow cytometry.
8. To analyze chromosome constitution, cells are treated with nocodazole for 5–6 h and cells with a round shape are collected from the culture dish. The cells are centrifuged at 300 g for 5 min, and 2 mL hypotonic solution is added to the precipitate and treated for 10 min at 39°C. The cell suspension is centrifuged, 2 mL fixative is added, and the cells are fixed for 10 min at room temperature. After centrifugation, 2 mL fixative is added, and the cells are mounted on a slide.

After drying, cells are stained with Giemsa for 10 min to count the chromosome number.

9. If necessary, 1 mL freezing medium is added to the cell precipitate in a 2-mL cryotube, directly placed into a deep freezer at –70°C and can be preserved for at least 1 mo. For thawing, cryotubes are plunged into water at 37°C for 30–45 s until half the ice melts and the contents are immediately transferred to a tissue culture tube with 10 mL DMEM that is cooled to 4°C and then centrifuge at 300*g* for 5 min. The cells are suspended in DMEM supplemented with 10% FBS and cultured.

3.5. Preparation of Microtools

3.5.1. Holding Pipet

1. Heat the glass capillary over a flame and pull rapidly by hand so that the outer diameter is 100–150 µm.
2. Set the capillary on a microforge vertically against the platinum–iridium wire filament.
3. Heat the filament of the microforge so that the capillary tip is reduced in size.

3.5.2. Enucleation and Injection Pipet

1. Pull thin-walled glass capillary using a pipet puller.
2. Set the capillary on a microforge horizontally against the wire filament.
3. Touch the tip of the capillary with an outside diameter of 10–25 µm to the glass bead on the tip of the wire.
4. Heat the wire slightly and stop the heating so that it breaks the capillary at the point of contact.
5. Set the capillary on a microforge vertically again and round the tip slightly using the filament.
6. Bevel the capillary tip at a 40°–45° angle on a rotating grinding wheel (*See* **Note 4**).

3.5.3. Needle

1. Heat the glass capillary over a flame and pull rapidly by hand. The diameter of the tip is not critical.
2. Set the capillary on a microforge vertically against the wire filament.
3. Pull the tip of the capillary down during low heating of the wire filament.
4. Pull the tip up and down two or three times during low heating of the filament, resulting in a fine glass needle. All microtools are slightly bent (30° angle) approx 1 mm from the tip by brief exposure to the heating wire, and, depending on the micromanipulator system used, one more bend must be made by hand with a small flame.

3.6. Nuclear Transfer

Unlike in other mammals, the zona pellucida of rabbit preimplantation embryos is necessary for normal development. Therefore, the slit on the zona

pellucida during NT should be small. EBSS supplemented with 10% FBS is used as the medium during micromanipulation.

*3.6.1. Removal of the Maternal Chromosomes Using the Conventional Method (*see ***Note 4****)*

1. Ovulated or in vitro matured oocytes are incubated with 7.5 µg/mL CB supplemented medium at 39°C for 15 min.
2. Oocytes are moved to a drop of CB-supplemented medium in a micromanipulation chamber (**Fig. 1**).
3. Hold the oocyte with a holding pipet using a micromanipulator under an inverted microscope (**Fig. 5A**).
4. Insert the beveled enucleation pipet into the perivitelline space and aspirate a small amount of oocyte cytoplasm close to the first polar body (**Fig. 5B**).
5. Stain the aspirated cytoplasm individually with 5 µg/mL Hoechst for 15 min and confirm the presence of the metaphase II chromosomes under ultraviolet light (**Fig. 5C**, arrow). The remaining oocyte whose aspirated cytoplasm has the chromosomes can be used as a recipient oocyte.

*3.6.2. Removal of the Maternal Chromosomes Using the Chemical-Assisted Method (*see *Note 5)*

1. When oocytes are treated with the medium supplemented with 0.6 µg/mL demecolcine for 30 min to 1 h at 39°C, membrane protrusion, where condensed maternal chromosomes are located, occurs (**Fig. 6A,B** , arrows).
2. Oocytes with a protruding membrane are moved to the medium supplemented with 7.5 µg/mL CB and 0.6 µg/mL demecolcine, and then the protrusion is removed with a beveled pipet (**Fig. 7A–C**).

3.6.3. Donor Nucleus Incorporation

1. Oocytes whose chromosomes have been removed are washed several times with medium and placed in a microdrop of the medium on a manipulation chamber.
2. Place the donor blastomeres or somatic cells in the second microdrop of the medium on the manipulation chamber (**Fig. 1**).
3. Aspirate donor blastomeres or somatic cells into the injection pipet and move the injection pipet to the droplet that contains recipient oocytes (**Fig. 8A**).
4. Insert the injection pipet into the perivitelline space of the recipient oocyte through the slit in the zona pellucida and introduce a single blastomere or somatic cell (**Fig. 8B,C**).
5. Press the donor cell carefully against the recipient ooplasm using the tip of the injection pipet.

3.6.4. Fusion of Donor Cell with Recipient Oocyte

1. The manipulated oocytes are allowed to equilibrate for 3 min in a 1:1 mixture of EBSS and Ca^{2+}-free Zimmerman cell fusion medium (*see* **Note 6**).

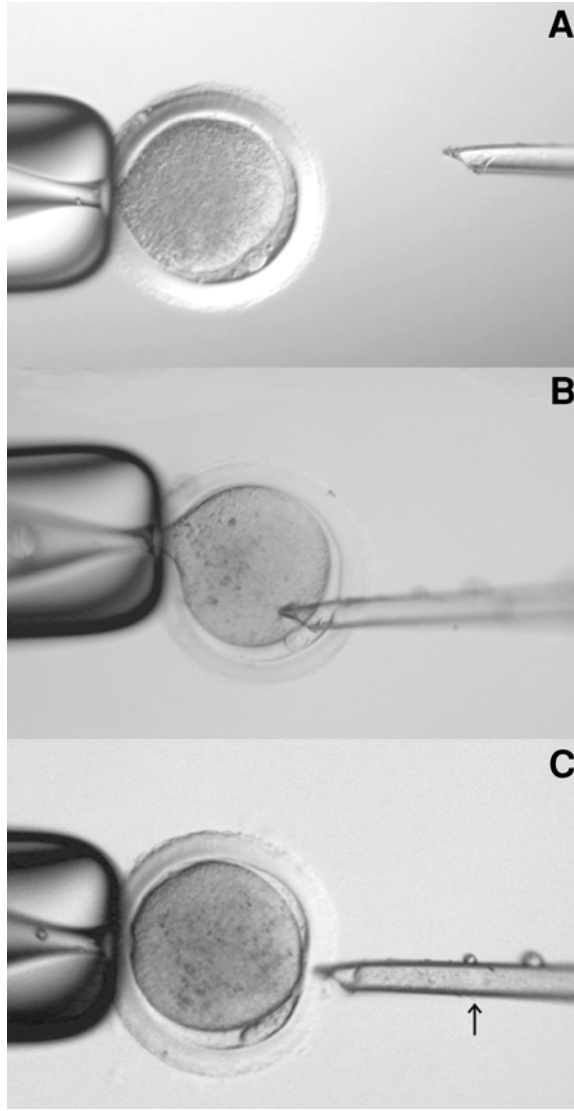

Fig. 5. Removal of chromosomes from oocyte (*see* text).

2. Move oocytes to 100% Ca^{2+}-free Zimmerman cell fusion medium for 3–5 min.
3. Move a single or several oocytes to a fusion chamber that is overlaid with fusion medium (**Fig. 9A**).
4. Move oocytes so that the agglutination plane of oocytes with donor cells is oriented in parallel with the electrodes using a glass needle attached to micromanipulator (**Fig . 9B**).

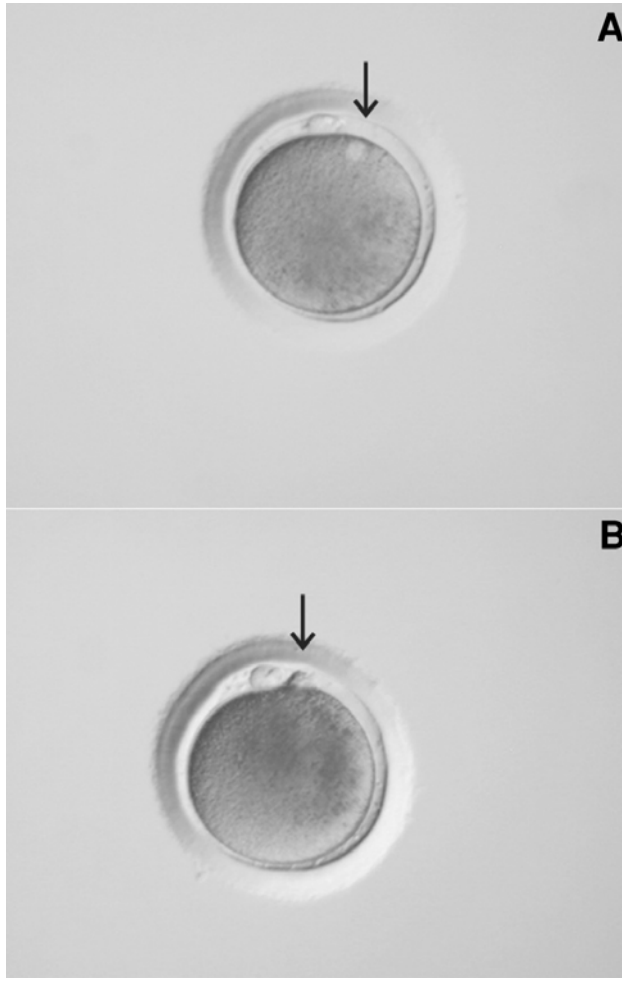

Fig. 6. Oocytes treated with demecolcine (*see* text).

5. Apply two1.5 kV/cm direct current pulses for 25 µs with a 0.1-µs interval to fuse donor cells with enucleated oocytes in Ca^{2+}-free Zimmerman cell fusion medium.
6. After electrical stimulation, reconstructed oocytes are washed in Ca^{2+} and Mg^{2+}-free EBSS medium containing 3 mg BSA/mL and are left for 15 min (*see* **Note 7**; **Fig. 9C**).

3.7. Parthenogenetic Activation

1. Before parthenogenetic activation, fused oocytes are cultured for 1–2 h in complete EBSS supplemented with CB to facilitate nuclear remodeling.

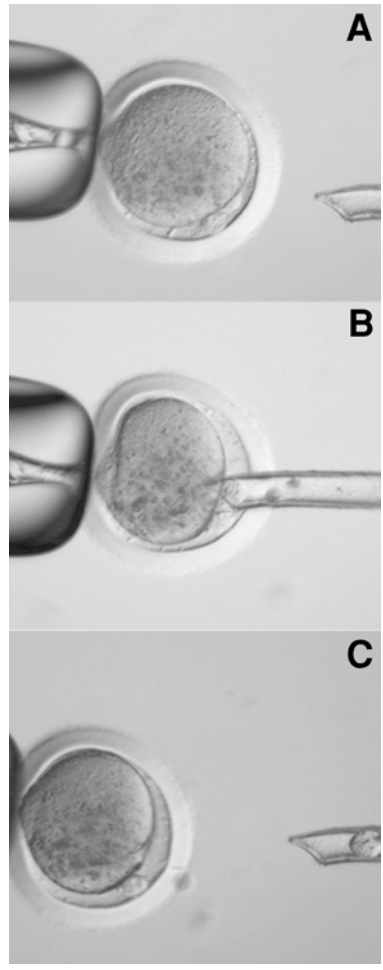

Fig. 7. Removal of chromosomes from oocytes treated with demecolcine (*see* text).

2. Activate fused oocytes by two 1.5-kV/cm direct current pulses for 60 μs at a 20-min interval in the cell fusion medium.
3. Culture activated oocytes in complete EBSS supplemented with 2 m*M* 6-DMAP for 2 h in an atmosphere of 5% CO_2 in air at 39°C.

3.8. In Vitro Culture

1. Wash reconstructed activated oocytes with complete EBSS several times.
2. Culture oocytes in complete EBSS for 5–6 d in an atmosphere of 5% CO_2 in air at 39°C.

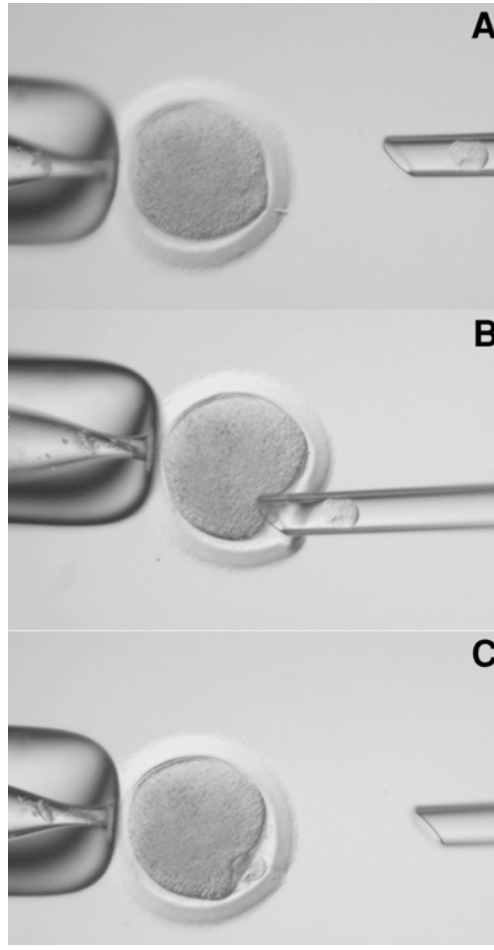

Fig. 8. Injection of donor cell into perivitelline space of enucleated oocytes (*see* text).

3.9. Embryo Transfer

Because the potential of rabbit embryos to develop into fetuses decreases drastically with the duration of in vitro culture, NT oocytes should be transferred to recipient females soon after NT.

1. Inject 30 IU of hCG intravenously into recipient females 12 h before embryo transfer.
2. Intravenously inject 1.5 mL nembutal solution into a marginal ear vein.
3. Shave the flank on both sides and clean with 70% ethanol.

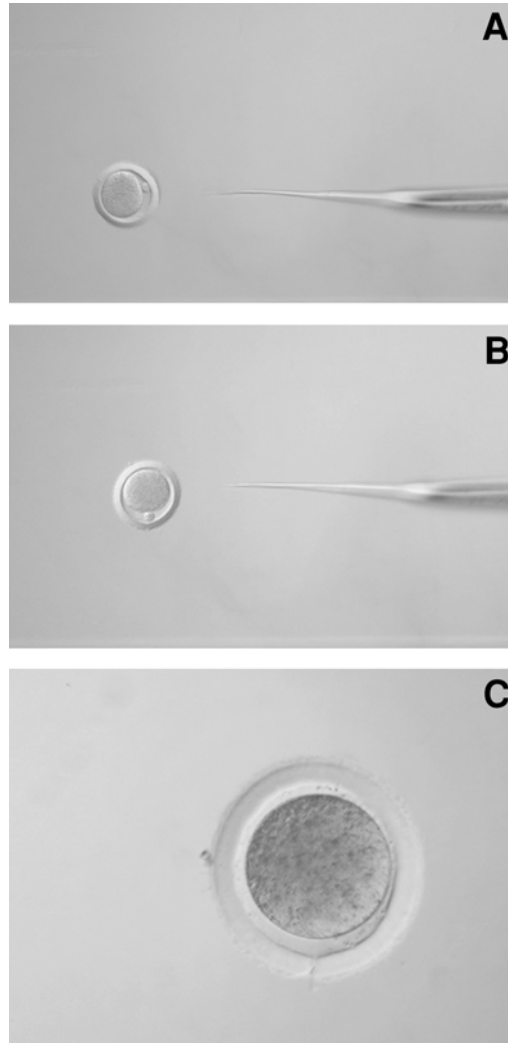

Fig. 9. Electric fusion of donor cell with enucleated oocyte (*see* text).

4. After flank incision, the oviduct is pulled to the exterior by grasping fatty tissue with forceps.
5. Aspirate NT oocytes with a minimum volume of the medium into the pipet.
6. Insert the pipet into the infundibulum and gently expel the oocytes. (**Fig. 10**).
7. A penicillin solution is sprayed into abdomen and the abdomen is closed.
8. Pregnancy diagnosis is possible after d 10 of pregnancy by palpation. If necessary, the presence of fetuses can be determined after d 15 of pregnancy by middle dissection.

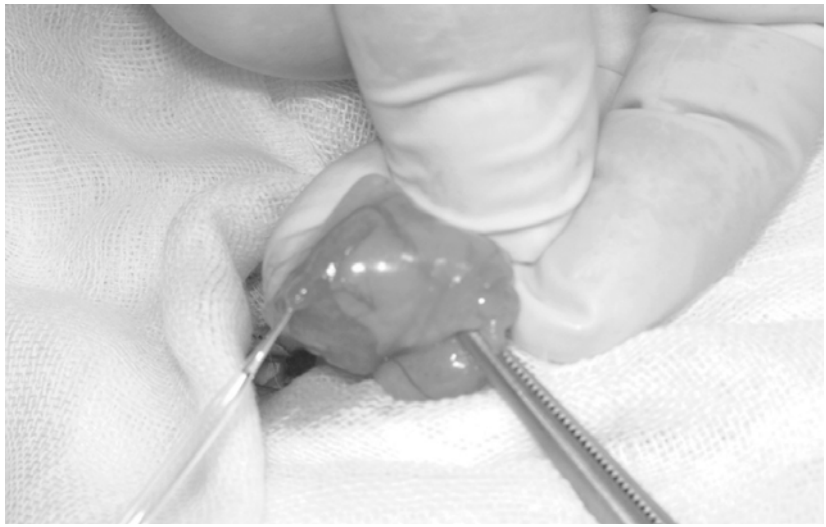

Fig. 10. Transfer of NT oocyte into the infundibulum (*see* text).

4. Notes

1. Because the timing of ovulation varies from 10.5–14.0 h following leutinizing hormone injection of (LH or hCG) ***(22)***, it is sometimes difficult to collect a large number of rabbit oocytes soon after ovulation. When oocytes are recovered 14 h after hCG injection, gently move ovaries with oviducts to the medium, rinse ovaries initially to recover oocytes sticking to the surface, and then flush oviducts with the medium.
2. The cell cycle of donor nuclei and condition of recipient oocytes are important for the normal development of NT oocytes ***(23)***. Two different combinations can be used for successful NT. One method is to fuse donor cells at the G1/G0 stage with oocytes of the second metaphase. The other method is to fuse donor cells at any stage of the cell cycle with previously activated oocytes. In the studies on NT of blastomeres of preimplantation rabbit embryos, no attention has been paid to the combination of donor cell cycle and the condition of recipient oocytes.
3. The direct exposure of donor chromosomes to nonactivated oocyte cytoplasm is essential for the reprogramming of somatic cell nuclei ***(24)***.
4. When thin-walled glass capillaries are used, a sharp point on the tip of the smooth bevel is unnecessary for penetration of the zona pellucida.
5. When oocytes are treated with demecolcine for 30 min–1 h, more than 70% of oocytes have a membrane protrusion, and the chromosome mass migrates to a cortical location ***(25)***. Removing the chromosomes with a small volume of cytoplasm is easy, and the success rate is high.
6. If manipulated oocytes are moved directly to fusion medium, some blastomeres come out from the slit in the zona pellucida.

7. If reconstituted oocytes are moved to EBSS containing Ca^{2+} and Mg^{2+} after electrical stimulation in a Ca^{2+}-free cell fusion medium, some oocytes might be parthenogenetically activated.

Acknowledgments

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12

Nuclear Transfer in Cattle

Gottfried Brem and Manzoor A. Nowshari

1. Introduction

For decades, a dream of animal breeders has been to clone (make exact genetic copies of) outstanding animals. This was first achieved by splitting embryos ***(1)***; however, with this technique, only a few animals of the same genetic origin could be produced ***(2)***. The second approach, which seemed to be more promising, was the embryo reconstruction using a single nucleus from an embryo to an enucleated one-cell embryo. This technique was based on an idea proposed by Spemann in 1938 ***(3)***. With only few exceptions, experiments on frogs and fish provided scientific evidence that all the cells in the body of an animal appear to contain the same genetic information, being contained in DNA, a molecule located in the nucleus of cells. Thus, within an animal, the DNA sequence in the mammary cells is identical to skin cells. These cells differ in their appearance and function because they utilize different parts of the genetic information, not because the total amount of information differs. Furthermore, all these cells have genetic information present in the one-cell embryo that develop into the animal. Therefore, if the nucleus of any of these cells were used to replace the genetic information in any one-cell embryo, an exact genetic copy of the animal whose cells donated the nucleus would develop. This theory was confirmed by successful experiments in frogs and fish ***(4,5)***. Using similar procedures and inner cell mass cells (ICM) as nuclear donors, Illmensee and Hoppe ***(6)*** reported the birth of live offspring (mice) in 1981. However, these results were never confirmed. In farm animals, similar procedures that resulted in the birth of live offspring were reported in 1986 ***(7)***. In the initial work on nuclear transplantation, embryonic blastomeres or ICM

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were used as donor nuclei because they were thought to be relatively undifferentiated, readily reprogrammable, and likely to support full-term fetus development ***(8)***. The most acclaimed example of animal cloning is the report by Wilmut et al. ***(9)***, the first to demonstrate that cloning of adult mammals is possible. These authors reported the birth of Dolly, a sheep, from an adult donor mammary cell. With the development of somatic cell cloning, there has been much interest in the technique, which principally enables both the multiplication of elite livestock and engineering of transgenic animals for various agricultural and biomedical purposes. The production of large number of low-cost genetically identical elite animals is still a very attractive goal for agriculturists. Large gains can be made in cattle if unlimited cloning of elite embryos or other cell types with an elite genetic background allows for the production and selection of superior clonal lines of animals. Cloning leads to automatic sex selection, thus of great interest to cattle breeders. Another agricultural use would be genome banking from domestic and wild animals. It is more convenient to freeze cells from chosen animals than semen or embryos. There is an increasing focus in improving various agricultural traits in livestock by gene targeting. The birth of calves following nuclear transfer (NT) using cultured transgenic fetal cell lines demonstrates the possibility of producing cloned transgenic cattle ***(10,11)***. For the production of transgenic animals, the donor cells have to be transfected with the desired expression vectors, or other procedures have to be used to genetically modify the cells (*see* **refs. *12*** and ***13***).

The NT technology is well advanced in cattle when compared to most of the domestic species. This is not only because of the numerous research programs focused on NT in cattle, but also because of the enormous base of knowledge that has been developed over the past 30 yr involving the application of assisted reproductive techniques in cattle. Successful and repeatable procedures for in vitro maturation, in vitro fertilization, and in vitro embryo culture are now well established in cattle. Each of these procedures represents a key step in the cloning process. The ability to access many oocytes at a relatively low cost also provides an opportunity to carry out numerous cloning attempts. As nuclear recipients, oocytes from ovaries collected at slaughterhouses are generally used; however, for experimental purposes, mature oocytes from superovulated donors collected by ovum pick-up can also be used ***(14)***. Various factors can influence the success of nuclear transplantation using embryonic, as well as somatic, cells as nuclear donors. Multiple differentiated cell types, e.g., fetal fibroblasts, cumulus cells, granulose cells, adult fibroblasts from mammary glands, skin and ear cells, have been used as nuclei sources for cloning cattle. Nearly all cell types tested thus far have resulted in live offspring, but with large differences in their cloning efficiency. Even subcultures

(batches) derived from the same biopsy may result in different cloning success. The relative cell cycle of the donor and recipient cell cycle has been recognized to be crucial to the success of NT experiments; therefore, the timing of individual steps and treatment of donor cells and recipient oocytes has to be given great consideration. The synchrony between the donor nucleus and recipient cytoplasm enhances the successful development of the reconstituted embryo. Despite significant technical improvements over the last decade, resulting in better preimplantation development of reconstructed embryos, the fetal and perinatal development remains low and highly variable. In cattle, in addition to a significant amount of perinatal mortality, a high abortion rate occurs during the last third of gestation. A higher prevalence of lethal syndromes has also been observed among the offspring born of NT embryos.

2. Materials

2.1. Equipments

1. Stereomicroscope fitted with a warm plate.
2. Inverted microscope equipped with differential interference contrast and epifluorescence.
3. Micromanipulators.
4. Microinjectors connected with syringes.
5. Osmometer.
6. pH-meter.
7. Incubators (gassed with CO_2; CO_2, and O_2).
8. Cell fusion machine.
9. Fusion chamber with two electrodes 250–300 μm apart.

2.2. Manipulation Chamber (Fig. 1)

1. Heat approx 20 g of wax until melted in a beaker.
2. With the help of cotton ear buds, apply a circle of wax on the glass coverslip and raise it to approx 3 mm by overlaying the wax on the circle.
3. Allow to cool.
4. Clean the coverslip with 70% ethanol, and keep it packed in a dust-free tissue paper until use.

2.3. Microtools

For micromanipulation, two types of pipets are used: one for holding oocytes/embryos and the other for enucleation/injection.

2.3.1. Holding Pipets (Fig. 2)

1. Pull glass capillaries by hand over a small flame.
2. Cut the pipet with a glass cutter to give it a diameter of 120 – 140 μm.

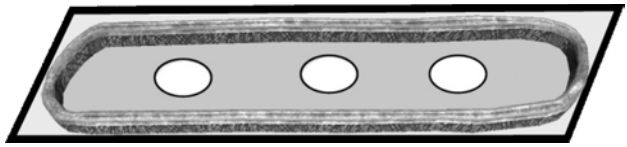

Fig. 1. A hand-made manipulation chamber with manipulation droplets.

3. Mount the capillary onto the microforge, and place the open end just in front of the glass bead on the filament. Apply heat until the open tip is almost closed, ensuring a smooth end with a diameter of approx 25 µm.
4. Make a bend approx 0.7 – 1 cm from the close to the tip of the pipet at an angle of 30°.

2.3.2. Enucleation/Injection Pipet (Fig. 2)

1. Pull glass capillaries using a pipet puller.
2. Mount a drawn capillary on the microforge, measure the diameter of the pipet using an eyepiece graticule, and break at the required size, usually between 15–20 µm. The pipet is broken by fusing it onto the glass bead, which is slowly heated until the glass melts and sticks to it. The power is then abruptly turned off, which results in breaking the pipet.
3. Mount the pipet onto the capillary holder of the microgrinder. The holder is brought slowly onto the grinding stone at an angle of 45° under visual observation through a binocular. Care should be taken to completely grind the pipet.
4. Mount the ground pipet in silicon rubber tubing with a diameter slightly more than that of the pipet, which has been fixed to a 5-mL syringe.
5. Wash the pipet by dipping into a 20% hydrofluoric acid under continuous blowing of air.
6. Wash with 70% alcohol and then twice with distilled water.

2.4. Solutions

2.4.1. Culture of Fetal and Adult Fibroblasts and Their Cyropreservation

1. Phosphate-buffered saline (PBS; without Ca^{2+} and Mg^{2+}): Add 8.0 g NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4 $2H_2O$, and 0.2 g KH_2PO_4 to milli-Q water. Adjust pH to 7.2 (*see* **Note 1**) and bring to a total volume of 1 L. Sterilize by filtration and store at room temperature.
2. Cell culture medium: prepare the medium ready-to-use by adding 5 mL glutamine (200 m*M* stock solution), 5 mL nonessential amino acids (100X stock solution), 5 mL sodium pyruvate (100 m*M* stock solution, 100X), 5 mL penicillin/streptomycin solution (10,000 IU/10 mg/mL), and 50 mL fetal calf serum (FCS) (tested batch and heat inactivated at 56°C for 30 min) to 500 mL Dulbeccos medium Eagle's medium (DMEM), filter and store it at 4°C.

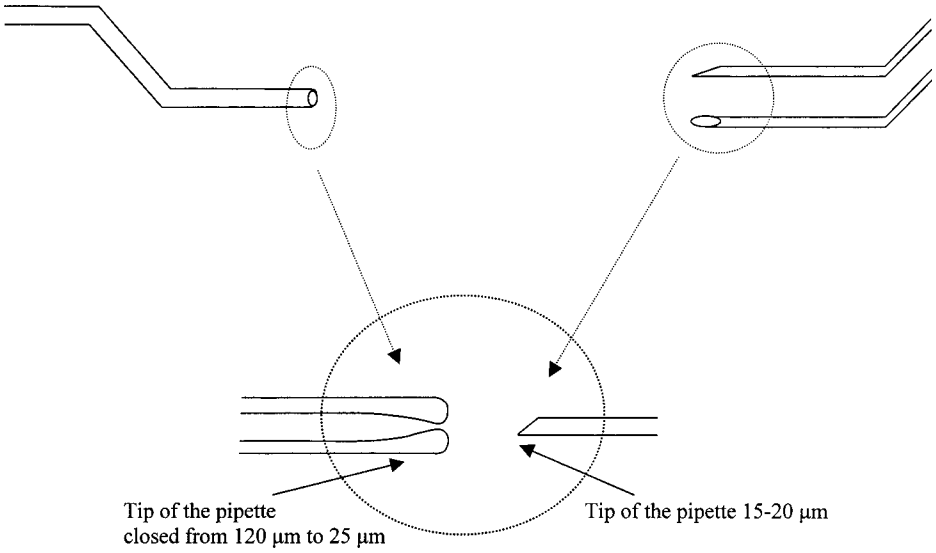

Fig. 2. A diagrammatic representation of the holding and enucleation/injection pipet.

3. Trypsin/EDTA: To make a solution of 0.25% trypsin and 0.2% EDTA, add 100 mg trypsin and 80 mg EDTA to 40 mL of PBS without Ca^{2+} and Mg^{2+}. Dissolve and filter-sterilize. Store at –20°C in 10-mL aliquots.
4. Freezing medium: To make 10 mL freezing medium, add 1 mL dimethyl sulfoxide (DMSO) to 9 mL FCS. Sterilize by filtering through 0.2-µm filter and store at 4°C.
5. Acid Tyrode's solution: To make 100 mL, add 8.0 g NaCl, 0.02 g KCl, 0.024 g $CaCl_2 \cdot 2H_2O$, 0.10 g $MgCl_2 \cdot 6H_2O$, 0.1 g glucose, and 0.4 g polyvinylpyrrolidone (PVP). Adjust pH to 2.5 with 5 *M* HCl, then filter-sterilize and store at 4°C.
6. Low -serum cell culture medium: cell culture medium (as above), but containing only 0.5% FCS.

2.4.2. Oocyte Handling/In Vitro Maturation

1. PBS: dissolve 8.0 g NaCl, 0.2 g KCl, 0.24 g $CaCl_2 \cdot 2H_2O$, 0.1 g $MgCl_2 \cdot 6H_2O$, 1.0 g glucose in Milli-Q water. Make volume to 1 L. Check the osmolarity (270–280 mosm) and pH (7.2). Filter-sterilize and store at room temperature.
2. For transportation of ovaries from a slaughterhouse, add 100 mg streptomycin and 30 mg penicillin to 1 L of PBS.
3. Modified PBS (mPBS): Add 36 mg sodium pyruvate, 10,000 IU/mL penicillin, and 10,000 µg/mL streptomycin to I L of PBS. Filter-sterilize and store at 4°C.
4. Gentamycin stock solution: Dissolve 50 mg gentamycin in 1.0 mL 0.9% NaCl and keep at 4°C for use.

5. Basic follicle-stimulating hormone (bFSH) stock solution: Dilute 50-U vial of bFSH with 25 mL M199, aliquot 25 µL into 0.5-mL microcentrifuge tubes, wrap tops with parafilm, and store at –20°C.
6. Basic luteinizing hormone (bLH) stock solution: Dilute 50-U vial bFSH with 25 mL M199, aliquot 25 µL into 0.5-mL microcentrifuge tubes, wrap tops with parafilm, and store at –20°C.
7. Estrus cow serum (ECS): blood is collected from cows in standing estrus, and serum is separated by centrifugation. Serum is then inactivated by heating at 56°C for 30 min. Filter-sterilize and aliquot in 1-, 5- and 10-mL tubes and store in –20°C.
8. Maturation medium (MPM): Make solution I by adding 10 mg L-glutamine, 80 mg $NaHCO_3$, 140 mg HEPES, 25 mg sodium pyruvate, 110 µL gentamycin stock solution 100 mL TCM 199, and solution II by adding 60 mg calcium lactate to 10 mL Milli-Q water. Mix solutions I and II, check the osmolarity (280 – 300 mOsm), and filter using 0.2-µm filter, and store at 4°C. Prepare every 2 wk fresh. Before, use add 1 mL ECS, and 25 µL bFSH stock solution, 25 µL bLH stock solution to 9 mL-medium. Filter and leave overnight in an incubator with 5% CO_2. The next morning, adjust pH to 7.3–7.4. Prepare fresh.
9. Wash medium: MPM without bFSH and bLH.

2.4.3. Oocyte manipulation, Fusion and Cultur

1. Hyaluronidase solution: Dissolve hyaluronidase in PBS to 1.0 mg/mL. Filter, aliquot, and store at –20°C.
2. Cytochalasin B stock solution: Dissolve 5 mg cytochalasin B in 500 µL DMSO. Distribute in 20 tubes at 25 µL and store at –20°C. Each tube can be used for 10 dilutions (2.5 µL/5 mL medium).
3. Bisbenzimide (Hoechest 33342) stock solution: Dissolve 1.0 mg/mL in PBS. Prepare 20-µL aliquots and store at –20°C.
4. Fusion medium: Add 23.96 g sucrose, 4 mg magnesium acetate·4 H_2O, 4 mg calcium acetate, 43.5 mg 7.75 mg potassium phosphate, glutathione to 250 mL of Milli-Q H_2O, then bring the pH to 7.0 with acetic acid (1–2 drops). Add 10 µL of Ozil-stock (10 mg $CaCl_2$/mL Milli-Q H_2O). Filter the solution and store at 4°C; 1 mo shelf life. Before use, add 6 mg BSA (fatty acid-free) to 30 mL fusion medium.
5. Ethanol alcohol solution: add 140 µL ethanol alcohol (absolute) to 1860 µL culture medium supplemented with 5% ECS.
6. Cycloheximide stock: Dissolve 5.628 mg cycloheximide in 1 mL Milli-Q H_2O, prepare 4 µL aliquots, and store at –20°C. Add 3.55 µL of this stock solution to 2.0 mL culture medium to a 10 µg/mL concentration.
7. Embryo culture medium: To 200 mL Milli-Q water, add 1258.2 mg NaCl, 106.8 mg KCl, 32.4 mg KH_2PO_4, 49.6 mg $CaCl_2 \cdot 2H_2O$, 19.2 mg $MgCl_2$. $6H_2O$, 421.2 mg $NaHCO_3$, 0.28 mg phenol red, 72.6 mg Na-pyruvate, 1 mL L-glutamine (200 m*M*), and 94.12 µL Na-lactate (1.3 g/mL). Check the osmolarity (270–280 mOsm). Filter and store at 4°C. Before use, add 200 µL essential amino acids,

100 µL nonessential amino acids, and 500 µL ECS to 9.5 mL medium. Adjust pH to 7.2 – 7.3, and filter-sterilize.
8. Hams's F-12 medium: To 45 mL of this medium add 5.0 mL FCS and 500 µL penicillin/streptomycin.

3. Methods

3.1. Preparation of Donor Cells/Blastomeres

3.1.1. Collection of Adult Cells

1. Skin samples are collected from the ear or any other site of the body by a biopsy needle.
2. Tissue pieces are washed in PBS and cut manually into small pieces.

3.1.2. Collection of Donor Fetal Fibroblasts

1. Collect fetuses from animals induced to abort or slaughtered on d 40–60 of pregnancy.
2. Clean the outer surface of the uterus with a sterile drapper.
3. Carefully make an incision and remove the fetus still in its amniotic sac, put in PBS, and transport to the laboratory on ice.
4. Transfer fetus (still in its amniotic sac) to a large empty Petri dish.
5. With the help of sterile scissors and forceps, the amniotic sac is opened and the fetus transferred to a large Petri dish containing PBS.
6. Dip the fetus in PBS and shake it to remove blood stains, and so forth. From large fetuses cut tail or neck portion for PCR analysis. From smaller fetuses cut whole head and preserve it at –80°C for future polymerase chain reaction (PCR)-analysis.
7. Incise the thoracic cavity of the fetus from sternum toward the upper extremity. Preserve the upper portion in a plastic envelop for future analysis.
8. Open the fetus medially and remove the inner organs.
9. Remove the cartilaginous tissue around the ribs. If the fetus is large enough, remove the ribs along with the cartilaginous tissue.
10. Wash the rest of the soft tissue four times in PBS for fibroblast preparation, and collect the tissue from the abdominal, back, hip, and flank region.
11. Wash these pieces of collected tissue in fresh PBS 1–2 times.

3.1.3. Culture of Donor Cells

1. Transfer the tissue pieces into a large Petri dish with 5–8 mL trypsin solution (0.25%), and cut into fine pieces with the help of a sharp scissors or scalpel knife, so that these can be pipetd in and out with a Pasteur pipet to form a suspension.
2. Transfer this suspension to a beaker, add a magnetic stirrer, and allow it to stir slowly for 10 – 15 min at 37°C. Alternatively, leave the suspension in an incubator, shake the suspension, and pipet a few times.
3. When the suspension is homogeneous, filter it in a test tube through a three-layered sterile gauze previously packed and autoclaved with 10 mL medium. Mix the filtrate thoroughly and centrifuge it for 5 min at 350*g*.

4. Discard the supernatant, resuspend the pellet in 3.4 mL medium, and centrifuge again.
5. Spread the cells in two or three large Petri dishes according to the size of the pellet. Gelatinized plates are preferred, but not necessary.
6. Leave the Petri dishes in an incubator and change medium after every 24 h (*see* **Note 7**).
7. Once confluent (after 5–7 d), freeze cells by making five or six vials from each plate.
8. For cell disaggregation, the medium is removed from the plates, and the cells are washed twice with PBS. Cells are covered with a thin layer of trypsin solution, and plates are incubated for 2– 4 min at 37°C. Once the cells are disaggregated, 3–5 mL PBS is added to the plate. The cell suspension is then transferred to a 15-mL centrifuge tube and centrifuged at 350*g* for 4 min. The supernatant is removed, and the cells are resuspended in 1–3 mL medium.
9. For freezing the cells, cool the cryopreservation medium along with the cryotubes on ice, transfer trypsinized and washed cells to a 10-mL centrifuge tube, centrifuge at 350*g* and remove the supernatant. Add freezing medium and mix them by pipetting slowly, aliquot in 1-mL cryotubes, and leave in a freezer at –80°C. After 24 h, transfer to liquid nitrogen container for storage.
10. Five days before use, the cells are thawed in a water bath at 37°C.
11. The cryoprotectant is removed by transferring cell suspension to 5 mL cell culture medium, mix and centrifuge at 350*g* for 4 min, remove the supernatant, and resuspend the pellet in 1 mL medium.
12. For a routine culture, thaw one vial and spread on a 8.5-cm plate. When confluent, passage the plate on 4 × 8.5-cm plates, which are again passaged (when confluent) on 4 × 8.5-cm plates.
13. The cells are then cultured for 4–5 d in low serum cell culture medium.

3.1.4. Preparation of Donor Blastomeres

Both in vitro as well as in vivo–produced embryos are used as donors for NT (production of donor embryos is out of scope of this chapter). Blastomeres from embryos at morula or earlier stage and ICM cells of blastocyst stage are used as donor cells.

1. Put several drops of PBS Ca^{2+}-, Mg^{2+}-free and several drops of acidified PBS, pH 2.5 in a Petri dish.
2. Transfer good-quality embryos selected as nuclear donors to a drop of PBS.
3. Pick up one embryo with as little medium as possible and wash through one drop of acid Tyrode's solution. Then transfer to a fresh drop of acidified solution.
4. Agitate the embryo in the acid solution while observing the zona dissolution.
5. Transfer the embryo into a fresh drop of PBS as soon as its zona pellucida is dissolved.
6. Repeat the procedure with the remaining embryos. Use new PBS drops and acidified PBS for each embryo.

7. Wash the embryos two to three times and transfer individually to drops of Ca^{2+}- and Mg^{2+}-free PBS.
8. Leave the Petri dish with embryos at 37°C (in incubator) for 30 min.
9. Pipet the embryos through a narrow pipet 8–10 µm id to separate the blastomeres.
10. To obtain ICMs, incubate zonae-free d 7 blastocysts in mPBS with 5 µg/mL cytochalasin B at 39°C for 10 min.
11. Wash the embryos twice in Ca^{2+}- and Mg^{2+}-free PBS.
12. Transfer to a drop of Ca^{2+}- and Mg^{2+}-free PBS with 0.25% trypsin and 0.2% EDTA for 5 min.
13. Isolate the ICMs from the surrounding trophectoderm (TE) with a fine glass pipet.
14. Separate the ICMs mechanically by using a fine pipet (approx 5 µm id).
15. Leave the separated blastomeres/ICM cells in a drops of Ca^{2+}- and Mg^{2+}-free PBS with 1 mg/mL PVP overlaid with paraffin oil.

3.2. In Vitro Maturation of Recipient Oocytes

1. Prepare maturation and wash media.
2. Make maturation droplets (400 µL) in four-well dish and cover with paraffin oil (*see* **Note 3**).
3. Equilibrate in incubator at least for 2 h.
4. Bovine ovaries are collected at the slaughterhouse and are transported to the laboratory in PBS at 25–30°C.
5. Wash ovaries prior to aspiration (warm PBS or saline is appropriate).
6. Aspirate small follicles (2–5 mm) using 18-gauge needle.
7. Put follicular fluid in a 50-mL conical tube to settle, and keep it on a warm plate (*see* **Note 4**).
8. Transfer the settled contents of the conical tube onto a large Petri dish.
9. Collect cumulus oocyte complexes (COCs) under stereomicroscope and transfer to a 35-mm Petri dish containing wash medium.
10. Wash the COCs twice in wash medium and twice in maturation medium.
11. Transfer 20–25 COCs to each maturation droplet and leave in an incubator for 18–20 h in an atmosphere of 5% CO_2 in air at 39°C.

3.3. Micromanipulation (Fig. 3)

3.3.1. Enucleation

1. Prepare manipulation chamber (Fig. 1), make three working droplets (approx 150 µL each) with medium PBS with FCS on the chamber, and cover with paraffin oil.
2. Remove the cumulus cells from the oocytes by vortexing them for 3–4 min after placing into warm (39°C) mPBS containing 1 mg/mL hyaluronidase (*see* **Note 5**). Remove rest of the attached cumulus cells by careful pipetting.
3. Wash the oocytes twice in mPBS medium.
4. Transfer batches of 20–25 metaphase II (MII) oocytes (*see* **Note 5**) at regular intervals to mPBS supplemented with 7.5 µg/mL cytochalasin-B for 10 min.

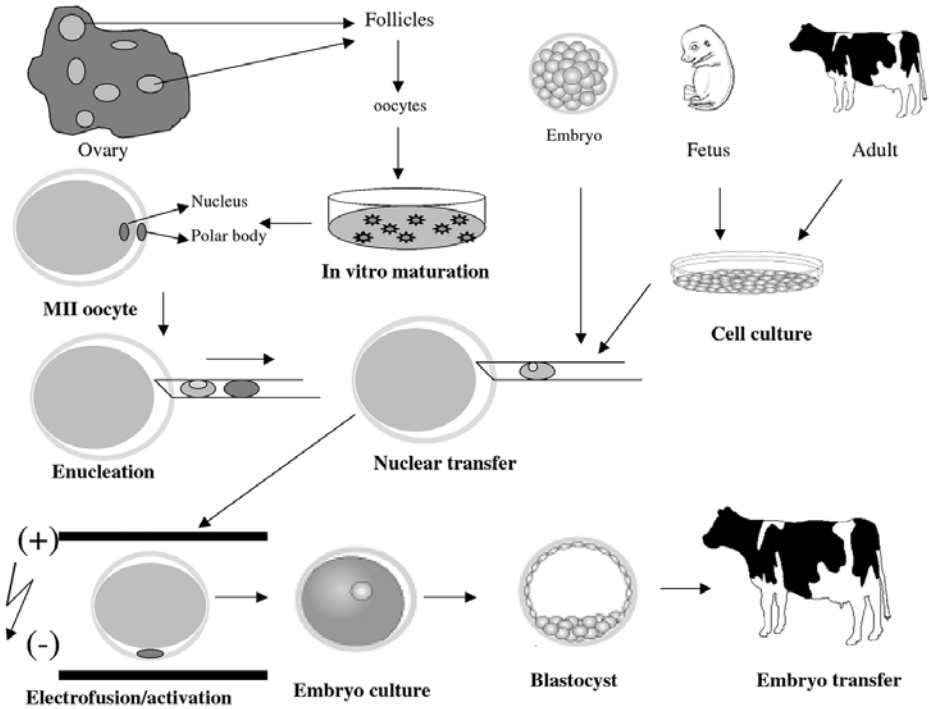

Fig. 3. NT using embryonic, fetal, and adult fibroblast cells as nuclear donors. Recipient oocytes are collected preferably from ovaries collected from slaughtered cattle and matured in vitro to MII stage.

5. After incubation, transfer each batch of cytochalasin-treated oocytes to the lower side of one of the medium droplet on the manipulation chamber.
6. Fix one oocyte with the holding pipet, under observation at ×40.
7. While focusing on the oocyte, increase the magnification to ×200.
8. Bring the enucleation pipet into focus.
9. Rotate the oocyte and bring the polar body to a 6 h position.
10. Penetrate the zona pellucida with the enucleation pipet and aspirate the polar body and cytoplasm adjacent to it.
11. Withdraw the enucleation pipet from the oocyte, then discard the aspirated material from the pipet.
12. Release the oocyte from the holding pipet on the upper side of the droplet.
13. After enucleating all the oocytes of the batch, transfer them to a drop of mPBS and leave on a warm plate (39°C).
14. Once all the oocytes are enucleated, transfer them to a small Petri dish containing mPBS medium with 10 µg/mL bisbenzimide. Cover the Petri dish with an almunium sheet to avoid exposure to light.

15. Wash the oocytes three times in fresh mPBS drops
16. Observe the oocytes under fluroscence microscope (*see* **Note 7**), then discard the oocytes with partial or complete nucleus (stained blue).

3.3.2. Reconstruction of Cell/Blastomere-Oocyte Couplets

1. Mount the holding and injection pipet on the micromanipulator.
2. Transfer 10–20 enucleated oocytes to a drop of the mPBS on micromanipulation chamber.
3. Transfer donor blastomeres or donor cells (*see* **Subheading 3.1.**) to the drop of medium containing enucleated oocytes on the micromanipulation chamber.
4. Fix the recipient enucleated oocyte with the holding pipet.
5. Pick up an individual cell or a blastomere with the injection pipet.
6. Penetrate the zona pellucida with the injection pipet, and deposit the cell or blastomere in the perivitelline space.
7. Withdraw the injection pipet from the oocyte.
8. Release the oocyte on the upper side of the chamber.
9. Continue injecting the cells or blastomeres to the enucleated oocytes.
10. Once the batch is finished, transfer the couplet batch to a drop of fresh mPBS, and continue the injections with the next batch of enucleated oocytes.

3.3.3. Fusion of Donor Nuclei and Recipient Cytoplast

1. Transfer 10–20 cytoplast/karyoplast couplets to fusion medium.
2. After 5 min, transfer the couplets to the fusion chamber, placing them in between two electrodes.
3. Align couplets manually using a hand-drawn capillary mouth pipet.
4. Apply two fusion pulses of 146 V for 60 μs (*see* **Note** 8).
5. Remove the couplets from the chamber after washing them in Ham's F-12 medium supplemented with ECS. Incubate the couplets in the same medium until activation.

3.3.4. Activation of the NT Couplets

1. Before activation, examine the couplets for successful fusion. Select the fused couplets for activation.
2. Transfer the couplets to culture medium with 7% ethanol alcohol and leave in this medium for 5 min.
3. Wash the couplets twice in the culture medium.
4. Transfer couplets to the culture medium with cycloheximide and incubate for 5 h.
5. After incubation, wash the couplets again in culture medium and transfer to drops of culture medium for further incubation in an incubator with 5% CO_2, 5% O_2, and 90% N_2 at 39°C.
6. Examine the embryos after overnight culture and remove noncleaved embryos.
7. Culture the embryos further for 6 d and transfer those developing to the blastocyst stage to recipients.

3.4. Embryo Transfer

NT embryos are transferred nonsurgically at the blastocyst stage into synchronized recipients.

Synchronized recipients: Recipients are selected among the herd of recipients that have been detected in standing estrus 7 d prior to embryo transfer (ET).

1. Confirm the ovarian status of the potential recipient (should have a corpus luteum).
2. Transfer selected embryos to a Petri dish containing 2 mL mPBS + 10% ECS.
3. Load one or two high-quality embryos in a 0.25-mL plastic straw along with minimal amount of medium (mPBS + 10% ECS).
4. Load the straw in an ET gun (Cassou gun).
5. Clean the vagina with lukewarm water.
6. The ET gun with the straw containing the embryo is penetrated through the cervix, and the embryo is deposited in the uterine horn ipsilateral to the corpus luteum.
7. The ET gun is removed.
8. Recipients are examined by ultrasonography on d 42 of gestation to record fetal development.
9. Pregnant cows are monitored by rectal palpation at regular intervals thereafter.
10. Commencing approx 2 wk before expected full term, pregnant cows are monitored daily by rectal and vaginal examination to determine fetal position and cervical dilation.
11. Parturition is induced with an injection of 20 mg dexamethasone (Dexadreson, Intervet).

4. Notes

1. All the media used for in vitro maturation, culture, and handling should be checked for pH and osmolarity before use.
3. Test each bottle of paraffin oil for its toxicity with mouse embryos culture system.
4. Try to keep the temperature constant during the manipulation. A high fluctuation in temperature may result in untimely and unwanted activation of the oocytes.
5. Do not expose the oocytes to hyaluranidase for more time periods than necessary to remove the cumulus cells.
6. Expose the oocytes to fluorescence light (while examining for enucleation) for minimal period of time (<1 s).
7. To achieve better enucleation rates, select the oocytes (MII stage) that are just extruding the polar body (approx 18 h after the start of maturation period).
2. To avoid the fluctuations in the temperature and gas atmosphere of the incubators, try to close the doors of the incubator as soon as possible.
8. The parameters for couplet fusion may need to be optimized for the size of nuclear donors used.

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In Vitro Fertilization and Embryo Transfer in Felids

Charles Earle Pope

1. Introduction

The genome of the cat is highly conserved. In fact, of the nonprimate mammalian species in which gene maps are developing, the cat genome exhibits the most similarities to that of the human ***(1)***. Biomedical studies in the domestic cat have contributed significantly to our knowledge in the areas of immunology, infectious diseases, genetics, neurophysiology, and cancer ***(2)***. Many of the heritable disorders of cats are analogous to those of humans, including hemophilia A and B, polycystic kidney disease, and several lysosomal storage diseases such as mucopolysaccharidosis, α-mannosidosis, and spingomyelinosis C. The close phylogenetic relationship, and the fact that many analogous genetic disorders have been identified and characterized, are important factors that demonstrate the advantages of domestic cats as biomedical research models for human disease when compared to other laboratory animals.

Although initial efforts to enhance reproductive rates of laboratory cats were reported more than 30 yr ago, most of the progress in development of assisted reproductive techniques (ART) has occurred during the past 10–15 yr and has been the subject of several reviews ***(3–6)***. A major focus of most recent work, including that of our laboratory, has been directed toward developing techniques that can be applied to the conservation of endangered felid species.

Initially, embryos were produced from in vivo–matured oocytes after retrieval by follicular aspiration from gonadotropin treated females. Although the first in vitro–derived kittens were produced from such oocytes ***(7)***, much of the recent work on in vitro embryo production has been done using in vitro–matured (IVM) oocytes. There are several reasons for this, not the least important of which is the greater accessibility of such material. Cat ovaries are easily

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obtainable from local veterinary clinics, making it possible to recover several hundred oocytes per week, particularly if the laboratory is located near a large metropolitan area. Furthermore, the ability to maximize in vitro embryo production using IVM oocytes becomes of utmost importance if the technology is to be of relevance for conserving endangered felids.

Tangible evidence of progress in the development of felid ART made during the last decade is found by listing the different types of domestic kittens that have been produced in our laboratory after transfer of in vitro–derived embryos to recipient females, as shown in **Table 1**. Most of our embryo transfers (ET) have been done on d 5 when the embryos are mid- to late-morulae, the age and stage at which uterine entry naturally occurs. Others prefer to do oviductal ET of early cleavage stage embryos, thereby minimizing the in vitro culture interval ***(7,8)***. Oviductal ET may be advantageous in certain circumstances and possibly results in slightly higher pregnancy rates. Our decision to concentrate on uterine ET stems from it being less technically difficult and, therefore, more likely to have practical applicability, particularly if additional kittens can be produced by *trans*-cervical ET into the uterus, as one report has shown ***(9)***. Such an approach requires that considerable attention be devoted to continual evolution and improvement of the in vitro culture environment. For example, even though the base of our culture media for in vitro fertilization (IVF) and in vitro culture (IVC) is still Tyrode's balanced salt solution ***(14)***, a comparison of the culture media we used during the early 1990s ***(10,11)*** with that used more recently ***(12,13)*** reveals several differences. Among the most notable changes are the addition of amino acids, delayed supplementation with fetal bovine serum (FBS) and a gas atmosphere of reduced oxygen (5%). Resulting from greater interest and effort in developing ART in felids, in vitro development has shown gradual, but steady, improvement, as shown by rates of blastocyst development at d 7 increasing from 15%–30% in our earlier studies up to approx 50% in recent reports.

This chapter describes the materials and methods used for the in vitro production of cat embryos, including IVM, IVF, IVC, embryo cryopreservation, and transfer of embryos to recipient females.

2. Materials

2.1. Components of Culture Media

2.1.1. Nondisposable Plastics or Glassware

1. All reusable plastic or glassware used for media preparation or holding/processing of gametes/embryos are washed in 1% 7X detergent (ICN, Aurora, OH) as described by the manufacturer.
2. The water used for soaking/rinsing (and media preparation) is fresh sterile filtered (0.22 µm) 18 *M*Ω water (Nanopure, Barnstead International, Dubuque, IA).

Table 1
Pregnancies Produced and Kittens Born After Transfer of Domestic Cat Embryos Produced In Vitro by our Laboratory

Embryo derivation	Specific treatment	Recipient Pregnant/total (%)	Kittens born, *n*	Reference
In vivo–matured/IVF	ET—d 4 or 5 ≥12 embryos/ET	11/26 (42)	21	***(10)***
In vivo–matured/IVF	ET—d 4 or 5 ≤12 embryos/ET	6/23 (26)	11	***(10)***
In vivo–matured/IVF	Frozen-d 2 ET—d 5/ICSI	2/4 (50) Micro-assisted	3	***(11)***
In vivo–matured/ICSI	Micro assisted IVF-intracytoplasmic ET—d 5	2/4 (50)	3	***(12)***
In vivo–matured/SUZI	Micro-assisted IVF-sub-zonal ET—d 5	1/4 (25)	1	***(14)***
IVM/ICSI	Microassisted IVF-intracytoplasmic ET—d 5	3/9 (33)	3	***(13)***
IVM/IVF	ET—d 5 or 6 Fresh trial 1	0/4 (0)	0	***(15)***
IVM/IVF	ET—d 5 or 6 Fresh trial 2	3/3 (100)	4	***(15)***
IVM/IVF	Frozen-d 4 or 5	4/10 (40)	3	***(20)***
	4°C ≥ 24 h pre-IVM ET—d 6 or 7	1 reabsorbing by d 21		***(24)***

3. After overnight soaking in the detergent, each item is scrubbed with a brush, rinsed, and allowed to soak overnight in water before additional multiple rinses.
4. After oven drying, each item is wrapped or bagged and sterilized using a tabletop autoclave filled with reverse osmosis water and dedicated to culture labware only.

2.1.2. Base Media

The base salt solutions and media to which additional supplements are added to make the media used for gamete/embryo holding, culture, and storage are as follows:

1. Tyrode's balanced salt solution (Ty, Irving Scientific, cat. no. 9282, Santa Ana, CA) is the base for IVF/IVC media and a HEPES-buffered medium for diluting/maintaining spermatozoa for IVF and embryos cryopreservation .

2. TCM 199, (Irving Scientific, cat. no. 9102, with $NaHCO_3$, without glutamine): base for IVM medium.
3. TCM 199, (Sigma, cat. no. 3769, without glutamine and $NaHCO_3$): base for HEPES buffered holding medium for oocytes/embryos outside of the CO_2 incubator.
4. TL Hepes solution, (Biowittaker, cat. no. 04-616F, Walkersville, MD): oocyte aspiration medium. 1–2 mL TL HEPES, with 10 µ/mL heparin and 50 µg/mL gentamicin added is preloaded into each 15-mL centrifuge tube to be used for collection of aspirated oocytes.

2.1.3. Additional Components

1. Gonadotropins. Equine chorionic gonadotropin (eCG; Calbiochem, cat. no. 367222, San Diego, CA) and human chorionic gonadotropin (hCG, Pregnyl, Organon Inc., West Orange, NJ) used in IVM medium. In a laminar flow hood, each vial of gonadotropin powder is reconstituted with sterile Ty so that each mL contains 100X final concentration used for culture (100X: eCG = 50 IU/mL; hCG =100 IU/mL). Aliquot into 1.5 mL microcentrifuge tubes, label, and store at –80°C until needed. Do not refreeze after thawing.
2. Supplement stocks. The energy sources and antibiotics in the IVF and IVC media are prepared as a 100X solution in Ty (**Table 2**). To prepare 10 mL 100X stock, weigh out 0.146 g glutamine, 0.040 g sodium pyruvate, 0.242 g calcium lactate, and 0.050 g gentamicin and solubilize in Ty. Sterilize by filtering into 1.5-mL tubes, label, and store at –80ºC until the day of use. Similarly, 10 mL of 100X supplement for IVM medium is prepared as described for IVF/IVC, except that glutamine is increased to 0.292 g and 0.200 g cysteine is added.
3. Bovine serum albumin (BSA; fraction V, Serological Proteins, Inc., cat. no. 82047, Kankakee, Il). BSA is added at a 6 mg/mL final concentration for IVF and 3 mg/mL for the first step of IVC (*see* **Note 1**).
4. FBS, Hyclone, Inc., cat. no. 30070, Logan, UT). BSA is replaced with 10% FBS as development approaches the early morula stage on IVC d 2 or 3. Each serum bottle is thawed upon arrival, aliquoted at 10 mL/sterile 15-mL centrifuge tube (polypropylene, Corning, Inc.,cat. no. 430052) and stored at –80°C. As needed, FBS is thawed and heat-treated at 56°C for 30 min before use.
5. Amino acids. Minimal essential medium (MEM) nonessential amino acids (NEAA) and MEM essential amino acids (EAA) are purchased as 100X and 50X solutions, respectively. Upon arrival, each 100-mL bottle is aliquoted into 1.0 mL/1.5 mL sterile microcentrifuge tubes and stored at –80°C until needed.
6. Epidermal growth factor (EGF, Sigma, cat. no. 9644). EGF powder is reconstituted with Ty to a 1000 ng/mL concentration (100X final concentration), filter sterilized into 1.5-mL microcentrifuge tubes, and stored at –80°C until needed.

2.2. Preparation of Media

2.2.1. Osmolality and pH

1. All culture/holding media are freshly prepared every week. **Table 2** outlines the quantities of each supplement to add to bicarbonate-based media for preparing a

Table 2
Quantities of Base Media And Supplements Used to Prepare 100 mL of Each Type of Culture Medium Used For In Vitro Production of Cat Embryos

Item	IVM	IVF	IVC-I	IVC-II
Ty balanced salt solution	–	89.5 mL	88.5 mL	76.5 mL
Water, type I, fresh	–	8.0 mL	8.0 mL	8.0 mL
IVF/IVC 100X supplement	–	1.0 mL	1.0 mL	1.0 mL
$NaHCO_3$, 7.5% solution	–	1.5 mL	1.5 mL	1.5 mL
100X MEM NEAAs	–	1.0 mL	1.0 mL	
50X MEM EAAs	–	–	–	2.0 mL
FBS	–	–	–	10.0 mL
BSA Fr V	0.3 g	0.6 g	0.3 g	–
TCM 199	9 6.75 mL	–	–	–
IVM 100X supplement	1.00 mL	–	–	–
hCG (1 IU/mL; 100 IU/mL stock)	1.00 mL	–	–	–
eCG (0.5 IU/mL; 200 IU/mL stock)	0.25 mL	–	–	–
EGF (10 ng/mL; 100X stock)	1.00 mL	–	–	–

total of 100 mL of each medium used to culture oocytes/embryos from IVM through development to the blastocyst stage.

2. Mixing and filtering are done using a horizontal laminar flow hood. After all components have been combined and thoroughly mixed for each medium, osmolality and pH values are checked.
3. Desired osmolality is between 285 and 295 mOsm. If the medium is higher than 295 mOsm, it is adjusted by adding fresh water.
4. Before gassing with 5% CO_2 in air, the pH of bicarbonate buffered media should be approx 7.7–7.8. Media not in that pH range are adjusted with 1.0 *N* NaOH or 1.0 *N* HCl, as necessary.
5. HEPES-buffered media are used for handling and processing of gametes when not maintained in a gas atmosphere of 5% CO_2. After recovery of cumulus oocyte complexes, either from excised ovaries or after laparoscopic follicular aspiration, they are maintained in a HEPES buffered TCM 199 medium made in the laboratory.
6. To prepare, 15 m*M* $NaHCO_3$, 15 m*M* HEPES, 3 mg/mL BSA, and 1% of 100X IVC supplement is added to the TCM 199 base medium described in **item 3 Subheading 2.1.2.**
7. HEPES buffered Ty (HeTy) is used for preparing embryo cryoprotectant medium and for extension and holding of sperm samples before IVF.
8. HeTy is prepared as described for He 199, except that $NaHCO_3$ is not added because it is contained in Ty (*see* **Subheading 2.1.2.**; **item 1**) as purchased.

2.2.2. Filtration

1. Each type of medium, after checking/adjusting osmolality and pH, is sterilized by syringe filtration (*see* **Note 2**) into 15-mL conical centrifuge tubes (Corning Inc., cat. no. 430052, Acton, MA) in a laminar flow hood.
2. No more than 5 mL bicarbonate-buffered culture medium is added per tube.
3. For a description of the filtration process for larger media volumes, as is done when the base medium is purchased in dry form and mixed in the laboratory *see* **Note 3**.

2.2.3. Gas Equilibration

1. Immediately after filtration, while still working in a laminar flow hood, each tube of medium is gassed with a mixture of 5% CO_2 in air (or 5% CO_2, 5% O_2, 90% N_2).
2. Insert an 18 g × 3.8 cm needle, attached to the gas mixture by tubing, into the tube while it is being held at a 15° angle.
3. Allow gas to blow over the media, but not into it, which prevents bubble formation.
4. After 45–60 s, the needle is quickly withdrawn, and the tube is capped tightly.

2.2.4. Media Storage

After gassing, the tubes of freshly prepared media are stored in a tilt rack at 4°C until use within 7 d.

2.2.5. Cryopreservation Solutions

1. The cryoprotectant solution consists of 1.4 *M* propylene glycol (PG), 0.125 *M* sucrose (S), 10% Dextran 70, and 10% FBS in HeTy (CPS).
2. Previously prepared HeTy medium can be used as the 'base' for the cryoprotectant solution.
3. However, preparation is easier if sucrose, then dextran, are added to and mixed withTy without BSA or FBS in it.
4. After sucrose and dextran are in solution, then 1% 100X IVC supplement, 15 m*M* HEPES and 1.4 *M* PG are added.
5. Next, add BSA (3 mg/mL) powder and swirl gently, avoiding bubbles, if possible.
6. After BSA is completely in solution, add 10% FBS to complete the CPA solution.
7. Sterilize by syringe filtration into sterile 5-mL cryotubes, label, and store at –80ºC until needed.

2.6. Artificial Vagina for Semen Collection

1. The artificial vagina (AV) consists of a sleeve and collection container prepared from a 2 mL latex bulb and a 1.5-mL microcentrifuge tube, respectively.
2. After washing, the closed-end portion of the bulb is cut off, leaving an approx 3-cm length of open tube.
3. The conical base of a microcentrifuge tube is cut away from the remaining portion and fitted into the end of the latex bulb that had been cut open.
4. The AV is bagged and autoclaved along with a 50-mL glass serum bottle.

5. To use, the bottle is filled with water warmed to approx 40°C, the AV is placed into the bottle, and the open end is folded over the lip of the bottle to hold it in place.
6. A small amount of nonoil-based sterile lubricant is smeared around the AV opening.

2.7. IVC of Oocytes and Embryos

1. Oocytes and embryos are placed in 500 µL preequilibrated IVM or IVC medium in four-well culture dishes (Nunclon, cat. no., 176740 Nunc, Denmark) and cultured in a closed system.
2. The platform of the closed system is the inverted lid of a micro titer plate (Corning, Inc., cat. no. 3513) onto which are placed the four-well dishes containing the oocytes or embryos next to a 60 × 15-mm Petri dish filled with approx 7 mL sterile water.
3. Several holes are drilled in the lid of the 60-mm Petri dish to provide humidity in the sealed bag.
4. The assembled system is placed inside of a 16.5 × 20.3 cm plastic bag (Kapak SealPAK pouch, cat. no. 402, Kapak Corp, Minneapolis, MN).
5. Then, a 16-gauge × 10-cm blunt-tipped needle, connected by tubing to a premixed tank of 5% O_2, 5% CO_2 and 90% N_2, is inserted into the open side of the bag, which, in turn, is placed in between the jaws of an impulse sealer (Model AIE-200, American International Electric, Whittier, CA).
6. The premixed gas is humidified during inflow by bubbling it through a flask containing 700 mL sterile water.
7. After the bag is inflated with the humidified gas mixture, the needle is quickly withdrawn, and the bag is heat-sealed.
8. During the sealing process, the bag and its contents sit on a heated block maintained at 38°C.
9. After checking for patency the bag containing the oocytes or embryos is placed into a water-jacketed incubator at 38°C (Forma Scientific, model 3130, Marietta, OH).

2.8. Gonadotropic Stimulation of Ovarian Follicle Development in Oocyte Donors

1. Porcine follicle stimulating hormone (FSH) (cat. no. 915, 50 IU/vial) and porcine leutinizing hormone (LH) (cat. no. 925, 25 IU/vial) are purchased from Sioux Biochemicals, Sioux Center, IA, and stored at 4°C until needed.
2. Immediately before use, lyophilized FSH is reconstituted by adding a sterile solution of 2% carboxymethylcellulose (CMC) and 1% Tween 20 (Tw20).
3. Initially, 10 mL CMC/Tw20 is added to the vial of powder.
4. To minimize bubble formation, direct flow down the inside wall, set the vial aside for a few minutes, then gently swirl for 30–60 s to ensure that all powder is completely dissolved.
5. Then, remove the 10 mL FSH solution and put into a sterile 50-mL centrifuge tube.

6. Add another 10 mL CMC/Tw20 to the original FSH vial and gently swirl before removing and adding to the 50-mL centrifuge tube.
7. Lastly, add a final 5 mL CMC/Tw20 to the FSH vial, aspirate it back into the syringe, and add it to the 50-mL tube. The final 25 mL vol of reconstituted FSH will have 2.0 IU/mL.
8. The FSH solution is then aliquoted into 3-mL glass tubes (Vacutainer, cat. no. 366381, sterile, no additive, Becton Dickinson, Franklin Lakes, NJ) and held at 4°C, if it is to be used within 2 d, or stored at –80°C for later use.
9. LH is similarly prepared, except that only 5 mL of CMC/Tw20 is added per vial to give a final 5 IU/mL concentration.

3. Methods

3.1. Animals

1. The cats in our facility are antibody-defined animals purchased from a US Department of Agriculture (USDA) approved, AAALAC accredited vendor (Liberty Research, Waverly, NY).
2. Potential oocyte donors are chosen from sexually mature females with most of them ranging from approx 1 to 6 yr of age.
3. They are group housed in rooms maintained at a temperature of 72–76°C, a relative humidity of 66– 67%, and 14/10 h of light/dark cycle.
4. The rooms are cleaned, and fresh food and water are provided daily.
5. In addition to the animal care personnel, a veterinary staff consisting of one veterinarian and two technicians provide full-time health care.
6. Semen for laboratory use is collected from two or three sexually mature tom cats that are individually housed in stainless-steel cages.

3.2. Hormonal Stimulation of Ovarian Follicular Development

3.2.1. Oocyte Donors

1. Potential oocyte donors are selected from females that are interestrus as determined by vaginal cytology and lack of behavioral signs of estrus.
2. Vaginal cells are recovered from nonsedated females with a moistened vaginal swab, then rolled onto a clean microscope slide.
3. After staining, each slide is microscopically evaluated, and those with a predominance of parabasal and intermediate cells are candidates for gonadotropin treatment.

3.2.2. Gonadotropin Treatment of Oocyte Donors

1. The standard total amount of FSH administered to potential oocyte donors previously untreated with exogenous gonadotropins is 3.0 IU given in decreasing doses 1 time/d for 4 d.
2. At 2.0 IU/mL, the total FSH volume of 1.5 mL is typically administered subcutaneously (sc) at the rate of 0.5, 0.4, 0.3, and 0.3 mL/d.

3. FSH is given during mid to late afternoon, and injections should be done at approx 24-h intervals. In the morning of the fifth day of hormone treatment, 3.0 IU LH is administered intramuscularly (im).
4. The LH injection is given 24 h before the scheduled oocyte retrieval.
5. For information about gonadotropin treatment for females that have previously undergone ovarian stimulation and oocyte recovery, *see* **Note 4**.

3.3. Oocyte Retrieval from Gonadotropin-Treated Donors

3.3.1. Pre-operative Procedure

1. Food and water are withdrawn on the day before laparoscopy.
2. Oocyte donors are sedated with injectable anesthetics, and the lower abdomen is closely clipped.
3. After intubation, anesthesia is maintained by inhalation of isoflurane and oxygen. The abdominal area is scrubbed with chlorohexidene and sprayed with 70% alcohol before sterile disposable drapes are placed over the animal, leaving only the surgical area exposed.
4. For information about disinfection/sterilization of instruments *see* **Note 5**.

3.3.2. Laparoscopic Procedure

1. Oocyte retrievals are done 24 h after LH administration.
2. Three abdominal entry sites are required for insertion of the Verres' needle and two ports, one for the telescope and the other for forceps.
3. To insufflate the abdominal cavity, a small skin incision is made on the right side 2.5–3.0-cm caudal to the umbilicus and 2.0-cm lateral to the midline.
4. Skin folds on both the cranial and caudal sides of the incision are raised, and Allis tissue forceps are attached for elevating the abdominal wall.
5. A 150-mm stainless-steel Verres needle (Olympus America, Inc., cat. no. A5150.1, Melville, NY) is positioned vertically at the incision site, and with manual pressure, is carefully inserted into the abdominal cavity.
6. Usually, a 'snapping' sound is heard as the blunt stylet tip returns to its full extension beyond the needle tip after entry into the abdominal cavity.
7. When the needle tip is confirmed to be properly located, both by feel and free inflow of saline, tubing attached to an automatic insufflator (Insufflator-15 L, Olympus America, Inc.) is connected, and the abdominal cavity is insufflated to a pressure of 10 mm Hg with 5% O_2, 5% CO_2, 90% N_2.
8. Then, a 5-mm safety trocar/cannula (Ethicon Endosurgery, cat. no. 355 SD, Cincinnati, OH) is inserted into the abdominal cavity through a skin incision approx 1-cm anterior to the umbilicus as a port for insertion of the rigid 5 mm telescope (O° angle, Olympus America, Inc., cat. no. A5290A).
9. A camera (Olympus America, Inc., cat. no. MH-972N) is connected to the telescope for visualizing the operative field on a 19"-color monitor mounted at eye-level and located at the tail-end of the operating table.

10. While visualizing the bladder and surrounding area, a second 5-mm safety trocar/cannula is inserted into the abdominal cavity through the skin incision in the midline between two most-posterior teats.
11. Even after insufflation, there is considerably less open area between the body wall and intestines at the lower puncture site.
12. Accordingly, it is important to be able to see the tip of the lower trocar as it enters the abdominal cavity to ensure that no organs or tissues are damaged during entry.
13. After the second-port entry, the surgery table is tilted (approx 15 to 20°) cranially downward so that intestines shift forward to improve access to the ovaries.
14. A 5-mm Babcock forceps (Olympus America, cat. no. A63040A) for lifting and stabilizing each ovary is inserted through the lower port.
15. If the ovaries are not immediately visible, the overlaying mesentery and/or intestinal loops are relocated using the Verres' needle.
16. Then, the forceps are placed around the base of the ovary, being careful to exclude fimbrial tissue before clamping and elevating into the correct position for visualizing and accessing follicles.

3.3.3. Follicle Aspiration

1. A 18-gauge, 6-cm stainless steel trocar/cannula is placed percutaneously above each ovary sequentially, not simultaneously, as a port for inserting the aspiration needle.
2. The sterile follicle aspiration units are custom -made in-house and consist of a 20 gauge (thin wall), 10 cm needle connected to a approx 0.8 m-length of 1.02 mm (id) × 1.16 mm (od) silicone tubing, the other end of which passes through a 17-mm silicone stopper with 2–3 cm of tubing extending through the exit side. The tubing is stabilized and sealed in the stopper with silicone adhesive.
3. The stopper also has a 16-gauge blunt tipped stainless-steel needle mounted in it for connecting via tubing to the vacuum pump.
4. For follicle aspiration, the stopper is seated into a 15-mL conical tube containing 1–2 mL TL HEPES solution with heparin.
5. Each visible follicle is punctured at 75 mmHg suction provided by a regulated vacuum pump specifically designed for follicle aspiration (Cook Veterinary Products, V-MAR-5115, Eight Mile Plains, Australia).
6. Most gonadotropin-stimulated follicles are from 2 to 4 mm in diameter when aspirated at 24-h post-LH injection.
7. The aspiration tubing is monitored carefully during the process to ensure that clotting has not occurred and that the fluid is flowing into the collection tube. Periodically (after 5–15 follicle punctures), the aspiration needle is removed, placed in a tube of TL HEPES + heparin to flush the contents down into the collection tube.
8. After all visible follicles have been aspirated, each ovary is rinsed with over 50 mL of sterile saline solution infused through either the aspiration cannula or the Verres' needle. The infused saline is removed by manual aspiration back into a 30-mL syringe connected to the Verres' needle.

3.3.4. Postlaparoscopic Procedure

1. When both ovaries have been aspirated, the surgery table top is returned to the level position, and any remaining infused saline is removed by aspiration.
2. The insufflation tube is disconnected, and the ports on each cannula are opened so that residual gas is exhausted out of the abdominal cavity.
3. At the puncture sites for the Verres' needle and trocars, the abdominal musculature is closed with an appropriately sized absorbable suture material in a simple interrupted pattern.
4. If the animal has a thick sc fat layer, it is closed with an absorbable suture material in a simple continuous pattern.
5. The skin is closed with an absorbable suture material in a buried subcuticular pattern. In cases where there is no closure of the sc fat layer (i.e., thin animals), the subcuticular suture is intermittently tacked to the abdominal musculature to decrease the likelihood of seroma formation.
6 Poliglecaprone 25 is the preferred suture material, owing to its low reactivity and long duration of strength.
7. A small amount of tissue adhesive (cyanoacrylate) is used at the completion of the skin suture to facilitate good-knot burial.

3.4. In vitro Oocyte Maturation

3.4.1. Ovary Collection and Transport

Domestic cat ovaries obtained from local veterinary clinics after ovariohysterectomy are transported to the laboratory at ambient temperature in HEPES-buffered saline solution (UltraSaline A Solution, Bio-Whittaker, Walkersville, MD) supplemented with 50 μg/mL gentamicin.

3.4.2. Oocyte Collection and Sorting

1. Within 2–6 h, ovaries are minced and cumulus-oocyte complexes (COC) collected into HEPES-buffered TCM 199 (He-199) at 35–38°C.
2. COC recovery and handling is done within laminar flow hoods using stereomicroscopes equipped with temperature-controlled stages set at 38°C.
3. After rinsing three times in He 199, COC are sorted into three groups according to ooplasm appearance and cumulus cell presence ***(15)***:
   a. Type A oocytes have uniformly dark, finely granulated ooplasm enclosed in a smooth, spherical oolemma.
   b. Ooplasm of type B oocytes have slightly lighter pigmentation and the ooplasm is less uniformly and evenly granulated.
   c. Oocytes of type C have pale ooplasm that is more coarsely granulated and more irregular in overall appearance.

3.4.3. IVC of IVM Oocytes

From 10 to 30 oocytes of each type/well are cultured using the closed system (*see* **Subheading 2.4.**) for 24 h in modified TCM 199 containing eCG/hCG

and BSA and the supplements described previously (IVM medium; *see* **Subheading 2.1.3.**).

3.5. Spermatozoa for IVF

3.5.1. Semen Collection

1. Semen is collected from adult domestic cats using an artificial vagina (AV, *see* **Subheading 2.2.** for description and **Note 6** for training information; ***16***).
2. The collection is done in a quiet area with minimal chance of disruption. As soon as the male mounts the 'teaser' female, the penis is gently directed into the AV unit so that natural mating does not occur.
3. Ejaculation usually occurs rather quickly, although, on occasion, males may require more time and patience to obtain a semen sample.
4. The volume of semen samples collected using an AV usually ranges from 30 to 60 µL, although samples of 80–100 µL are obtained from some males.

3.5.2. Semen Extension and Cooling for Temporary Storage

1. The semen sample is held at room temperature during processing.
2. A 3–6 µL aliquot of the fresh semen sample extended in 35 µL HeTy provides more than enough spermatozoa for IVF.
3. The remainder of the semen sample is combined with 500 µL TEST yolk buffer (Refrigeration Medium, Irving Scientific, Santa Ana, CA) in a 1.5-mL microcentrifuge tube and gradually cooled to 4°C by putting the tube in a 100-mL screw-top bottle containing room temperature (22–23°C) water, then placing the sample in a 4°C refrigerator.
4. To stabilize the tube within the water bath, a hole slightly larger than the diameter of the microcentrifuge tube is drilled in the middle of the lid.
5. The bottle is completely filled with water so that the contents of the tube will be submerged during cooling.
6. Cooled semen is used for IVF for up to 3 d after collection/storage, whereas fertilizing ability is maintained for at least 7 d at 4°C ***(17)***.
7. To use spermatozoa held at 4°C, a 3–6 µLaliquot is pipetted directly from the visible sperm pellet formed by sedimentation after cooling when motility is temporarily suspended.

3.5.3. Estimation of Sperm motility and Concentration

1. The percentage of motile spermatozoa in the diluted sample is estimated subjectively using bright-field microscopy, and sperm concentration is measured photometrically (SpermaCue, MiniTube of America, Veronica, WI).
2. To measure sperm concentration, an aliquot of semen sample extended in HeTy is further diluted 1:1 in a formal citrate solution consisting of 2.9% (w/v) trisodium citrate dihydrate and 0.1% (v/v) formaldehyde in deionized water ***(18)*** to inhibit motility.

3. A 25-µL aliquot of the sperm sample diluted in formal citrate is loaded into the photometer cuvette, the cuvet is placed in the chamber of the photometer, and the concentration is measured as $n \times 10^6$ sperm/mL.
4. Then, 10 µL of the extended sample is further diluted with HeTy to 10×10^6 motile sperm/mL immediately before IVF.

3.6. IVF

1. The IVF dishes are prepared by pipetting 4–40 µL drops of modified Ty containing 6 mg/mL BSA (IVF medium; *see* **Subheading 2.2.1.** and **Table 2**) into a 35 × 10 Petri dish (Falcon, cat. no. 1008) and overlaying with approx 3 mL of mineral oil (Sage BioPharma, cat. no. 4008, Bedminster, NJ).
2. From 5 to 15 oocytes are pipetted into each drop in approx 5 µL medium.
3. For IVF, 5 ± 1 µLof the aliquot previously diluted to 10×10^6 motile sperm/mL is added to each droplet of IVF medium to give a final motile sperm concentration of 1×10^6/mL.
4. Oocytes and spermatozoa are coincubated in 5% CO_2 + air at 38°C.

3.7. IVC

1. At 5–7 h or 15–18 h postinsemination, in vivo matured and IVM oocytes, respectively (*see* **Note 7** for explanation of insemination durations), are rinsed four times in He 199 and cultured in 500 µL of modified Ty containing NEAA and BSA (IVC-1 medium) at 38°C in sealed bags filled with 5% CO_2, 5% O_2, and 90% N_2.
2. Then, on d 2 or 3, uncleaved oocytes are removed and embryos are placed into fresh modified Ty containing NEAA, EAA, and 10% FBS, instead of BSA (IVC-2, *see* **Subheading 2.2.1.** and **Table 2**).
3. Those embryos that are not transferred to recipients on d 5, 6, or 7 are evaluated on d 7 by visually determining blastocyst development using a stereomicroscope.
4. Additional information, such as number of cells per embryo, can be obtained after fixation using any of the standard staining methods, such as aceto-orcein-stained wet mounts, Giemsa/air-dryed preparations, or fluorescence.
5. A simple chemically defined differential staining technique can be used to determine inner cell mass and trophectoderm cell numbers in cat blastocysts ***(19)***.

3.8. Embryo Cryopreservation

Initially, embryos were frozen on IVC d 2 at the two to four-cell stage ***(11)***, but the protocol is equally effective for embryos frozen on d 3, 4, or 5 ***(15,20)***. In fact, we are currently freezing embryos on d 5.

3.8.1. Equilibration and Freezing

1. The cryoprotectant solution consists of 1.4 *M* PG, 0.125 *M* S, 10% dextran 70, and 10% FBS in HeTy (CPS).

2. On d 2 (4–8 cells), d 4 (early morulae) and d 5 (morulae) of IVC, embryos are exposed to CPS at 22°C after a two-step equilibration in two parts of HeTy: 1 part CPS (1/3 CPS) and one part HeTy: two parts CPS (2/3 CPS) with 3 min/step.
3. During the 10–15 min equilibration in CPS, embryos are loaded into 0.25- mL nonirradiated straws (Ag Tech, cat. no. B4-2400, Manhattan, KS) and after heat-sealing the tip, each straw is placed into the chamber of a controlled rate-freezing unit (CryoLogic, model CL-863, Victoria, Australia) at 20°C.
4. Embryos are cooled at 2.0°C/min to –6.0°C.
5. During a 10 min hold at –6.0°C each straw is manually seeded with a cotton swab.
6. Cooling is then resumed at 0.3°C/min to –30°C, and after a 10-min hold, embryos are plunged into liquid nitrogen for storage.

3.8.2. Thawing and Culture

1. Straws containing the embryos are thawed for 2 min in air at 22°C, the heat-sealed tip is cut off, and the contents are expelled into a 35-mm Petri dish.
2. Cryoprotective agents are removed at room temperature using a five-step rinse consisting of 3 min each in HeTy plus: 0.95 *M* PG/0.25 *M* S; 0.95 *M* PG/0.125 *M* S; 0.45 *M* PG/0.125 *M* S; 0 PG/0.125 *M* S; 0 PG/0.0625 *M* S.
3. After a brief rinse in He 199 at 38°C, thawed embryos are cultured in IVC-2 medium until transfer to a recipient female on d 5, 6, or 7, or until evaluation/staining on d 7 or 8.
4. Such embryos are usually starting to form blastocysts by d 6 and up to half should reach the blastocyst stage by d 7, providing a convenient visual estimate of the developmental potential of the embryos being transferred.

3.9. ET

1. The recipient female is anesthetized as described earlier for laparoscopy.
2. D 5–7 in vitro-derived embryos are transferred by mid-ventral laparotomy into the uterus of gonadotropin-treated females from which follicular oocytes had been aspirated 5–7 d previously.
3. Using the aseptic technique, a section of one uterine horn is exteriorized through a 1.5-cm incision.
4. An entry site for the catheter is made by puncturing the exposed uterine horn near the anterior tip with a sterile 16-gauge stainless steel trocar with the tip ground to a round short-beveled point.
5. Embryos are loaded in approx 80 µL culture medium into a sterile 14-cm, 3.5 fr, open-ended tom-cat catheter (The Kendall Co., cat. no. 703021, Mansfield, MA) attached to a 1-mL all-plastic syringe.
6. The tip of the catheter is carefully threaded through the puncture site into the uterine lumen approx 4 cm toward the uterine body before expelling the embryos by a quick push of the syringe plunger.
7. The catheter is removed, flushed with culture medium, and the medium is checked with a stereomicroscope to ensure that no embryos are present.

8. Closure of the incision and postoperative care is the same as described for laparoscopy.

4. Notes

1. Each new BSA lot is tested for its ability to support in vitro cleavage of cat oocytes and development to the blastocyst stage. If in vitro embryonic development is similar to that usually obtained in our laboratory, then we request that the company set aside several bottles for us to purchase as needed.
2. Low protein binding syringe filters (Acrodisc, Pall Corp., cat. no. 1492, 0.22 μm, Ann Arbor, MI) are used for sterile filtration of all culture media prepared weekly. As a precautionary measure, for each new filter, dispose of the first 500 μL medium before collecting into sterile tubes. If using a single filter for more than one type of medium, discard the first 0.5–1.0 mL new medium.
3. If the base medium is purchased in dry form, after mixing with water, the medium (usually prepared in 1-L batches) is filtered using positive pressure. For these larger volumes (>1 L) of media, a peristaltic pump (Cole Parmer, cat. no. 77000-30, Chicago, IL) having a flow rate of 100 mL/min. with 3.9-mm id silicone tubing is used. The medium is pumped through a filter unit (AcroCap, Pall Corp. cat. no. 4480, 0.2 μm) attached to the sterile tubing and collected into 100-mL sterile glass screw-top bottles prepared as described previously. Each bottle is labeled and stored at 4°C until use.
4. The total FSH amount to be given to previously treated donors is based on their earlier follicular response to hormone treatment. As a general rule, if 20 or more oocytes were recovered during the last laparoscopic retrieval, then the total dose is not increased. However, if fewer than 20 oocytes were recovered from the potential oocyte donor at one or more previous retrievals, then the total FSH dose is increased to 3.6–4.0 IU or even 5.0 IU if only 5–15 oocytes were recovered. Another selection criterion is based on a minimum interval of 6 mo since the previous stimulation with exogenous gonadotropins. Swanson et al. ***(21)*** reported that repeated treatment (two or three times) of domestic cats with 150 IU eCG followed 84 h later with 100 IU hCG at intervals of 49–57 d resulted in development of ovarian refractoriness to follicular development and recovery of fewer oocytes at each attempt. The decrease in follicle development did not occur if the interval between eCG/hCG treatments was increased to 130–135 d. Accordingly, a minimum of 6 mo is allowed between FSH/LH treatments. Our recent data on number of oocytes recovered after repeated oocyte retrievals following follicular stimulation with FSH/LH agrees with the earlier report in which eCG/hCG was used. As shown in **Table 3**, in both the 3X group and the 4X group, the mean number of oocytes/retrieval is similar at each retrieval.
5. Before each laparoscopy, the reusable equipment, such as the telescope, fiber optic cable, camera, and connecting cable, are disinfected by soaking for 60–90 min in a solution of 0.05% chlorohexidene (2% chlorohexidene diacetate, Nolvasan Solution, Fort Dodge Animal Health, Fort Dodge, IA). The instruments are

Table 3 Effect of Repeated Gonadotropin Stimulation and Laparoscopic Oocyte Retrievals on Number of Oocytes Recovered from Domestic Cats

			Oocyte retrieval, *n*			
Group	Donors, number	Age (years), mean	1*	2*	3*	4*
3X	36	6.3	20.1 ± 10.5	22.8 ± 12.9	20.0 ± 12.5	–
4X	16	7.2	17.3 ± 8.6	19.4 ± 7.6	15.9 ± 11.5	21.6 ± 12.2

* Mean number ± SEM.

then rinsed thoroughly in deionized water sterilized by autoclaving. The other instrumentation, such as Babcock forceps, 5-mm trocar/cannula, tubing/needle sets for follicle aspiration, and insufflation needles are sterilized by autoclaving or ethylene oxide. After each use, the telescope, camera, and cables are rinsed and dried. The other instruments are rinsed, cleaned by sonication in 7X detergent (1%), and rinsed thoroughly again before drying and repackaging for sterilization.

6. Ideally, training for AV semen collection should begin before sexual maturity, although sexually experienced mature males are quite trainable. An estrous female, who is compatible and cooperative with the male is used as a teaser. Also, semen can be collected from some males using a stuffed 'dummy' cat. Using an artificial 'teaser' cat eliminates the necessity of finding a cooperative female in estrus, which can occasionally be problematic.
7. IVM oocytes are coincubated with spermatozoa for 15–18 h mostly for practical reasons. Because the ovaries do not arrive in the laboratory until mid-afternoon after pick-up from the veterinary clinics, the oocytes are not placed into culture until late afternoon. Most developmentally competent cat oocytes complete IVM in approx 24 h and they are inseminated during late afternoon the following day. So, rather than returning to the laboratory later that night, spermatozoa are coincubated overnight with oocytes. Then, the next morning, approx 15 to 18 h postinsemination, oocytes are rinsed and placed in culture. On the other hand, in vivo–matured oocytes are aspirated from gonadotropin-treated donor females during the morning, and IVF is done by around mid-day. Over 90% of metaphase II cat oocytes examined between 0.5 and 3.0 h postinsemination (IVF) had undergone sperm penetration into the ooplasm ***(10,22)***. Furthermore, cleavage frequencies of oocytes following 6 vs 15 h coincubation with spermatozoa were not different ***(23)***.

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14

Production of Chimeric Chickens

Mitsuru Naito and Takashi Kuwana

1. Introduction

Somatic and germline chimeric chickens are very useful for studying various biological functions of cells, such as immune rejection of donor cells in recipient embryos and chickens, or analyzing the developmental fate of transferred donor cells into recipients. Production of germline chimeric chickens makes it possible to manipulate germline cells in vitro (e.g., DNA transfer or cryopreservation), and the manipulated germline cells can allow viable offspring by germline chimeric chickens. It is expected that endangered avian species can also proliferate via interspecific germline chimeric chickens.

Primordial germ cells (PGCs) in chickens originate from the epiblast ***(1)*** and are located in the ventral surface of the area pellucida at stage X ***(2,3)***. Then, they translocate to the dorsal side of the hypoblast and are anteriorly carried to the germinal crescent region. Subsequently, they enter the developing blood vascular system and temporarily circulate throughout the embryo. Finally, they migrate to the germinal ridges, future gonads, and differentiate into oogonia in the female embryo and spermatogonia in the male embryo ***(4–6)***.

Germline chimeric chickens can be produced by transfer of stage X blastodermal cells ***(7–17)*** or PGCs isolated from the germinal crescent region ***(18)***, embryonic blood ***(19–24)***, or embryonic gonads ***(25–27)***. Thus far, introduction of exogenous DNA into PGCs for producing transgenic chickens ***(18,23,28–30)*** and cryopreservation of PGCs for preserving genetic resources ***(21,27)*** have been carried out through the application of PGC manipulation.

This chapter describes the protocol (**Fig. 1**) for producing chimeric chickens, particularly germline chimeric chickens using chicken embryo culture techniques ***(31,32)***. The developmental stages of the chick embryos from first

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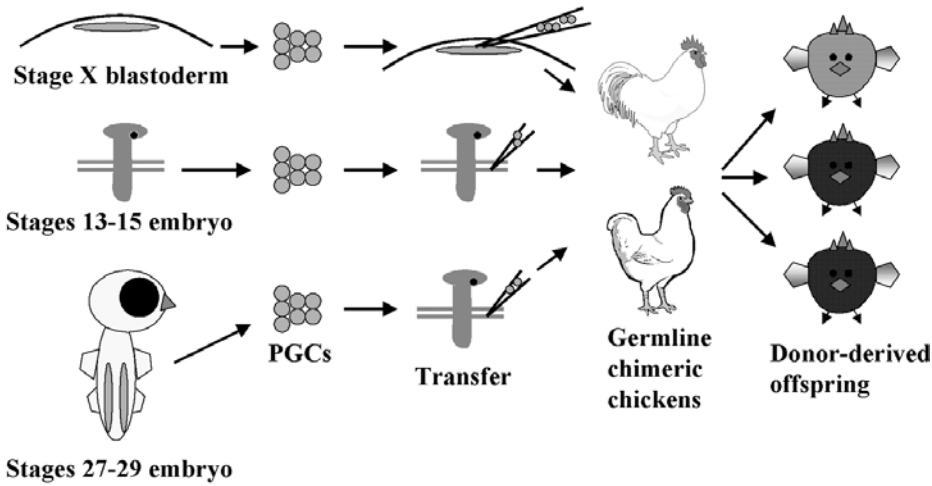

Fig. 1. Production of germline chimeric chickens.

cleavage to primitive streak formation (stages I–XIV) are designated by Roman numerals ***(2)***, and from prestreak stage to hatching (stages 1–46) by Arabic numerals ***(33)***.

2. Materials

2.1. Embryo Culture (see *Note 1)*

1. Thin albumen: Collect from freshly laid eggs. Store at 4°C.
2. Egg shells: Cut off the sharp end of a normal size egg at the 33 mm diameter and discard the contents (system II), and cut off the blunt end of a large egg (egg weight 80–90 g) at the 40 mm diameter (system III) by microgrinder.
3. Plastic rings: 55-mm outer diameter, 35-mm inner diameter, and 5-mm thickness with four screws in the outer side diagonally.
4. Cling films: cut 4.5 × 4.5 cm.
5. Incubator: P-008B (Showa Furanki, Japan).
6. Microgrinder: Minitor Jet.

2.2. Collection and Transfer of PGCs

1. DPBS(–): Dulbecco's phosphate-buffered saline without Ca^{2+} and Mg^{2+} (Dainippon Pharmaceutical Co., Ltd., Japan). Store at room temperature.
2. KAv-1 medium: α-minimal essential medium (α-MEM; Gibco-BRL, USA) supplemented with 1 m*M* D-glucose, 5×10^{-5} *M* 2-Melcaptoethanol, and 10 m*M* EPPS (Wako Chemicals, Japan) containing 5% of fetal bovine serum (FBS) and 5% of chicken serum. Adjust the medium, pH 8.0, by Na_2CO_3 ***(34)***. Store at 4°C.

3. Ficoll solution: 16% and 6.3% Ficoll (type 400 DL, Sigma, USA) in KAv-1 medium. Store at 4°C.
4. Trypsin solution: 0.25% trypsin-EDTA (Sigma).
5. Glass micropipet: Pull 1.0 mm siliconized microcapillary tubing and bevel the tips (30°) to an outside diameter of 30 μm for PGC injection, 60μm and 80μm for blastodermal cell injection and blood collection.

3. Methods

3.1. Chicken Breeds

1. White Leghorn (WL) and a colored breed, such as Barred Plymouth Rock (BPR), are used. For the autosomal pigment inhibitor gene, WL is homozygous dominant (*I/I*) and BPR is homozygous recessive (*i/i*).

3.2. Preparation of Donor PGCs

3.2.1. PGCs or their Immediate Precursor Cells from Stage X (EG&K) Blastodermal Cells

1. Break the unincubated fertilized eggs, remove the thick albumen capsule from the yolk, and rotate the yolk to position the blastoderm on top of the yolk.
2. Place the filter ring paper on the vitelline membrane above the blastoderm, and cut the vitelline membrane along the side of the filter ring paper.
3. Pick up the filter ring paper with vitelline membrane and blastoderm, place them in DPBS(–), and peel off the blastoderm layer from the vitelline membrane by a gentle stream of pipetting.
4. Separate the central disc of the area pellucida from the whole blastoderm using a plastic straw by pressing from above.
5. Dissociate blastodermal cells by trypsin treatment at 37°C for 5 min, and wash the cells with KAv-1 medium.
6. Disperse the collected blastodermal cells in a small volume of KAv-1 medium and place on a plastic dish.

3.2.2. PGCs from Stages 13–15 (H&H) Embryonic Blood

1. Incubate the fertilized eggs at 38°C and 50–60% relative humidity for approx 53 h to obtain embryos at stages 13–15, when most of the PGCs circulate in the bloodstream.
2. Break the egg and collect embryonic blood from the dorsal aorta with a fine-glass micropipet. Disperse the collected blood in KAv-1 medium.
3. Wash the collected blood with KAv-1 medium, disperse the cells in 1.8 mL of 16% Ficoll solution, and overlay 200 μL of 6.3% Ficoll solution in a centrifuge tube.
4. Centrifuge at 800*g* for 30 min then recover the PGC-rich fraction located in the layer between 16% and 6.3% Ficoll solution, and wash with KAv-1 medium.
5. Disperse the collected PGCs in a small volume of KAv-1 medium and place on a plastic dish (*see* **Notes 2** and **3**).

3.2.3. PGCs from Stages 27–28 (H&H) Embryonic Gonads

1. Incubate the fertilized eggs at 38°C and 50–60% relative humidity for 5–5.5 d to obtain embryos at stages 27–28.
2. Isolate the embryo from the yolk and place in a dish filled with DPBS(–). Fix the head and tail of the embryo to the plastic dish with fine needles, and remove the embryonic gonads using a needle.
3. Cut the gonads in small pieces, dissociate cells by trypsin treatment at 37°C for 5 min, and wash with KAv-1 medium (*see* **Note 4**).
4. Disperse the collected PGCs in a small volume of KAv-1 medium and place on a plastic dish (*see* **Note 5**).

3.3. Preparation of Recipient Embryos

3.3.1. Stage X Blastoderm

1. Break the fertilized eggs, remove the thick albumen capsule from the yolk, and rotate the yolk to position the blastoderm on top of the yolk (*see* **Note 6**).

3.3.2. Stages 14–15 Embryo

1. Break the fertilized eggs, remove the thick albumen capsule from the yolk. Then, transfer the yolk to the recipient egg shell, fill with thin albumen, seal with cling film, and secure with plastic rings and elastic bands.
2. Incubate the reconstituted eggs (system II) at 38°C and 50–60% relative humidity for approx 53 h with rocking to obtain embryos at stages 14–15.
3. Remove the plastic rings and cling film from the reconstituted egg, and remove the blood (4–10 μL) from the dorsal aorta of the embryo using a fine-glass micropipet (*see* **Note 7**). Then, fill with a small amount of thin albumen, again seal with cling film, and secure with plastic rings and elastic bands.
4. Incubate the reconstituted eggs with rocking until PGC injection.

3.4. Transfer of PGCs into Recipient Embryos and Embryo Culture

3.4.1. Stage X Blastodermal Cells

1. Pick up the blastodermal cells (500–1000 cells) under the microscope using a fine-glass micropipet.
2. Inject the blastodermal cells into the subgerminal cavity of the recipient blastoderm by inserting the micropipet from the central part of the area pellucida.
3. Transfer the manipulated embryo (yolk) to the recipient egg shell, fill with thin albumen, seal with cling film, and secure with plastic rings and elastic bands. Incubate the reconstituted eggs (system II) at 38°C and 50–60% relative humidity for 3 d with rocking.
4. Transfer the contents of the egg to the large recipient egg shell (system III), cover with cling film, and further incubate at 37.8°C and 50–60% relative humidity for 14 d. Then, move the eggs to the hatcher and incubate at 37.6°C and 60–70% relative humidity for a further 4 d until hatching.

5. Peel off the cling film from the egg shell just before hatching. Help the chick to come out of the egg shell when the chick stays within it.

3.4.2. cPGCs from Embryonic Blood and gPGCs from Embryonic Gonads

1. Pick up the PGCs (e.g., 200 cells) under the microscope using a fine-glass micropipet.
2. Inject the PGCs into the dorsal aorta of the recipient embryos of stages 14–15, from which blood was removed before the injection (*see* **Note 8**).
3. Transfer the manipulated embryos to the large recipient egg shells (system III), cover with cling film, and incubate until hatching.

3.5. Test Mating

1. Mate the mature putative chimeric chickens with BPR (*see* **Note 9**). When WL (*I/I*) is a donor and BPR (*i/i*) is a recipient, white offspring (*I/i*) indicate that the offspring are derived from the donor PGCs (WL), and black offspring (*i/i*) indicate that the offspring are derived from the recipient PGCs (BPR). When BPR is a donor and WL is a recipient, white offspring (*I/i*) are recipient-derived, and black offspring (*i/i*) are donor-derived. The results of the test mating show the germline transmission of donor PGCs into recipient gonads (*see* **Note 10**).

4. Notes

1. Embryo manipulations can be done by opening a small hole in the egg shell. By using chicken embryo culture techniques, embryo accessibility is increased because the whole embryo is exposed.
2. The collected cell population by Ficoll solution contains PGCs and erythrocytes. PGCs are easily distinguishable by their remarkably large size and the presence of considerable numbers of refractive granules (lipids) in the cytoplasm as observed using a phase-contrast microscope. Picking up the PGCs only from the cell population under the microscope is possible through careful observation.
3. PGCs obtained from embryonic blood can be cultured for a limited period on feeder cells ***(39)***.
4. PGCs obtained from 5–5.5 d incubated embryos can be cultured in vitro with stroma cells of the gonads for several d ***(25,26)***. Cultured PGCs give rise to viable offspring by transferring into the bloodstream of recipient embryos of stages 14–15. Then, the cultured PGCs reenter the gonads of recipient embryos and bring about viable offspring via germline chimeric chickens.
5. Embryonic germ (EG) cells can be produced by culturing PGCs obtained from embryonic gonads on feeder cells in vitro ***(40)***. Chicken EG cell colonies are uniformly round, multilayered, and well-delineated. Chicken EG cells transferred into recipient embryos differentiate into germ cells and various kinds of somatic cells.
6. Embryo sexing can be done by removing a cluster of cells from the area opaca of the blastoderm by inserting a needle underneath the blastoderm layer ***(15,24)***.

After DNA extraction from the collected blastodermal cells, W-chromosome specific repeating sequences are amplified by polymerase chain reaction (PCR), and the sex of embryos is determined ***(38)***. Embryo sexing makes it possible to produce same-sex or mixed-sex chimeric chickens.

7. Recipient embryos can be compromised by exposing the embryos to γ-radiation (500–700 rads; ***11***), soft X-ray irradiation ***(36)***, or removal of a cell cluster from the central disc of area pellucida ***(15)*** at stage X prior to donor cell injection. These treatments enhance the germline transmission of donor cells into the germline of chimeric chickens.
8. Success of blood collection from the embryos and PGC transfer into recipient embryos is dependent on the fineness of the glass micropipet. Making good glass micropipets is important.
9. Somatic and germline chimeric chickens are produced by the transfer of stage X blastodermal cells, because stage X blastodermal cells contain somatic cells and PGCs (or their immediate precursor cells) in the cell population. Sometimes somatic chimerism is shown in the feather color. Feather chimerism and germline chimerism are not directly correlated ***(35)***. On the other hand, only germline chimeric chickens are produced by the transfer of PGCs isolated from embryonic blood or gonads. Germline chimerism is usually determined by test mating.
10. The efficiency of obtaining donor-derived offspring from the chimeric chickens is high when the donor is WL and the recipient is BPR ***(22)***. Similar results are obtained when germline chimeric chickens are produced by transfer of stage X blastodermal cells. The combination of WL donor and BPR recipient produces W-bearing spermatozoa more efficiently than the reverse combination ***(37)***. Perhaps, the apparent dominance of WL over BPR in the competition of germ cell proliferation in the recipient gonads reflects the difference in the egg-laying performance between WL and BPR.

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15

Production of Haploid and Diploid Androgenetic Zebrafish

Bruce P. Brandhorst and Graham E. Corley-Smith

1. Introduction

1.1. Zebrafish as an Important Vertebrate Model System

Zebrafish (*Danio rerio*) are a popular vertebrate model system, particularly useful for research in developmental genetics and neurobiology. The adults are easy to obtain and rear; the generation times are only a few months; and large clutches of big embryos are produced (100–1000 per mating). The embryos develop externally and are nearly transparent, facilitating microscopy, experimental manipulations, and screening for morphological mutants. High-density genetic linkage maps have been produced for visible and DNA markers, and the genome is being sequenced. Several useful genetic tools have been developed. Large-scale mutagenesis screens have detected genes involved in morphogenesis and other developmental processes ***(1,2)***. These screens involved a classical three-generation crossing strategy to detect recessive lethal mutations revealed in homozygous diploid mutants. Similar screens are done using insertional mutagenesis to facilitate cloning of the disrupted genes ***(3)***. Many useful molecular markers of differentiating cells have been characterized, and gene expression can be effectively manipulated by the use of morpholino antisense oligonucleotides or expression of dominant-negative mutant proteins. Much information concerning zebrafish mutants, strains, and methods can be found at http://www.zfin.org.

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1.2. Uniparental Inheritance: Gynogenesis and Androgenesis

A useful genetic tool for zebrafish (and some other vertebrates) is the production of offspring with uniparental inheritance. Gynogenotes inherit all their chromosomes from the mother, while androgenotes inherit all their chromosomes from the father. To produce gynogenotes, eggs are inseminated with sperm that were irradiated to eliminate the paternal genome; gamete fusion activates embryonic development. Fertilization of eggs that have been irradiated to destroy the maternal genome with normal sperm produces androgenotes ***(4)***. Haploid gynogenotes or androgenotes complete embryonic development, but die as larvae. They are very useful for genetic mapping (e.g., **refs. *5–9***). Haploids can be useful for mutant screens ***(10,11)***, but note the common abnormalities of haploids summarized in **Subheading 1.4.1.** Screening haploids allows for a rapid identification of parents bearing a mutation in a parental P or F1 screen, avoiding the necessity of producing one or two more generations that require much more time and holding-tank space. Diploid homozygous androgenotes and gynogenotes can be produced by inhibition of the first mitotic division of the zygote; this is accomplished by imposition of a brief heat-shock treatment ***(12)***. Diploid gynogenotes can also be produced using hydrostatic pressure to inhibit the extrusion of the second polar body of the zebrafish oocyte, which is arrested in meiosis II until fertilization ***(13)***. These diploids are homozygous for all genes proximal to the breakpoint closest to the centromere on each chromosome. If these diploids are not homozygous for recessive deleterious mutations, they develop normally and are fertile. Owing to extensive inbreeding and the use of clonal lines recessive lethality has been virtually eliminated from laboratory stocks of zebrafish. A second round of gynogenesis or androgenesis produces a clonal line ***(12)***. Diploid gynogenotes have been used effectively in mutant screens for zygotic and maternal effect genes ***(14,15)***.

1.3. Strategy for Production of Androgenetic Zebrafish

Several technical problems must be solved in order to produce androgenotes. The irradiation must eliminate the maternal genome without severely damaging cytoplasmic components required for embryonic development. It must be possible to hold eggs following collection from the mother long enough for the irradiation prior to fertilization. During normal mating, fertilization occurs almost immediately upon oviposition. Exposure of the egg to fresh water initiates elevation of the chorion and cytoplasmic streaming, culminating in the formation of the blastodisc under the micropyle through which the fertilizing sperm passes. These responses begin within 1 min of contact with water and quickly prevent fertilization. Even if eggs are extruded into a dry dish, they

remain fertilizable for less than 10 min, whereas irradiation often requires longer treatment times. Finally, a method is required to suppress the first mitotic division for the production of diploid androgenotes.

We found that ovarian fluid, which surrounds oocytes in the ovary, of Pacific salmon (*Oncorhynchus* spp.) can be used to hold zebrafish eggs in a fertilizable state for up to 6 h ***(4)***. To the best of our knowledge, salmon ovarian fluid is not commercially available, and collecting it requires access to a hatchery. Many hatcheries produce rainbow trout (*O. mykiss*), an appropriate source of ovarian fluid ***(16)***. We have described how to collect and store ovarian fluid from salmonids ***(17)***. Fortunately, a defined medium has been described that permits holding eggs for delayed fertilization ***(18)***.

Gamma rays (e.g., ^{137}Cs and ^{60}Co) and X-rays can be used to fragment maternal DNA; they are a relatively safe source of irradiation that is highly penetrating. Although ultraviolet (UV), which crosslinks DNA, is useful for irradiation of sperm, UV irradiation is less penetrating and can result in damage to the cytoplasm of eggs; thus, we chose not to use it (*see* **Subheading 1.6.**)

The dose of irradiation was selected by performing a Hertwig dose–response experiment ***(19)***. Clutches of eggs were irradiated, fertilized, and scored for survival to the larval stage. As the dose increased, the survival of batches of eggs declined to nearly zero, and then increased again before declining again to nearly zero, as expected from Hertwig's experiments. The interpretation is that survival declines as the increasing dose of irradiation results in increasing aneuploidy. As the irradiation dose is increased, the irradiated chromosomes become genetically inactivated by severe fragmentation, and euploidy (haploidy) can be restored by fertilization, producing androgenotes. The decrease in survival at even higher doses is likely the result of damage to the cytoplasm and mitochondria from excessive irradiation. Thus, the second survival peak was chosen for the production of androgenotes.

Heat shock is used to inhibit cytokinesis for the first mitotic division of the haploid zygote; division of the replicated chromosomes occurs, but separation of the two sets of chromosomes into two cells is inhibited, resulting in a single homozygous diploid cell, which then undergoes subsequent mitotic divisions to produce the embryo. Although easy to apply in a consistent manner, and highly effective in inhibiting division, heat shock imposes stress on the zygote that sometimes results in developmental abnormalities or arrest. Hydrostatic pressure or chemical treatments may be a useful alternative for preventing the first division.

In summary, to produce androgenetic haploid zebrafish larvae, eggs are extruded from female fish, irradiated, and in vitro-fertilized. To produce diploid androgenetic zebrafish, the first cytokinesis is inhibited by heat shock.

1.4. Properties of Zebrafish Androgenotes

1.4.1. Haploid Syndrome

Figure 1 shows typical haploid androgenotes when compared to wild type embryos and larvae. The percentage of haploid androgenotes that form larvae with an optimal haploid appearance ranged from 5 to 60% for different experiments when using X-rays; it was as high as 80% when using gamma rays ***(4,17)***. Some other larvae had minor imperfections, such as bent tails or eye deformities, and some were severely abnormal or deceased. Microscopic comparison of haploid gynogenetic and androgenetic larvae revealed no consistent differences, indicating that the irradiation does not cause severe irreversible damage to the egg cytoplasm ***(4,17)***. Haploid larvae display a characteristic syndrome: they survive for about 4 d, but do not feed ***(11)***. Consistently, they have stockier bodies with shorter tails and a shorter distance between the yolk sac margin and the anal pore (*see* **Fig. 1**), and they do not inflate their swim bladders. The neural tube of haploid laevae is kinked and less clearly defined; the eye is incompletely formed at the choroid fissure; and the otic vesicles are sometimes duplicated. Their pericardial cavity is swollen, and there is poor circulation through blood vessels with reduced diameter ***(11)***. Because the mid-blastula transition is delayed by one-cell cycle ***(20)***, haploids have more numerous and smaller cells, an effect especially noticeable for pigment cells. However, the schedule of early development is normal for haploids, and all of the major organs are present, although sometimes slightly abnormal. Clutches of haploid gynogenetic and androgenetic embryos (but more so), always include a variable fraction of defective embryos. The frequency and characteristics of the defects are influenced by the genetic background of the parent contributing the chromosomes, because deleterious recessive genes are unmasked. This is also influenced by egg quality and the mother's health. Generally, these variable abnormalities are easily distinguished from mutant embryos with a consistent phenotype sought in mutant screens. Haploid embryos and larvae are particularly useful for genetic screens involving in situ hybridization or immunostaining to assess cellular differentiation or for analysis of DNA extracts (e.g., in searches for deletions of cloned genes ***11***).

1.4.2. Maternal DNA Transmission

We detected no transmission to haploid or diploid androgenotic zebrafish larvae of many maternal-specific DNA markers using polymerase chain reaction (PCR) amplification and fluorescent detection on an ABI 373A DNA sequencer ***(4)***. The test was sufficiently sensitive to be confident in detection of a DNA marker present in 2% of the cells from which the DNA had been extracted (clipped fins or whole larvae). To test the possibility that maternal

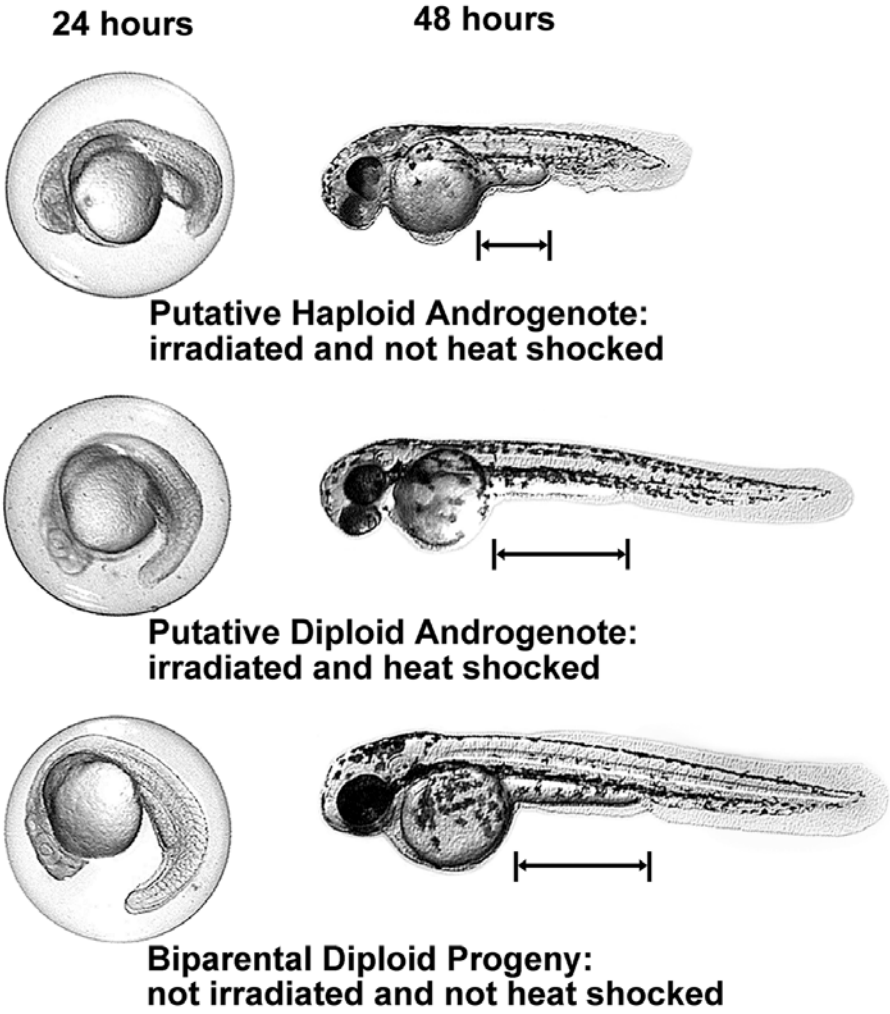

Fig. 1. Haploid and diploid androgenetic embryos and hatched larvae. Photographs were made at 24- and 48-h after fertilization. The androgenotes are referred to as putative because they have characteristic appearances, but transmission of maternal genes was not tested. In our experience, larvae with such appearance and produced in the way described always lack maternal DNA markers when tested. The bars show the distance between the posterior yolk sac margin and the anal pore, which is always reduced in haploids. The apparent difference in number of eyes is dependent on the angle of photography and is not a phenotypic difference. Reproduced from **ref. *4*** with permission of the Genetics Society of America.

genes might be inherited by a small fraction of somatic cells in a mosaic fashion in androgenotes, we also used a visible genetic marker ***(9,17)***. *Golden* (*gol*^{bl}) is a recessive pigment mutation. Homozygous mutant embryos lack pigmented melanocytes 48 h post fertilization, whereas wild-type embryos have many pigmented cells. Androgenotes were produced by fertilizing gamma (^{137}Cs)-irradiated eggs of wild type females with sperm of *golden* males. Androgenotes lacking pigment at 48 h inherited no gol+ allele from the mother. At low irradiation doses, only abnormal larvae formed as expected from the Hertwig effect, but they were pigmented ***(4,9)***. At the higher dose (40,000 R) used to produce androgenotes, the well-formed haploid larvae lacked pigmented cells, whereas some of the abnormal embryos contained a few pigmented cells. This indicates that fragments of maternal DNA, including functional genes, can be inherited in a mosaic manner, but this is rare at higher doses and may not occur at all when the maternal DNA has been sufficiently fragmented to allow normal development of (euploid) androgenotes.

1.4.3 Recessive Mutations and the Efficiency of Androgenotes Production

Our initial investigations of androgenotes were performed on males of the *AB line selected to produce good gynogenetic haploids and is lethal-free, but not entirely homozygous ***(11,21)***. We also used a modestly inbred line (SFU) of zebrafish derived from pet stores and were able to produce haploid androgenotes of normal appearance at a higher efficiency than for the *AB line. The average number of recessive lethal mutations per individual in wild populations of zebrafish is 1.4 ***(22)***. Clearly, this frequency would make the production efficiency of viable haploid larvae low. There has probably been selection against lethal and deleterious recessive mutations in pet-store stocks.

The production efficiency of diploid androgenotes with a normal appearance was approx 2%, while approx 10% has been reported for gynogenotes using pressure ***(11)***. Because the efficiency of production for haploid androgenotes is generally much higher in the same clutch of eggs, the low production rates of diploids likely results from the trauma of the heat shock. Ploidy can be verified by preparing chromosome spreads ***(23)***, but haploids are clearly distinguishable from diploids (*see* **Note 3** and **Fig. 1**).

1.4.4. Sex of Androgenotes

All the diploid androgenotes we have produced and raised to maturity were phenotypic males. We have crossed some of these males with wild-type females: progeny were of both sexes, although the sex ratios were sometimes highly skewed. It is probable that the irradiation or manipulation of the eggs or zygotes resulted in a stress response that caused male sexual differentiation

(11,24). The basis of sex determination and sexual differentiation is not understood in zebrafish, which lack heteromorphic sex chromosomes ***(23,25)***. We have performed extensive random-amplified polymorphic DNA (RAPD) screens on bulked DNA samples from males or females. No sex-linked DNA markers were consistently identified, making it impossible to identify the genetic sex, if any.

1.5. Utility of Androgenesis for Genetic Screens and Mapping

Mutant hunts using haploids permit a first-generation screen for recessive alleles and have been performed using gynogenotes ***(11)***. Although the production of androgenetic haploids is less efficient and more difficult than gynogenetic haploids, androgenesis is sometimes advantageous for genetic screens. For instance, gamma irradiation of diploid blastula-stage embryos (3 h post fertilization [PF]) mutagenizes the four or so progenitors of the germline, but irradiated female embryos are converted to males in a dose-dependent manner ***(11,24)***. The excess males can be used for androgenetic, but not gynogenetic, haploid screens. Young adult males can be efficiently mutagenized with ENU ***(2,12)*** or gamma rays ***(11)***, allowing for haploid androgenetic screens. Mutagenesis by gamma irradiation is especially useful when deletions of a cloned DNA sequence are sought in a reverse genetic screen, which can be performed by PCR amplification of DNA for haploid androgenotes ***(26)***. Although the haploid androgenetic embryos used in screens cannot be bred, interesting mutations can be recovered from the males carrying clones of mutagenized spermatogonia. Deletions can be quickly mapped and used in noncomplementation screens for new alleles ***(27)***.

An attractive feature of mutagenesis of the male germline for use in androgenetic haploid screens is that spermatozoa can be cryopreserved ***(28)***, allowing the recovery of (viable) mutations long after the screen is complete (or ready to be repeated) without maintaining parental or progeny lines. For this purpose, cryopreservation of spermatozoa from testes dissected from mutagenized adult males provides more volume than manual extrusion from adults ***(28)***. We are unaware of a method for cryopreservation of teleost eggs.

Gynogenotes have been used to produce several zebrafish genetic maps based on recombination during female meiosis (e.g., **refs. *6–8***). To produce a genetic linkage map based on recombination during meiosis in males, a panel of haploid androgenotes was produced from a single male produced from a cross of two inbred strains carrying many different polymorphic DNA markers ***(9)***. DNA was extracted from the haploid larvae and analyzed for 274 loci. Male recombination was found to be greatly suppressed, especially near centromeres, in comparison with the female map. Such differences are common in animals with heteromorphic sex chromosomes, but was not predictable for

zebrafish for which no heteromorphic sex chromosome has been detected. The difference in recombination rates in males and females has a variety of practical implications for genetic analysis.

For species in which it is not practical to produce inbred lines, androgenesis can be helpful for genetic linkage mapping. For instance, homozygous parents produced by androgenesis or gynogenesis from two strains of *O. mykiss* were crossed. Sperm from the hybrid line were used to produce diploid androgenotes (doubled haploids) that were used to efficiently place DNA markers on a linkage map ***(29)***.

1.6. Alternative Sources of Irradiation

Several radiation sources have been used to successfully block the inheritance of chromosome sets including gamma rays, X-rays, and ultraviolet (UV) rays. We have used X-rays (10,000 R) and gamma rays (40,000 R for ^{137}Cs) to produce androgenetic zebrafish. Gamma irradiation appears to result in higher survival and more normal development, with up to 80% of haploid androgenotes forming hatched larvae ***(9,17)***. Gamma rays have higher energy and lower energy transfer than X-rays, accounting for the higher dose required for the second peak of the Hertwig effect. Gamma and X-rays fragment DNA, whereas UV covalently crosslinks it. Crosslinking may be preferable because it should prevent the mosaic inheritance of fragments of chromosomes, and it has been used for irradiating sperm for production of gynogenotes. We were concerned that UV might not sufficiently penetrate the large eggs to crosslink nuclear DNA and that UV-sensitive maternal cytoplasmic components required for normal development would be damaged ***(30–32)***. UV irradiation of eggs for production of androgenetic haploid zebrafish has recently been reported ***(33)***. A 254-nm UV lamp (model UVG-11, Ultraviolet Products, San Gabriel, CA) was used at a 9-cm distance for 4 min, resulting in a dose of 1440 J/m^2. A similar UV dose was reported to produce androgenetic muskellunge embryos ***(34)***. The androgenetic embryos produced by UV irradiation of eggs have sufficient cells for extraction of DNA for PCR screens. However, it appears that UV irradiation of eggs is more likely than X or gamma irradiation to produce abnormal androgenotes, although we have not performed comparisons on irradiated eggs from the same clutch or strain.

1.7. Application to Other Species and Sperm Banking

In principle, it should be possible to use irradiation to eliminate the maternal genome from the eggs of many animals, performing a dose-response curve to identify the second peak of the Herwig effect. In practice, development of

androgenetic embryos of some species will be impaired. In mice ***(35)***, and likely in all mammals ***(36)***, genomic imprinting based on parent-of-origin prevents the production of uniparental offspring. We have proposed that cryopreservation of sperm and androgenesis using eggs of a related species may provide a useful avenue to restore endangered species or populations following extinction ***(37)***. Efficient production of hybrid androgenetic larvae between two species of cyprinid fishes (goldfish and carp) has been reported ***(38)***. A problem with this approach is that homozygous androgenotes may be of only one sex; however, it may be possible to reverse the sex using hormone treatments. Another serious concern is the frequency of recessive lethal alleles that may be uncovered in duplicating the paternal genome. Although there have been few estimates of the number of recessive lethals per individual in wild populations, it ranges between 1 and 2 for several animals having quite different genome sizes, estimated gene numbers, and generation times ***(22)***. This number may differ for small populations of endangered species. It may be feasible to produce diploid androgenotes without homozyogosity, such as by intracytoplasmic injection of a second sperm pronucleus after fertilization of the irradiated egg. This would ameliorate the problems of sex determination and recessive deleterious mutations.

1.8 Obtaining Good Eggs for Androgenesis and In Vitro Fertilization (IVF)

Zebrafish are photoperiodic and breed every morning shortly after start of the light phase. Breeding adults (25–50 per 45-L tank) are maintained at 28.5°C on a 14 h light/10 h dark cycle and should be well-fed. Males (slimmer and more yellowish, especially on the abdomen) are kept separately from females (plumper when gravid with more silvery abdomens). To be bred should adults be well-fed for the week prior to breeding, including brine shrimp in the diet. They should be fed approx 30 min before the end of the light cycle on the previous day and transferred to a clean tank, maintaining sexual isolation. IVF requires high-quality eggs, which can be obtained in the following way. Shortly after the beginning of the light part of the photocycle, place a gravid female in a 18-L or 36-L tank with one to three males. Observe the fish, and when breeding activity commences, remove the female from the tank and squeeze her to obtain eggs (*see* **Subheading 3.1.**). Good eggs are slightly granular, translucent, and yellowish in color. Although not always completely spherical, the best eggs look full. The best batches contain few, if any, broken eggs and no whitish or withered eggs. The chorion of good eggs quickly elevates when ovarian fluid is diluted with water, even without fertilization.

2. Materials

2.1 X-Ray Source

The X-ray source should produce at least 150 keV. Lower-energy X-rays (soft X-rays) are believed to cause more cytoplasmic damage at the dose required for androgenesis. We use a Torrex 150D cabinet style X-ray inspection system (Faxitron X-Ray Corp., Buffalo Grove, IL, cat. no. 708-465-9729). The instrument has a built-in 1.2-mm beryllium window. We use no extra filters, as they extend the time required to deliver the required dose. For an X-ray source with higher output, a 0.5-mm aluminum or copper filter will selectively remove soft X-rays (low keV) suspected of causing more cytological damage than the hard X-rays that are more selective in targeting DNA. X-ray dosimetry was performed with a MDH1515 dosimeter using a MDH model 10X5-180 ion chamber (paddle chamber), which was calibrated with a known ^{137}Cs source.

2.2. Water Baths

Two water baths are needed, one to maintain water at the permissive temperature of 28.5 ± 5°C for holding eggs prior to heat shock and the other at 41.4 ± 0.05°C for heat shocking eggs. A calibrated thermometer is required to ensure this accuracy (e.g., Fisher Scientific, cat. no.15041A, with an uncertainty certified not to exceed 0.03°C). Both water baths contain beakers holding fish water (*see* **Subheading 2.3.1.**). To promote heat transfer, the water in the beakers is stirred. Temperatures should be measured in the beakers. Because the time of the first mitotic division is temperature-dependent, accurate temperature control of the cooler water bath is also important for producing diploid androgenotes. To transfer eggs between hot-water baths and allow abrupt thermal changes to be applied to the eggs, they are placed in a heat-shocking tube (*see* **Note 1).**

2.3. Solutions

1. Fish water (for holding fish and embryos): Deionized (or glass distilled) water (dH_2O) filtered through activated charcoal, supplemented with 60 mg/l aquarium sea salts (e.g., Instant Ocean or Coral Reef Salt) and aerated..
2. Hank's saline: 0.137 *M* NaCl, 5.4 m*M* KCl, 0.25 m*M* Na_2HPO_4, 0.44 m*M* KH_2PO_4, 0.3 m*M* $CaCl_2$, 1.0 m*M* $MgSO_4$, 4.2 m*M* $NaHCO_3$. The bicarbonate is added to autoclaved Hank's premix from a 100X stock (0.35 g $NaHCO_3$ in 10.0 mL dH_2O) prepared on the day of use. The premix can be made up from 10X (NaCl +) or 100X stock solutions of the salts.
3. Egg-holding solution: Hank's saline supplemented with 0.5% bovine serum albumin (BSA: Sigma fraction V) ***(18)***.

4. Sperm extender: 80 m*M* KCl, 45 m*M* NaCl, 45 m*M* sodium acetate, 0.4 m*M* $CaCl_2$, 0.2 m*M* $MgCl_2$,10 m*M* Hepes, pH 7.7. The solution is filtered through 0.22-μm pores and stored at 4°C.
5. Tricaine anesthetic (3-aminobenzoic acid ethyl ester methanesulfonate; tricaine methanesulfonate tricaine mesilate; MS-222; Sigma cat. no. A5040. A 100X stock solution is 0.20% (w/v) tricaine in dH_2O, adjusted to pH 7.0 with 1.0 *M* Tris-HCl, pH 9.0, or fresh sodium bicarbonate, if required. This solution is toxic if not properly buffered. Dilute 1:100 (to 20 ppm) with fish water just prior to use. Store stock as frozen aliquots. Tricaine powder gradually declines in quality.

2.4. Supplies

1. A 5-cm long kitchen sponge with 3-cm long V-shaped slit on top, approx 0.5 cm wide at the top.
2. Silanized capillary tubes: Kimax-51, Kimble Products cat. no. 34502, 0.8-1.1mm id, 100-mm length. To silanize the tubes, place them on their sides in a vacuum dessicator along with 1 mL dimethyldicholorsilane in a small beaker. Using a water aspirator or pump with a trap, draw a vacuum sufficient enough to cause boiling of the silane solution and hold until most of the solution is evaporated prior to releasing vacuum (four times for approx 10 min each once overnight). Bake tubes at 180°C for 2 h before use.
3. 2- and 5-mL micropipets: Drummond cat. nos. 2-00-002 and 2-00-005.
4. Plastic spoon.

3. Methods

3.1. Collection of Zebrafish Eggs for Irradiation and Delayed IVF

1. Place approx 100 μL egg-holding solution (or salmon ovarian fluid) in 50-mm diameter plastic Petri dishes at room temperature. The fluid should form a small dome near the center of the dish. Place top of Petri dish on bottom to reduce evaporation.
2. Anesthetize a female zebrafish by immersion in 100-mL fish water containing 20 ppm tricaine in a 250-mL beaker at room temperature. Within 2–4 min, swimming will slow, and the fish may turn onto its side; movement of the operculum will slow, but should not cease, allowing continued gas exchange via the gills. It should be possible to remove the now docile fish with a plastic spoon without its tail thrashing. The tricaine concentration may need to be adjusted empirically; if the fish are still actively swimming after 4 min, double the concentration. At appropriate concentrations of tricaine, a few fish can be anesthetized at a time and gametes collected from each in succession without killing any fish.
3. Using the plastic spoon, lift the anesthetized fish from the beaker and place it belly up in the V-shaped slit of a damp sponge. Place under a stereo microscope.
4. Carefully dry the belly and genital pore near the anal fin with facial tissue or Kimwipe. Contact of gametes with water must be avoided, it activates oocytes and sperm, allowing only a brief period for fertilization.

5. Draw some egg-holding solution from the dish into a silanized glass (Kimax-51) capillary tube and expel back into the dish. This prewets the tube, reducing friction and reducing the chance of rupturing eggs.
6. Squeeze the fish gently with moistened fingers of one hand or Millipore forceps, and carefully draw eggs into the glass capillary tube held with the other hand. A mouth tube can be used to control the eggs entry into the capillary.
7. Gently expel eggs into the 100-µL dome of egg holding solution in the Petri dish. Avoid placing eggs on a dry dish, which may reduce subsequent fertilization rates.
8. Observe eggs under the stereo microscope to assess quality by appearance.
9. Return the fish to fish water. They should survive the trauma, but the fish should not be squeezed again for at least 1 mo.
10. Place a lid on the Petri dish to reduce evaporation. Although possibly unnecessary, black plastic sheets are placed over the dishes to shield out light. Eggs are held at room temperature until fertilized. The fertilization rate declines as eggs are held longer. Try to fertilize eggs after collection as soon as possible, usually within 30 min.

3.2. Collection of Sperm for Delayed IVF

1. Put 50-µL sperm extender into 500-µL microcentrifuge tubes on ice.
2. Perform **steps 2–4** as described above for female fish.
3. Gently squeeze male fish and take up milt into a 2- or 5-µL glass capillary tube (Drummond Microcaps) by capillary action; this tube does not have to be silanized. Gently expel milt from one male into a microfuge tube containing sperm extender solution using approx 1–2 µL sperm per 50-µL sperm extender.
4. Gently swirl to mix sperm and sperm extender.
5. Store on ice until needed (*see* **Note 2**).

3.3. Irradiation of Eggs

1. Spread approx 100 zebrafish eggs in approx 100-µL egg-holding solution into a monolayer in the center of a 50-mm plastic Petri dish with a minimum layer of solution just covering the eggs.
2. Place the dish 23 cm from the focal point of the X-ray beam of the Torrex 150D (shelf 8), which is the shelf closest to the irradiation source.
3. Initiate irradiation using the maximum settings for the instrument: 145 kV and 5 mA, producing a dose of 12.2 R/s. To achieve the desired dose of 10,000 R, irradiate for 820 s at room temperature. After irradiation, fertilize eggs in vitro as described in the following subheading.

3.4. IVF

1. Spread 5–15 µL of sperm extender solution containing sperm evenly over all the eggs in the Petri dish.
2. Immediately add 0.5 mL fish water to activate sperm and gently swirl to mix.

3. After 1 min, very gently add fish water at 28.5°C to three-fourths of the Petri dish, and leave undisturbed at 28.5°C for 1 h. To promote gas exchange, ensure there is a space between the top of water and the dish cover. After 1 h, add 0.02% stock solution of methylene blue to a final concentration of approx 0.3 ppm to inhibit fungal growth. Keep eggs separated from contact to reduce spread of fungus growing on eggs that may die.
4. Place dish in 28.5°C incubator, and after 24 h, remove dead embryos and flush out methylene blue, the use of which is optional.

3.5. Production of Diploid Androgenotes by Heat Shock

1. Start timer as soon as 0.5 mL of 28.5 ± 0.5°C fish water is added to milt and eggs; this is time zero.
2. Place the Petri dish in a 28.5°C incubator or on a shallow ledge in a beaker containing 28.5°C water. After 1.0 min, very gently add 28.5°C fish water to three-fourths full.
3. At 5 min, transfer eggs to a heat-shocking tube and suspend tube in beaker containing 28.5°C fish water in water bath. The tube should be suspended, not rested on bottom of beakers, and should be left uncapped.
4. At exactly 13.0 min, transfer the heat-shocking tube containing eggs to a beaker containing 41.4°C fish water in the second water bath.
5. At 15.0 min, very gently transfer heat-shocking tube containing eggs back to 28.5°C beaker and leave there undisturbed for 1.5 h.
6. After 1.5 h, transfer eggs very gently into Petri dishes three-fourths full of water and place in a 28.5°C incubator.
7. At 24 h, remove dead eggs and inspect developing embryos under a stereo microscope (*see* **Notes 3**,**4**).

4. Notes

1. The heat-shocking tube is made by cutting off the bottom of a 50-conical plastic centrifuge tube, then melting the cut lip by pressing onto a piece of nylon mesh (e.g, 153-μm mesh Nitex) placed on the shining side of a piece of aluminum foil placed on a hot plate. Slide the tube off the foil, allow to cool, and trim off excess mesh. Eggs are retained by the mesh while solutions drain rapidly.
2. Sperm are best used fresh. When feasible, collect sperm while eggs are being irradiated. However, sperm can be collected before collecting eggs and stored in extender solution for several hours. When diluted with water, sperm begin to swim actively and are capable of fertilization for approx 1 min. The sperm quality can be assessed microscopically by dilution of a sample with water; they should swim actively for approx 1 min following the addition of water and have normal appearance.
3. Production of the following types of embryos facilitates the evaluation of successful of the procedures: (1) normal diploid control group embryos produced by

delayed IVF; (2) irradiated and fertilized, but not heat-shocked embryos (putative haploid androgenotes); (3) irradiated, fertilized, and heat-shocked animals (putative diploid androgenotes; *see* **Fig. 1**). Fertilize all groups of eggs at the same time, and keep in a 28.5°C incubator except during manipulations. The irradiated, but not heat-shocked, embryos are included to ensure that the irradiation dose is adequate to prevent inheritance of the maternal genome in each experiment. If no diploid phenotypes are observed in this group of putative haploids, it is highly likely that embryos in the irradiated and heat shocked group with diploid phenotypes are diploid androgenotes. Confirmation of exclusive paternal inheritance requires investigating the inheritance of parentally polymorphic DNA and/or visible markers to putative androgenetic progeny ***(4,39)***. The lack of homozygous maternal-specific markers in the progeny is strong evidence that supports sole paternal inheritance; however, it does not rule out the possibility some maternal genes being inherited by some cells (*see* **Subheading 1.4.2.**).

4. Haploid androgenetic embryos exhibit the haploid syndrome: a shortened body, kinked neural tube, and small melanocytes in comparison with normal diploid embryos (*see* **Subheading 1.4.1.**). The shortened body is noticeable at 24 h and obvious at 48 h, whereas the difference in melanocyte size is noticeable at 48 h. Haploid larvae rarely feed, do not develop swim bladders, and usually die when 4–5 d old. The development of androgenetic diploid embryos is initially retarded in relation to diploid control embryos.

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16

Construction and Detection of Fluorescent, Germline Transgenic Zebrafish

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1. Introduction

Zebrafish transgenesis was first demonstrated over 10 yr ago; however, the intensity of technological development has significantly increased in the past few years with the popularity of the zebrafish model. By transgenesis we are referring to germline transgenesis—the introduction of new genetic material into the germline, such that it is passed down to progeny and inherited in a Mendelian manner. This chapter describes the procedures used to produce transgenic zebrafish expressing fluorescent reporter genes via DNA microinjection. These procedures were modified from those developed by members of this laboratory during the establishment of the transgenic mouse facility at Duke Comprehensive Cancer Center. This chapter aims to aid others in efficiently producing transgenic zebrafish.

The development of transgenic technology in zebrafish appears to have mirrored the early development of that in mice. Observations and manipulations from several different laboratories were necessary to determine conditions that would routinely yield detectable transgene expression in mice. Two fundamental modifications that facilitated the generation of transgene-expressing mice are (1) the elimination of plasmid sequences from the introduced transgene, because these sequences could interfere with or provide faulty expression patterns of the transgene ***(1–4)***; and (2) the addition of at least one intron to the transgenic construct ***(5)***. These same modifications have proven useful in the generation of transgene-expressing zebrafish ***(6–10)***.

As with zebrafish, some of the first transgenes introduced into mouse embryos were viral in origin. Both SV40 and Moloney murine leukemia virus

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(MMLV) were introduced into the mouse germline ***(11)***. However, it became clear that these viral sequences were not transcriptionally adequate in either the early mouse embryos or in the early embryonal carcinoma cells ***(12–14)***. As mouse genes and promoters were identified, the field of expression transgenesis developed rapidly and resulted in transgenic mouse facilities being commonplace on most university campuses. Presently, the introduction of ectopic expression via the germline is being eclipsed by technology in which embryonic stem (ES) cells that carry specific mutations or deletions within selected genes are reintroduced to generate knockout mice. A similar technology for zebrafish is currently under analysis by Collodi and colleagues ***(15–17)***.

Although there are now various methods for generating transgenic zebrafish, we describe here the protocols that have worked well in our laboratory. The emphasis in this chapter is on techniques that can elicit detectable transgene expression in fish, with a particular focus on fluorescent reporter genes. We discuss the fundamentals of plasmid design, injection set-up, and choice of detection methods that have worked in our experience—stressing what we believe to be the most important aspects of the technique.

2. Materials

2.1. Plasmids

1. pW1 (*see* **Fig. 1**).
2. fluorescent reporter genes; *see* Living Colors™ products at the Clontech website (http://www.clontech.com).
3. Qiagen gel purification kit (cat. no. 28704).
4. Phenol red (1% = 10X solution).

2.2. Injection Pipets

1. Thin thin-walled borosilicate pipets with filament, 1-mm od, 0.78-mm I.D., 10 cm long (Sutter Instruments, cat. no. BF100-78-10 or World Precision Instruments [WPI] cat. no. TW100F-4).
2. Sutter Instruments Brown-Flaming pipet puller.
3. Sutter Instruments K.T. Brown pipet beveler with fine diamond abrasive plate, cat. #104D.
4. Lumina Fiber optic lamp (Chiu Technical Corporation, WPI).

2.3. Embryo Holder

1. Microhematocrit capillary tubes (Fisher, cat. no. 22-362-574).
2. Agarose (any commercial electrophoresis agarose).
3. Double-sided tape.
4. 15-cm Petri dish.

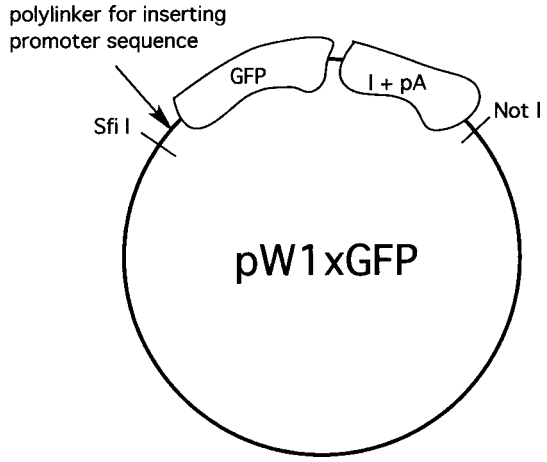

Fig. 1. Transgene plasmid design. The pW1xGFP plasmid contains a number of useful properties including a polylinker for cloning in promoter/enhancer regions, an intron (I) and polyadenylation site (pA), and *Sfi*I and *Not*I restriction sites for easy removal of plasmid vector sequences. Restriction sites in the polylinker (following *Sfi*I) are as follows: *Sac*I, *Sal*I, *Eco*RI, *Xba*I, *Hin*dIII, *Bgl*II, *Xho*I, *Bam*HI.

2.4. Embryo Injection

1. Standard dissection scope with ×10 zoom control and transmitted light illuminator.
2. Micromanipulator—we use either the Leitz Micromanipulator M or the Narishige three-axis micromanipulator, M-152.
3. Medical Systems Corporation PLI-90 microinjector with pipet holders for 1-mm outer diameter pipets.
4. Compressed nitrogen tank to provide pressure for the PLI-90 microinjector.
5. 30% Danieau solution (final concentrations: 17.4 m*M* NaCl, 0.21 m*M* KCl, 0.12 m*M* $MgSO_4$, 0.18 m*M* $Ca(NO_3)_2$, 0.15 m*M* HEPES).

2.5. Fluorescent Analysis>

1. Epifluorescent microscope—we use Zeiss Axioverts or an inverted Nikon TE300 equipped with high numerical aperture objectives (e.g., Zeiss Fluar objectives: ×5, N.A. = 0.25, ×10, NA = 0.5, ×20, N.A. = 0.75 or comparable Nikon Superfluor objectives). (*see* **Note 1**).
2. Fluorescent filters sets for measuring green fluorescent protein (GFP); exciter D470/20; emitter D510/20; beamsplitter 490 dclp, Chroma), (yellow fluorescent protein (YFP; JP-2 set) and (red fluorescent protein; RFP, CZ 915 set, Chroma). *See* also http://www.chroma.com/pdf/gfpnotes.pdf for Chroma's application notes on fluorescent protein applications.

3. Bioptech Delta T4 culture dish controller for maintaining temperature control during time-lapse experiments.
4. Scanalytics IPLab Spectrum software for fluorescent image analysis and automated time-lapse acquisition.
5. Uniblitz model D122 shutter driver.
6. Goldseal 48 × 60-mm no. 1 coverslips for screening (VWR, cat. no. 48404-142).
7. 0.2 m*M* 1-phenyl-2-thiourea in 30% Danieau solution (toxic; prepare 20-m*M* stock, aliquot, and store at –20°C)
8. 0.17 mg/mL Tricaine (3-aminobenzoic acidethylester) in 30% Danieau solution.
9. 3% Methylcellulose and/or 0.3–1% agarose in 30% Danieau solution.

2.6. Polymerase Chain Reaction (PCR) Analysis

1. extraction buffer: 0.1 *M* Tris-HCl, pH 7.4, 0.02 *M* disodium EDTA, 0.2 mg/mL Proteinase K, 0.2% Triton X-100.
2. MJ Research Inc. PTC100 PCR machine-heated lid and with 96-well template.
2. Red *Taq* DNA polymerase and 10X buffer (Sigma, cat. no. D4309).
3. dNTPs (20 m*M* each).
4. GFP primer oligonucleotides: CGT CCA GGA GCG CAC CAT CTT CTT and ATC GCG CTT CTC GTT GGG GTC TTT (*see* **Note 2**).
5. zEf1α primer oligonucleotides: TCT GTT GAG ATG CAC CAC GA andT T G GAA CGG TGT GAT TGA GG.

3. Methods

3.1. Design of Transgene

3.1.1. Plasmid Sequences

Based on our experience in making mouse transgenics, we routinely use a simple plasmid modified in several ways to reconstruct the transgene. In **Fig. 1** is the basic pW1 plasmid used for making transgenes ***(18)***. It is derived from the Promega plasmid vector pGEM9z and has the following useful characteristics: (1) an SV40 small t intron and polyadenylation signal; (2) a polylinker for inserting regulatory sequences and reporter genes; (3) a high-copy number plasmid for producing reasonable DNA amounts; and (4) rarer restriction sites flanking the polylinker and SV40 sequences so that a transgene-containing fragment can be purified away from the plasmid vector sequences before injection (*see* **Note 3**).

We have successfully generated stable transgenic lines using not only native zebrafish promoter/enhancers, but also lines using heterologous promoter/enhancer combinations. For instance we have generated retinoic acid responsive transgenic lines in which promoters from either the Herpes simplex virus thymidine kinase gene or the zebrafish GATA-2 gene are coupled to regulatory sequences to which the retinoic acid receptor ***(9)*** binds and functions to regulate transcription (RAREs). Additionally, we have generated similar lines

using estrogen-responsive elements (EREs) with either the viral or zebrafish basal promoters (unpublished results). We have also generated lines from a 1-kb promoter/enhancer region of the rat GAP43 gene that express the transgene resembling the endogenous zebrafish GAP43 gene ***(10)***. Finally, we have generated lines using natural promoter/enhancers, such as the zebrafish HuC promoter (plasmid containing the zebrafish HuC promoter courtesy A. B. Chitnis).

3.1.2. Reporter Genes

This laboratory has experience working with various fluorescent reporter genes in both embryos and mammalian cells. Most of our work has been with GFP-derived genes, which can be obtained with several useful genetic modifications: (1) mutations that allow for better folding at 37°C; (2) mutations affecting the wavelength for fluorescent excitation; and (3) mutations affecting the emission wavelength (i.e., color of fluorescence). As a starting point we recommend the use of either the enhanced *YFP* gene or the enhanced *GFP* gene because both gene products fold rapidly without toxic effects on the embryo and emit bright stable fluorescence signals that can be detected using standard fluorescein-isothiocyanate (FITC) filter sets. Although fluorescent gene reporters are available from several of companies, we routinely obtain them from Clontech because of the variety of options they offer.

In addition to the useful commercially available GFP mutant variants, there are also plasmids expressing a variety of fluorescent fusion proteins altered with regard to half-life or intracellular targeting. The GFP reporters are long-lived, therefore, interpretation of transgenic expression patterns must consider that fluorescence may be detected for several hours after transcription of the mRNA has ceased ***(19)***. The half-life of the GFP protein can be shortened by fusion to peptides or proteins with sequences that facilitate faster protein degradation. Fluorescent reporters are also available with signal sequences that can target the fluorescent protein to the nucleus, mitochondria, or other subcellular structures. This can be useful for concentrating the fluorescent signal to a smaller subvolume, thereby increasing the sensitivity of signal detection. We have also found that by using a version of the RFP, dsRed, targeted to mitochondria, we can abrogate some of the deleterious effects when it is expressed in the cytoplasm of mammalian cells.

As previously mentioned, our transgene plasmids have been designed to easily purify transgene sequences from the plasmid vector sequence by restriction digestion and gel purification. (We use the Qiagen gel purification kit for this procedure.) DNA is collected at a 100ng/µL concentration and stored at –20°C. We have had success injecting DNA resuspended in a variety of solutions, including dH_2O, TE (10 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA), Danieau

solution (58 m*M* NaCl, 0.7 m*M* KCl, 0.4 m*M* $MgSO_4$, 0.6 m*M* $Ca(NO_3)_2$, 5 m*M* HEPES, pH 7.6), or a solution made up of 0.1 *M* KCl, 5 m*M* Tris-HCl, pH 8.0, and 0.5 m*M* EDTA. It is useful to add dilute phenol red (0.1% final concentration) to the DNA solution prior to injection in order to visually monitor the site of injection.

3.2. Making Injection Pipets

Because we microinject through the chorion into one-cell embryos, we require injection pipets that are strong enough to pierce the chorion but sharp enough not to rupture the membrane of the embryo (*see* **Note 4**). We have found that beveled injection pipets with an 8 micron outside diameter fulfill these requirements. The wider tip provides the strength to pierce the chorion and the bevel allows for easier penetration of both the chorion and the embryo. It should be stressed that having a good pipet is critical to efficient injection. If the pipet does not pierce the chorion and embryo smoothly or cannot be easily withdrawn, the injections will not be successful.

We produce our injection pipets from 1 mm outside diameter pipets that have a filament running through the inside (**Subheading 2.2.**; *see* **Note 4**). We pull the pipets on a Sutter P77 Brown-Flaming pipet puller but pipets for injection could be pulled on a variety of different types of pulling equipment. The pipet puller provides us with two pipets per pulling event. We transfer the pulled pipets to a piece of parafilm on a dissection microscope stage and break the end with a razor blade. We then select pipets that are 8 microns in diameter by examining the cut tip on a microscope equipped with a 10X objective and an eyepiece micrometer.

Pipets with a sharp angle (30–45°) and 8 micron outer diameter may be used without further processing. However, we have more reproducible results and longer injection pipet lifetimes if we follow this up by beveling the tip of the pipet with a Sutter K. T. Brown type beveler. We use the holder to place the pipet at a 30 degree angle to the horizontal. We attach it with a 1 mm inside diameter tubing that is attached to a fiber optic lamp (*see* **Subheading 2.2.**). The bevel is ground on a fine diamond surface grinding plate specifically made for 2–20 micron outside diameter tips. By having the fiber optic light directed out of the tip of the pipet one can gauge when the tip of the pipet reaches the surface of the grinding wheel (the reflection of light on the grinding surface gets smaller as the tip reaches the surface). This allows one to monitor the progress of the beveling process.

The tip and bevel are then re-examined on the microscope with the micrometer to determine the approximate width of the tip. Pipets are stored in a 15-cm Petri dish with double-sided tape on the bottom to affix the pipets to the plate.

We usually do not keep such pipets for more than a week. We find the best results are obtained when pipets are made one d prior to injection.

3.3. Microinjecting

3.3.1. Molded Embryo Holders

We use an agarose embryo holder to arrange 1-cell stage embryos for injection. We mold these holders in the following manner: 1) 20–30 micro-hematocrit capillary tubes are placed next to each other and taped the bottom of a 15-cm Petri dish using double sided tape. The ends of the pipets are aligned with a razor blade before taping them to the dish. A 2% agarose solution is poured over the pipets to approx a 1/4 inch thickness. After solidifying, the agarose is cut into a rectangle. By turning the agarose over and placing the poured top side on the bottom, one then has a mold with several short grooves for holding individual embryos but with a backstop beyond the groove to hold the embryo from further movement when the injection pipet enters the embryo. The holder is diagrammed in **Fig. 2A,B** (*see* **Note 5**).

3.3.2. Injection Station Setup

We have three injection stations that consist of: 1) a dissection scope with transmitted illumination; 2) a micromanipulator; and 3) a foot-pedal operated Medical Systems Corporation PLI-90 microinjector connected to a compressed nitrogen tank (**Fig. 3**). The tip of the injection pipet is backfilled using a Pasteur pipet that has been drawn out in a flame so that its outside diameter will fit within the injection pipet. The inner filament of the injection pipets helps draw the liquid to the tip (*see* **Note 6**). The filled injection pipet is placed in a pipet holder that is attached through tubing to the microinjection apparatus. The PLI-90 allows us to create a backpressure on the fluid in the micropipet tip so that cytoplasm is not drawn into the micropipet by capillary action when the tip enters the embryo. This backpressure has to be adjusted slightly for each pipet because it is determined by the inside diameter of the individual injection pipet.

3.3.3. Embryo Injection

Embryos are collected as soon as possible after fertilization, to inject at the 1-cell stage (*see* **Note 7**). Fertilized embryos are lined up on the agarose holder under a dissecting microscope. The micromanipulator with pipet holder is placed next to the microscope. Different individuals prefer different embryo orientations as diagrammed in **Fig. 2C**. However the orientation of the embryo and the injection pipet should allow for minimum movement of the embryo during the injection process. The DNA is injected into the yolk-free cytoplasm of the embryo by pressure pulsing the DNA solution into the embryo as the pipet pierces the embryo (*see* **Note 8**). The pipet holder is moved laterally

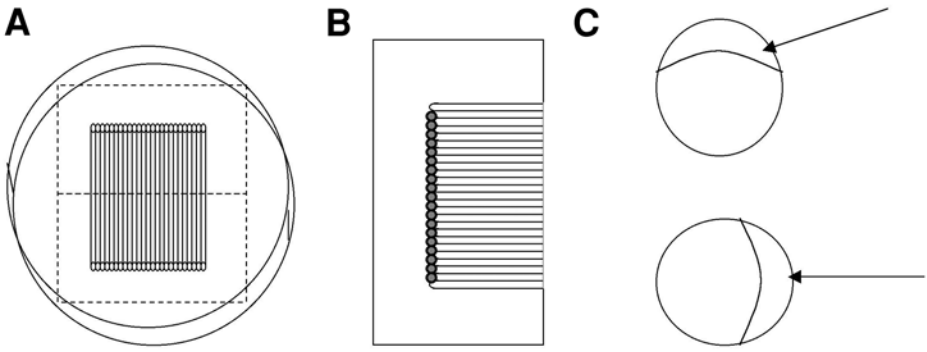

Fig. 2. Egg holder diagram. **(A)** Affix microhematocrit capillary tubes to the bottom of a 10-cm Petri dish using double sided tape, pour 2–3% agarose over pipets to a 1/4-inch thickness, and let agarose harden. Use a scalpel to cut along the dotted lines and a thin weighing spatula to pry up agarose and turn over for molded egg holder. **(B)** Line up fertilized eggs one/groove. **(C)** Yolk-free cytoplasm can be oriented either on top or parallel to the plane of the holder. Arrows represent positions of the injection pipets.

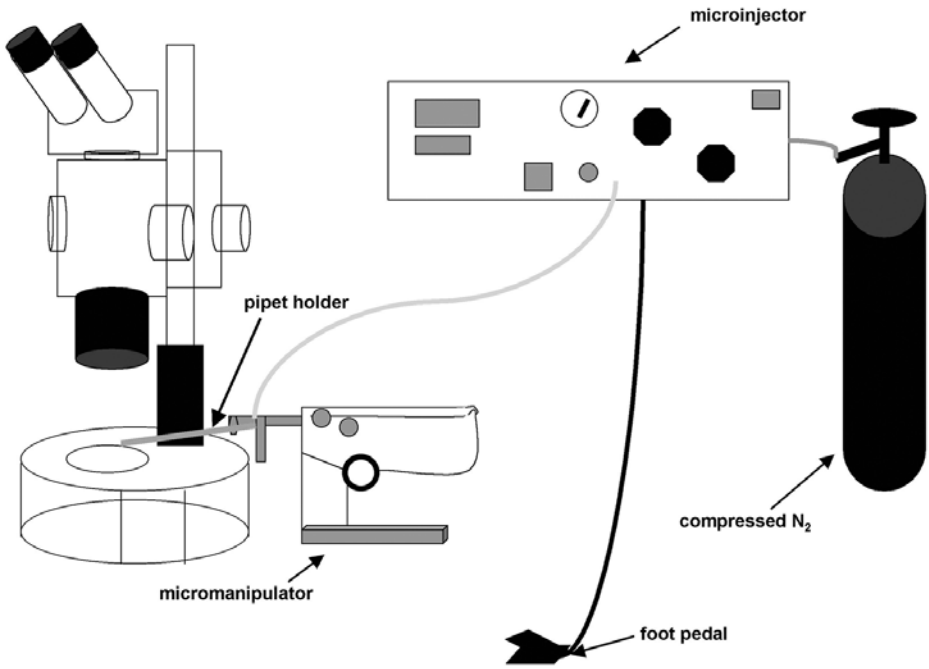

Fig. 3. Schematic of microinjection station.

to the next embryo and the procedure is repeated. Once a row of eggs has been injected, use a transfer pipet to rinse them off the holder into a Petri dish with fresh 30% Danieau solution.

The pulse volume of liquid can be adjusted by varying injection time or pressure controls on the PLI-90 injector. We inject roughly the equivalent of one cell volume of an 8-cell embryo (approx 3–4 nL) into the one-cell embryo. We recommend that only one cell stage embryos are injected. Since the embryos will divide to two cells in less than 1 h following fertilization, there is a limited time at which one can do this before getting a fresh stock of one cell embryos. This procedure takes a little practice and it is advisable to practice with a reliable marker of injection (*see* **Note 9**).

3.4. Microscopy

It should be underlined that at a concentration of 100 ng/uL, 1 nL of injected DNA introduces tens of millions of DNA molecules into the embryo. Therefore, there are many more DNA substrates for expressing the transgenic reporter than will eventually be present once the transgene is integrated into the genomic DNA. This means that the signal observed in injected embryos will most likely be much stronger than that observed in embryos that are expressing integrated transgenes. It is essential, therefore, to use extremely sensitive detection equipment to screen for and analyze germline transgenic progeny. First of all, we suggest the use of a compound research microscope rather than an epifluorescent dissection microscope, as the latter is useful for detecting expression from only the strongest promoters. We typically use high numerical aperture, low power objectives (5× and 10×) to screen fluorescent embryos. These high quality lenses allow us to detect low intensity signals that are restricted to only a few cells per embryo - signals that cannot be detected with low N.A. objectives or epifluorescent dissection microscopes. We stress the importance of high numerical aperture lenses since fluorescence intensity is proportional to the 4th power of the numerical aperture of the objective ***(20)***. Numerical aperture (NA) values to consider for increased sensitivity are as follows: 1) for a 5× objective, an NA of 0.25, 2) for a 10× objective, an NA of 0.5, 3) for a 20× objective, an NA of 0.75.

Another enhancement is to ensure that the fluorescent microscope is equipped with filter cubes that match the spectral qualities of the particular fluorescent protein employed. Both Chroma and Omega have considerable experience in working with fluorescent proteins. As mentioned previously, we tend to use YFP in preference to GFP because of lower background fluorescence, however the use of YFP is only an advantage if we use a YFP specific filter set. The high degree of background fluorescence elicited at the

excitation maxima for GFP (490 nm, similar to fluorescein), is significantly reduced at the excitation maxima for YFP (515 nm). Therefore by using a filter set such as Chroma's JP-2 set, we can excite with wavelengths above 500nm and reduce our auto-fluorescent background .

For imaging of fluorescent transgenic embryos, unless one is working with an extraordinarily strong promoter such as the beta-actin promoter, the images should be captured with a sensitive digital camera such as a cooled CCD camera controlled by a personal computer with good imaging software. Scanalytics produces a reasonable software package (IP Lab Spectrum) for both Windows and Macintosh computers. We use both of these software packages. Packages such as this can control image collection, stage movement and camera shutter, and thus can be used for automated time-lapse image capture.

3.5. Screening Embryos

Although it is tempting to screen for transgenic embryos only by fluorescence, we strongly advise against this as the only screening step. Some of the promoters express during oogenesis, thereby providing the oocyte with fluorescent signal in the form of mRNA and/or protein. In some cases, this occurs before the first meiotic division, resulting in oocytes that are GFP-positive by fluorescence, but do not have the *GFP* gene in their genome. If fluorescence is used as the sole screening method, it is advisable to examine the embryos for fluorescence prior to the transcription of the embryonic genome (500–1000 cell stage). Embryos fluorescent at this stage are GFP positive owing to oocyte contribution. Thus, although the parent is a germline transgenic, not all the fluorescent progeny may have the transgene in their germline. Because the "inherited" fluorescence from the female can obscure the specificity of the embryo-generated fluorescent signal, we suggest comparing fluorescent signals from transgenic embryos generated separately from transgenic male and female parents.

We generally screen for germline transgenesis by pooling embryos from potential "founder" animals, extracting their DNA and then running a PCR series using primer sequences derived from our fluorescent reporter gene.

The protocol is provided below.

3.5.1. DNA Extraction

1. collect eggs from breeding pairs (we usually mate two potential founders together to more efficiently survey larger numbers of adults), the majority of which will be negative.
2. Clear debris from Petri dish with embryos.

3. Collect hatched embryos between 3 and 7 d of age.
4. Use a Pasteur pipet to transfer fish to 1.5-mL microfuge tubes (10 fish/tube) with as little liquid as possible (10 tubes, appropriately labeled, should be prepared for each potential founder).
5. Draw a fired Pasteur pipet to a fine point and use to quickly draw out the remaining liquid in each tube.
6. Quickly add 100 µL of extraction buffer before the larvae die (use aerosol tips).
7. Cap tubes and incubate at 55°C for 2–5 h.
8. Transfer tubes from the 55°C bath to 94°C for 5 min to denature the Proteinase K.
9. Spin at maximum speed in a high-speed microfuge (12,000–14,000*g*) for 3 min to produce a tight pellet.
10. Remove supernatant, transfer to a fresh tube, and store at –20°C until ready to use in PCR reactions.

3.5.2. PCR Protocol

1. Thaw DNA samples and thaw, vortex, and microfuge reagents. *Taq* and dNTPs should be kept on ice after thawing.
   When conducting PCR analysis for diagnostic purposes, we run PCR reactions in 25-µL reactions to conserve reagents. We also use pipet tips with filters to prevent aerosol contamination of the mixes. The components are scaled down and then multiplied by the number of samples:

Reagent	Use	For 96 Samples
10X Red *Taq* PCR buffer	2.5 µL	240 µL
dNTP's (20 m*M* each)	0.25 µL	24 µL
40 pmol/µL Primers	0.5 µL	48 µL
DNA (variable)	1 µL	–
1 U/µL Red *Taq* DNA Polymerase	0.5 µL	48 µL
ddH_2O	20.25 µL	1944 µL
	25 µL total	

   A mix for 96 samples does not fit into one Eppendorf tube, so the mix is divided in half and put into two Eppendorf tubes instead of using a larger tube. This prevents possible pipet contamination from inserting the tip all the way into the liquid of a larger tube. DNA is not added to the mix because 96 separate samples are tested for the DNA to be added individually.
2. Two controls are used: (1) a "doped" GFP control, GFP plasmid DNA added to a sample of embryo-extracted DNA to confirm the efficacy of the mix and PCR program; and (2) a zebrafish Ef1α-positive gene control PCR to ensure DNA quality. The products should be approx 350 bases for the GFP primers and 750 bases for Ef1α. If either control fails, a negative result of the samples being tested cannot be interpreted as negatives and the PCR has to be repeated.

The Ef1α control uses a separate set of primers that calls for a separate mix:

Reagent	Use	For 4 Samples
10X Red *Taq* PCR buffer	2.5 µL	10 µL
dNTPs (20 m*M* each)	0.25 µL	1 µL
40 pmol/µL Primers	0.5 µL	2 µL
DNA (variable)	1 µL	–
RedTaq DNA Polymerase	0.5 µL	2 µL
1 U/µL ddH_2O	20.25 µL	81 µL
	25 µL total	

3. It is useful to arrange the samples in a specific manner and the pattern to be maintained for subsequent PCR screenings. Each tube should be carefully labeled.
4. Add 1 µL of each respective DNA sample to the 96-well plate. Select four samples to use in the Ef1α controls. Be sure to thoroughly mix the DNAs with the mix in the 96 well plate.
5. We normally run a PCR program as described below:

Step 1	30 s	95°C
Step 2	30 s	55°C
Step 3	1 min	72°C
Step 4	Return to step 1	29 times
Step 5	7 min	72°C
Step 6	99 h, 59 min, 59 s	4°C
Step 7	End	

6. With our PCR apparatus, the program takes approx 1 h and 45 min.
7. Load 2.5 µL of 10X DNA loading buffer into the reactions.
8. Load the samples into a 1.5% agarose gel (*see* **Note 10**). We add DNA markers to the first and last wells.
9. The gel is run at 100 V until the dye marker is two-thirds the length of the gel bed.

3.5.3. Establishing a Transgenic Line

Potential "founder" fish, identified by PCR-positive embryos should be individually crossed to wild-type fish, and embryos from these crosses should be subjected to the same PCR protocol as above. This will determine which injected parent is transmitting the transgene to the progeny. Once this is determined, the positive parent should again be crossed with a wild type fish and the resulting embryos should be screened for fluorescence (*see* **Subheading 3.6.**). For screening purposes, we place individual embryos in drops of Danieau solution on large coverslips in a 4 × 5 array and screen using low-power high NA objectives (*see* **Subheading 3.4.**). Remember, transgenic zebrafish are highly chimeric, and it is possible that only 2–3% of the F1 generation may express the transgene; so many clutches of embryos may need to screened to obtain a

few expressing embryos. Fluorescent embryos should be pooled and raised to maturity, then, these fish will transmit the transgene to 50% of their progeny.

3.6. Microscopic Characterization of Transgenic Embryos

3.6.1. General Recommendations

Once transgene positive, fluorescence-positive progeny are identified, there are a few basic considerations for describing and organizing your analysis. The validity of the expression pattern is difficult to prove with only a single transgenic founder. Having several independent founder animals and comparing their expression pattern to show that they are qualitatively the same is an important step in transgene characterization. If the promoter is from a zebrafish gene comparing the transgene expression with that of the endogenous gene, using either *in situ* hybridization analysis or *in situ* antibody localization study is useful for validating the transgenic expression pattern.

Unfortunately, most zebrafish strains produce pigment within 24 h of embryonic growth. This pigment can absorb and consequently quench the fluorescent transgenic signal. Therefore, for more detailed expression analysis, we either make our transgenics in an albino/albino background or cross the transgenic fish into an albino/albino background. Alternatively, in nonalbino strains, pigment formation may be limited by adding a tyrosinase inhibitor (0.2 m*M* 1-phenyl-2-thiourea final concentration) to embryos at 10-h postfertilization ***(21)***. We also try to breed our transgenic lines to homozygosity to obtain adult fish that give rise to only transgene-positive embryos. Generally, we have not had much difficulty getting our transgenic lines in homozygous form, suggesting that they have not integrated into necessary genes.

3.6.2. Microscopic Techniques

In order to collect images from a transgenic embryo that does not produce a strong fluorescent signal, in addition to employing the most sensitive equipment as discussed earlier, it is also necessary to immobilize the embryo because the actual exposure times may be long. We routinely anesthetize the embryos from the 18-somite stage onward with 0.17 mg/mL tricaine. The natural orientation of the anesthetized embryo depends on the age ***(22)***. To position the embryo in a different orientation, we embed the anesthetized embryos in 3% methyl cellulose or 0.3–1% agarose. The methylcellulose provides more flexibility, because the same embryo can be easily repositioned for different views. Conversely, immobilizing in agarose physically fixes the embryo in one position and is best for time-lapse imaging in a single orientation. Embryo development becomes retarded in 1% agarose; therefore, we recommend a lower percentage agarose for extended time-lapse studies.

Our fluorescent microscopes are all inverted microscopes so we place our embryos on coverslips and orient the specific region visually desired just adjacent to the coverslip. If the embryo becomes embedded without its surface touching the coverslip, the optical quality of the image will be limited. If using an upright microscope to image embryos, a bridge should be made just the height of the embryo so that the coverslip is right next to the top edge of the embryo. Bridges can be made by cutting coverslip strips with a diamond-tipped pen and layering them on top of each other so that thin layers on each coverslip end hold a second coverslip above the embryo ***(21)***. Alternatively, high quality water immersion lenses may be used to image embryos without a coverslip on an upright microscope.

3.6.3. Image Capture Options

Most commonly, fluorescent microscopes with mercury arc lamps are used for image capture. In this case, it is important to optimize filter combinations for the fluorescent reporter gene in use because the mercury arc does not have a large-intensity peak at the excitation wavelength for GFP-derived flurorophores. However, most confocal microscopes are equipped with a 488-nm argon laser line that is quite effective for the eGFP reporter gene and can be used for the *YFP* gene, but with slightly less effectiveness. Some argon lasers have a 514-nm line may be more effective for YFP, but most confocal microscopes are not set-up to use this line.

As the size of zebrafish embryos limits how deeply one can visualize fluorescence without light scatter becoming a serious problem, two- or multiphoton microscopy is a useful option if such a facility is available because it allows deeper probing into biological tissue.

3.7. General Recommendations

This chapter outlines how we currently make transgenic zebrafish. We do not proclaim that these are the best procedures, only that they work reliably for us and should allow others to produce expressing transgenic zebrafish at a reasonable frequency. Listed below are some important points to consider when pursing transgenesis research with zebrafish:

1. Inheritance of the transgene in successive generations should be assayed by PCR and not just by fluorescence. Several promoters can express during oogenesis, and because most fluorescent reporters are fairly stable proteins, GFP expressed during oogenesis can result in oocytes with GFP mRNA and/or protein ***(8)***. This expression can remain even after fertilization, resulting in embryos that are GFP-positive, but not transgene-positive. This maternally expressed GFP can be distinguished from embryonically expressed GFP by examining progeny embryos for fluorescence prior to the 1-k cell stage (approx 3 h postfertilization) when the

embryonic genome begins to be transcribed. Embryos that are GFP positive at this stage are positive due to carryover from the oocyte.

2. Most transgenic founder fish that we have made are chimeric for the transgene resulting in progeny that have been as low as 3% positive for the transgene. Thus, it is essential to screen at least 100 potential transgenic embryos (most efficiently via PCR analysis in pools) to test whether the parent is passing the transgene to its progeny.
3. On average we inject approx 1.5×10^8 molecules of DNA (assuming a 2-kb transgene) into the one-cell stage embryo. Hence, when the DNA is transcribed, it usually results in a reasonably strong fluorescent signal. On those occasions when the DNA integrates into a primordial germ cell to produce a germline transgenic founder, the resulting fluorescent signal likely could be several-fold lower in intensity. This demands the optimization of both the microscope and camera to detect weaker fluorescent signals.
4. Related to optimization, it is important to remember that fluorescent intensity from an optical element is proportional to the fourth power of an objective's NA divided by the second power of its magnification ***(20)***. Therefore, screening of fluorescent embryos is best performed on a research microscope equipped with high NA, low-power objectives as outline above in **Subheading 3.4.** Dissection scopes with epifluorescent units should not be used to screen fluorescent embryos unless working with a remarkably strong regulatory sequence like the β-actin promoter.
5. The early developers of mouse transgenics found that plasmid sequences could be inhibitory to developmental expression or could confer expression to regions that normally would not express the gene from which the regulatory sequence was isolated ***(1–4)***. Thus, we recommend constructing transgene plasmids in which the transgene can be easily separated from plasmid vector sequences, which is why we continue to do all of our work within the context of the pW1 plasmid (*see* **Fig. 1**). We suspect the smaller the regulatory sequence being analyzed, the greater the importance of removing extraneous sequences from the injected DNA.

4. Notes

1. We have used Zeiss 5× and 10× Fluar objectives on an Olympus-inverted microscope to increase the fluorescent signal at low magnification.
2. Check the sequence of these GFP primers with the specific DNA sequence of the GFP-related reporter because there have been many different silent and nonsilent mutations interposed in the different GFP-related reporter genes (BFP, CFP, GFP, and YFP).
3. Although transgenic zebrafish have been made successfully by maintaining the plasmid sequences in their injected transgene, evidence exists that the plasmid can affect expression. This might be more evident if using smaller regulatory sequences.
4. Some laboratories dechorionate embryos prior to injection using pronase and therefore use injection pipets with a finer outer diameter. Conversely, generation

of transgenic lines in fish with thicker chorions, such as Fundulus heteroclitus, requires pipets made from a thicker harder glass.

5. A recent modification to this protocol by another laboratory uses computer ribbon wire to form the grooves for the agarose mold (i.e., the individual grooves separating each wire in the ribbon are large enough to form a mold for pouring agarose over to form the holder; Schroeter, Eric, personal communication Washington University, St. Louis, MO).
6. Pipet tips may also be filled by placing the back edge of the pipet with the filament into a 1-μL droplet of DNA solution, allowing it to be drawn up by capillary action. If this is done, be careful not to submerge the whole end of the pipet into the droplet.
7. Some laboratories use in vitro fertilization to control this step.
8. Rather than using pulses of pressure to inject embryos, some prefer to have sufficient backpressure to allow a very slow and constant flow of DNA solution out of the pipet tip during injection.
9. When learning how to inject it is useful to practice with reagents that will give one a fluorescent signal. A simple reagent would be a fluorescent dextran. Inject this and examine the embryos in the fluorescent microscope immediately and also the next day (examining the next day provides a sense of how many embryos have survived the injection process).
10. Using a PCR machine with a 96-well format, the whole procedure of processing the samples for gel electrophoresis can be made efficient by using a multiwell pipettor (12 wells) and having agarose-well combs made so that each well is spaced the same distance as the tip-tip distance of the multiwell pipettor to add 12 samples at a time to the agarose gel.

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17

Production of Zebrafish Germline Chimeras from Cultured Cells

Lianchun Fan, Annette Alestrom, Peter Alestrom, and Paul Collodi

I. Introduction

Because of its many favorable characteristics, the zebrafish has become a popular model for studies of vertebrate development *(1,2)*. To further enhance the utility of the zebrafish model for the genetic analysis of embryogenesis, we have been working to establish methods for cell-mediated gene transfer and targeted mutagenesis using pluripotent zebrafish embryonic stem (ES) cells *(3–5)*. Successful ES cell–mediated gene transfer requires that the cultured pluripotent ES cells are able to produce viable germ cells after being introduced into a host embryo *(6,7)*. ES cells are genetically altered in culture by the incorporation of foreign DNA, and, following in vitro selection and expansion, the cells are introduced into the host embryo where they participate in normal development and contribute to the germ cell lineage. When sexually mature, the germline chimeric animal possesses eggs or sperm derived from ES cells, making it possible to pass the genetic alteration on to subsequent generations to establish the transgenic or knockout line *(6,7)*. This chapter describes a protocol for the derivation of germline-competent pluripotent zebrafish ES cell cultures, along with methods for introducing foreign DNA into cells by electroporation and for using the cultured ES cells to generate germline chimeric embryos.

1.1. Zebrafish ES Cells Remain Germline-Competent for Multiple Passages in Culture

We have developed methods for the derivation of zebrafish ES cell lines that maintain the ability to produce viable germ cells after they are introduced

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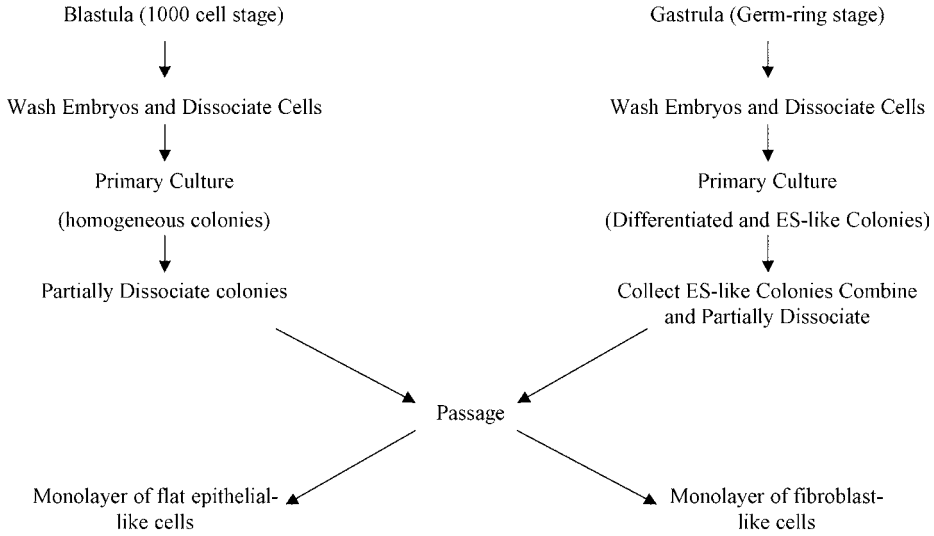

Fig. 1. General protocol for the derivation of zebrafish ES cell lines initiated from blastula- and gastrula-stage embryos. All of the cell aggregates present in the blastula-derived primary culture possess an ES-like morphology and are harvested for the first passage, whereas individual ES-like aggregates must be selected from the differentiated cells present in the gastrula-derived primary culture.

into a host embryo (**Fig. 1**). Germline competent ES cell lines have been initiated from blastula- and gastrula-stage zebrafish embryos, and both lines have been used to generate germline chimeric fish after at least five passages (4–6 wk) in culture (**Fig. 2A,B**) ***(4,5)***. ES cell contribution to the germline of the host fish was confirmed by breeding the chimera to produce F1 embryos that possess a marker gene and pigmentation pattern donated by the cultured cells (**Fig. 2C**) ***(4,5)***. Culture of the ES cells on a feeder layer of rainbow trout spleen cells (RTS34st; ***8***) was necessary for maintaining germline competency ***(4,5)***.

1.2. Plasmid DNA Introduction into Zebrafish ES Cell Cultures by Electroporation

In order to use the zebrafish ES cells for the production of transgenic and knockout lines of fish, methods must be established for the efficient introduction of vector DNA into the cells and selection of stable transfectants. Using the pDsRed2-N1 plasmid-containing genes encoding the red fluorescent protein (RFP) and bacterial aminoglycoside phosphotransferase (*neo*) under the control of the cytomegalovirus (CMV) and simian virus 40 (SV40) promoters, respectively, we have optimized electroporation conditions for the zebrafish ES cell cultures (***5***; **Fig. 3**). The frequency of transient expression in the ES cell

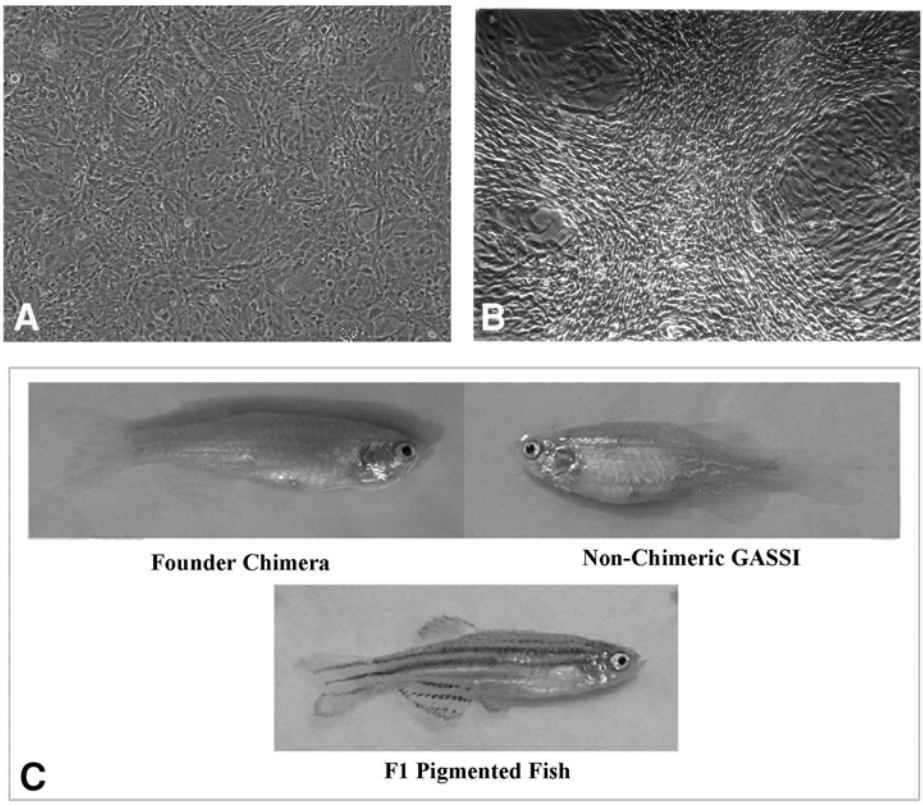

Fig. 2. Zebrafish ES cell cultures at the fifth passage derived from **(A)** blastula- and **(B)** gastrula-stage embryos. Both of the cultures were used to generate zebrafish germline chimeras. **(C)** Contribution of the ES cells to the germline of a host embryo was confirmed by breeding the sexually mature chimera to a nonchimeric GASSI mate and screening individuals of the F1 generation for the presence of a pigmentation pattern donated by the cultured ES cells.

cultures was approx 1%, determined by the number of RFP cells present 72 h following electroporation. The frequency of stable expression was approx 10^{-4}, estimated by the number of G418-resistant colonies that formed 2 wk after electroporation.

1.3. The Zebrafish ES Cell Cultures Contribute to the Germ Cell Lineage of a Host Embryo to Generate Germline Chimeras

Zebrafish germline chimeras have been produced by introducing the cultured ES cells into host embryos by microinjection ***(4,5)***. Host embryos at the blastula stage of development (1000 cells) were injected with approx 50–100

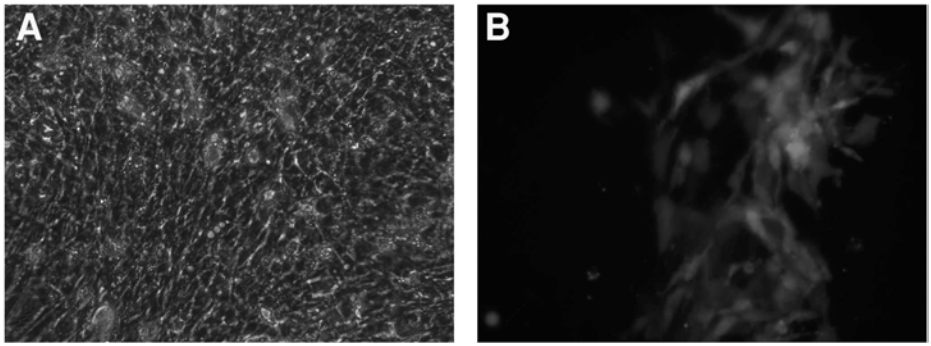

Fig. 3. Zebrafish ES cells expressing RFP following electroporation of the pDsRed2-N1 plasmid. Photomicrograph of the same field under **(A)** light and **(B)** UV showing cells exhibiting stable expression of RFP.

ES cells harvested after long-term culture. Using the techniques described in this chapter, a relatively large number of zebrafish embryos can be rapidly injected with ES cells (300/h), resulting in a 50% survival rate. ES cell contribution to the germline of the host embryo is confirmed by the production of F1 individuals possessing a pigmentation pattern and marker gene donated by the ES cells (**Fig. 2C**). The frequency of zebrafish germline chimera production using multiple-passage ES cell cultures is approx 2% ***(5)***. To facilitate the identification of germline chimeric individuals, ES cell cultures were derived from a transgenic line of fish expressing EGFP under control of the β-actin promoter ***(5,9)***. Owing to the transparency of the zebrafish embryo, potential germline chimeras can be identified 2 d post injection by visually screening for the presence of EGFP-expressing cells in the gonad region ***(5)***. This rapid visual screening method allows the identification of the germline chimeras very soon after injection, eliminating the need to raise all of the injected embryos to sexual maturity for F1 screening. In our experience, approx 40% of the embryos identified in this manner were later confirmed by F1 screening to be germline chimeras. Polymerase chain reaction (PCR) analysis of adult chimeric fish has revealed that ES cell cultures derived from either the blastula- or gastrula-stage embryos contribute extensively to many tissues of the host embryo ***(5,10)***. In addition to the gonad, the ES cells are able to contribute to the liver, muscle, fin, head, and gut.

2. Materials

1. Leibowitz's L-15 (cat. no. 41300-039), Ham's F12 (cat. no. 21700-075) and Dulbecco's modified Eagles' medium (DMEM; cat. no. 12100-046) are available from Gibco-BRL, Grand Island, NY. One liter of each medium is prepared separately by dissolving the powder in double-distilled (dd)H_2O and adding HEPES

buffer (final concentration 15 m*M*, pH 7.2), 120 µg/mL penicillin G, 25 µg/mL ampicillin, and 200 µg/mL streptomycin sulfate.
2. LDF medium is prepared by combining Leibowitz's L-15, DMEM, and Ham's F12 media (50:35:15) and supplementing with 0.180 g/L sodium bicarbonate and 10–8 *M*. sodium selenite The medium is filter-sterilized before use.
3. Phosphate-buffered saline (PBS): 2.7 m*M* KCl, 1.5 m*M* KH_2PO_4, 137 m*M* NaCl, 8 m*M* Na_2HPO_4, pH 7.0.
4. TE buffer : 10 m*M* Tris-HCl, 1 m*M* EDTA, pH 8.0.
5. Fetal Bovine Serum (FBS; Harlan Laboratories, cat. no. BT-9501-500, Indianapolis, IN).
6. Trout serum (East Coast Biologics, Inc., North Berwick, ME).
7. Trypsin (Sigma, cat. no. T-7409, St. Louis, MO). Trypsin/EDTA solution is prepared by dissolving 1 g trypsin and 0.186 g EDTA in 500 mL PBS. The solution is filter-sterilized before use.
8. Human epidermal growth factor (EGF; Invitrogen, cat. no. 13247-051, Carlsbad, CA). EGF stock solution is prepared at 10 µg/mL ddH_2O.
9. Human basic fibroblast growth factor (bFGF; Gibco-BRL, cat. no. 13256-029). bFGF stock solution is prepared at 10 µg/mL in 10 m*M* Tris-HCl, pH 7.6.
10. Bovine insulin (Sigma, cat. no. I-5500). Stock insulin is prepared at 1 mg/mL in 20 m*M* HCl.
11. Bleach (Chlorox) solution is prepared fresh at 0.5% in ddH_2O from a newly opened bottle.
12. The pDsRed2-N1 plasmid (Clonetech, cat. no.6973-1, is available from Palo Alto, CA).
13. Zebrafish embryo extract is prepared by homogenizing approx 500 embryos in 1 mL LDF medium. Centrifuge at 20,000*g*, 10 min to remove the debris. The supernatant is collected, filter-sterilized, and the protein is measured. The extract is diluted to 10 mg protein/mL and stored frozen (–20°C) in 0.2-mL aliquots.
14. RTS34st cells are cultured at 18°C in Leibowitz's L-15 medium (Sigma, cat. no. L5520) supplemented with 30% FBS. The RTS34st cells are growth arrested by irradiation (3000 rad) before they are used as a feeder layer. Conditioned medium is prepared by adding fresh L-15 plus 30% FBS to a confluent culture of RTS34st cells and incubating for 3 d. The medium is removed, filter-sterilized, and stored frozen (–20°C).
15. Geneticin (G418 sulfate); Gibco-BRL, cat. no. 11811-031. G418 stock solution is prepared at 100 mg/mL in ddH_2O and filter sterilized before use.
16. Pronase (Sigma, cat. no. P6911) and is prepared at 0.5 mg/mL in Hank's solution.

3. Methods

3.1. Derivation of ES Cell Cultures from Zebrafish Embryos

3.1.1. Derivation of Cultures from Blastula-Stage Embryos

Protocols for the basic care and breeding of zebrafish are available ***(11)***. To initiate cell cultures, the embryos first must be sterilized by repeated rinsing in

the medium followed by treatment with dilute bleach solution. After the embryos are enzymatically dechorionated and dissociated, the cells are seeded onto a feeder layer of growth-arrested RTS34st cells. Because the zebrafish blastula has not begun to differentiate, the primary culture will consist of pluripotent cells that form dense homogeneous aggregates within 24 h following plating. The ES cell line is established by harvesting all of the cell aggregates contained in the primary culture during the first passage.

1. Collect embryos from wild-type fish ***(11)***, and rinse several times with clean water to remove debris.
2. Incubate the embryos at 28°C until they reach the blastula stage of development (approx 4 h postfertilization).
3. Divide the embryos into groups of approx 50 individuals, each group contained in 35-mm Petri dishes.
4. Rinse each group of embryos five times using 3 mL of LDF culture medium for each rinse.
5. Soak each group of embryos in 3 mL of bleach solution for 2 min followed by three rinses with LDF culture medium.
6. Repeat **step 5** (*see* **Note 1**).
7. Remove the chorions by incubating each group of embryos in 3 mL pronase solution for approx 30 min followed by gentle swirling to release the embryo from the digested chorion. Remove the pronase solution along with the floating chorions, and rinse the dechorionated embryos with LDF medium.
8. Incubate each group of embryos in 3 mL trypsin/EDTA for 1 min while pipetting gently to dissociate the cells.
9. Collect the cells by centrifugation (500*g* for 5 min) and resuspend the pellet obtained from each group of approx 50 embryos in 1.8 mL LDF medium.
10. Transfer the 1.8 mL cell suspension to a single well of a six-well tissue culture plate containing a confluent monolayer of growth-arrested RTS34st cells.
11. Let the plate sit undisturbed for 30 min to allow the embryo cells to attach to the RTS34st monolayer. After the cells have attached, add the following factors to each well: 150 µL FBS, 15 µL zebrafish embryo extract, 30 µL trout serum, 30 µL insulin stock solution, 15 µL EGF stock solution, 15 µL bFGF stock solution and 945 µL RTS34st conditioned medium.
12. Incubate the six-well plate for 5 d (22°C), during which time the cells form tight aggregates that increase in size as the cells proliferate (*see* **Note 2**).
13. Harvest the cells from each well by adding 2 mL trypsin solution/well and incubating 30 s before transferring the cell suspension to a plastic centrifuge tube. Pipet the cell suspension up and down several times to partially dissociate the cell aggregates, and add 0.2 mL FBS to stop the trypsin action.
14. Collect the cells by centrifugation (500*g* for 5 min) and resuspend the pellet in 3.6 mL LDF medium.
15. Add 1.8 mL cell suspension to each of the two wells on a six-well plate containing a confluent monolayer of growth-arrested RTS34st cells, and add the factors listed in **step 11** to each well.

16. Incubate the six-well plate for 5 d at 22°C, and harvest the cells as described in **step 13**. Combine the cells harvested from two wells, collect the cells by centrifugation, and resuspend the cell pellet in 3.6 mL of LDF medium. The suspension still contains a large number of cell aggregates (*see* **Note 3**).
17. Add the cell suspension to a 25 cm^2 tissue culture flask containing a confluent monolayer of growth-arrested RTS34st cells. Let the flask sit undisturbed for 1–3 h to allow the cells to attach to the feeder layer.
18. Add the following factors to the flask: 300 µL FBS, 30 µL of zebrafish embryo extract, 60 µL trout serum, 60 µL bovine insulin stock solution, 30 µL EGF stock solution, 30 µL bFGF stock solution, and 1.890 µL RTS34st-conditioned medium.
19. Incubate the flask for 7 d at 22°C, then harvest the cells in trypsin/EDTA as described previously and seed them into two flasks that contain a confluent monolayer of growth-arrested RTS34st cells. With each passage, the cell aggregates become easier to dissociate, and fewer aggregates are present in the culture. Continue to passage the culture about every 7 d.

3.1.2. Derivation of Cell Cultures from Gastrula-Stage Embryos

ES cell cultures are initiated from zebrafish gastrula-stage embryos using the same basic methods as those described for cultures derived from blastulas (**Subheading 3.1.1.**). One difference is that because cell differentiation has begun to occur in the zebrafish gastrula, a majority of the cell aggregates forming in the primary culture will possess a morphology indicative of a differentiated phenotype. The differentiated colonies appear heterogeneous and are comprised of loosely adherent cells that may contain pigmented melanocytes or neurites that extend from the periphery. Although the majority of cell aggregates are differentiated, pluripotent ES cell cultures can be established from the gastrula-derived primary cultures by selecting individual aggregates that maintain a homogeneous ES-like morphology.

1. Collect embryos from wild-type fish and rinse several times with clean water to remove debris.
2. Incubate the embryos at 28°C until they reach the germ-ring stage of development (approx 6 h postfertilization).
3. Follow **step**s 3–12 in **Subheading 3.1.1.**
4. Use a drawn-out Pasteur pipet to remove cell aggregates from the primary culture that possess a very dense homogeneous appearance without morphological indications of differentiation (*see* **Note 4**). Combine 30–50 of the isolated aggregates in a sterile 2-mL centrifuge tube containing LDF medium.
5. Collect the cell aggregates by centrifugation at 500*g* for 5 min, and resuspend the pellet in 1 mL of trypsin/EDTA solution. Incubate 2 min with occasional gentle pipeting to partially dissociate the cell aggregates.
6. Add 0.1 mL FBS to stop the action of the trypsin, and collect the cells by centrifugation at 500*g* for 5 min.

7. Resuspend the cell pellet in 1.8 mL of LDF medium, and add to a single well of a six-well plate containing a monolayer of growth-arrested RTS34st feeder cells.
8. Let the plate sit undisturbed for 5 h to allow the embryo cells to attach, and add the factors listed in **step11**, **Subheading 3.1.1.** The culture consists of small-cell aggregates and some single embryo cells attached to the RTS34st cells.
9. Incubate the plate at 22°C for 7 d during which time the cells proliferate and the aggregates become larger without exhibiting morphological signs of differentiation.
10. Passage the culture as described in **steps 13–15**, **Subheading 3.1.1.** dividing the cells into two wells of a six-well dish.
11. Incubate the plate for 7 d and then passage the culture, combining the cells from two wells of the six-well dish into a single 25-cm^2 flask.
12. Passage the culture every 7 d. The cell aggregates will become easier to dissociate with each passage, and, eventually, the cells will grow as a monolayer.

3.2. Injection of Cultured ES Cells into Host Embryos to Generate Germline Chimeras

1. Collect zebrafish embryos ***(11)*** from the GASSI strain of fish, and incubate the embryos at 28°C until they reach the 1000-cell blastula stage. The GASSI strain of zebrafish lack melanocyte pigmentation on the body and can be easily distinguished from the heavily pigmented wild-type zebrafish ***(12)*** (*see* **Note 5**).
2. Dechorionate the embryos in pronase solution as described in **Subheading 3.1.1.**, **step 7**.
3. Place approx 50 embryos in a shallow depression made in 1% agarose contained in a 60-mm Petri dish filled with water.
4. Harvest (**Subheading 1.3.1.**, **step 13**) a culture of zebrafish ES cells derived from wild-type embryos, and suspend the cells in LDF medium at a density of 2–3 × 10^6 cells/mL.
5. Draw approx 1 µL cell suspension into the tip of a drawn-out Pasteur pipet that is connected to a Pipet-Aid pipetor with Tygon tubing (**Fig. 4A**). The opening at the end of the Pasteur pipet should be approx 20 µm.
6. Deliver approx 50–100 cells into the cell mass of each blastula sitting on the agarose (**Fig. 4B**; ***13***).
7. Allow the embryos to recover for 2 h before transferring them from the agarose to a Petri dish containing water. Incubate the embryos at 26°C for 7 d, replacing approx 50% of the water daily.
8. Transfer the embryos to a finger bowl (14-cm diameter × 6-cm deep) containing water and incubate at 26°C for 7 d.
9. Transfer the embryos to a 2.5 gal tank and rear to sexual maturity.
10. Test for germline chimerism by individually breeding the sexually mature fish developed from the injected embryos with noninjected GASSI fish and screening the F1 generation for pigmented individuals (**Fig. 2C**). If a large number of fish are to be tested, the initial screen can be conducted by breeding groups of 25–30 injected fish. Once a group containing a germline chimera is identified, each member of that group can be examined by pairwise breeding.

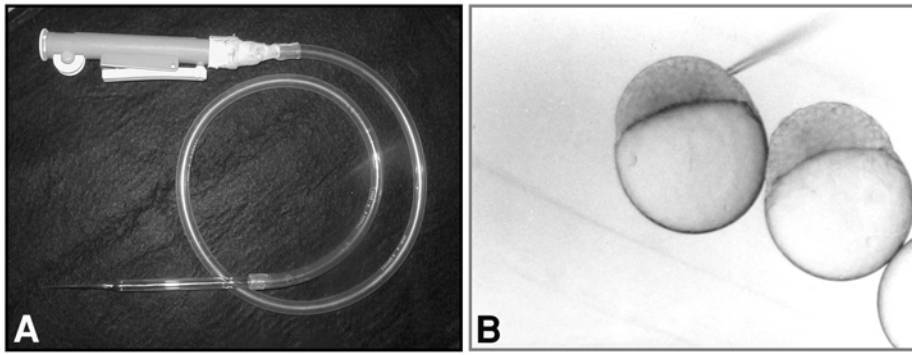

Fig. 4. Microinjection of ES cells into zebrafish host blastula *(12)*. **(A)** Microinjection apparatus consists of a PipetAid connected to a drawn-out glass Pasteur pipet with Tygon tubing. **(B)** Dechorionated host embryos are immobilized in a trough made in agarose, and the ES cells are delivered into the center of the embryo cell mass.

3.3. Plasmid DNA Electroporation into Zebrafish ES Cells

1. Harvest ES cells by trypsinization, wash two times with PBS, and suspend 6×10^6 cells in 0.75 mL of PBS in a 0.4 cm electroporation cuvet.
2. Add 50 µg sterile, linearized, plasmid DNA dissolved in 50 µL TE buffer. In addition to the gene of interest, the plasmid should contain a selectable marker gene, such as *neo* under the control of a constituitive promoter similar to one derived from CMV or SV40.
3. Electroporate the cells using the following conditions: 950 µF, 300 V with a time constant of 11.6 ms. Cell mortality should be approx 50% as measured by trypan blue staining 0.5 h after electroporation.
4. Plate the cells into two 100 mm diameter culture dishes containing the culture medium described in **Subheading 3.1.1.**, **step 11**.
5. Add 5 µL/mL of the G418 stock solution 24 h after electroporation. Change the medium every 2 d. Colonies will begin to appear 2–3 wk after selection in G418 is initiated.

4. Notes

1. If bacterial contamination continues to be problematic, a very brief ethanol treatment of the embryos can be included in the protocol. After completing the bleach treatments, briefly (5 s) submerge the embryos in 70% ethanol, and then immediately rinse them 3–5 times in LDF medium.
2. It is important not to allow the embryo cell aggregates to become too large, or the cells will begin to differentiate. Five days is usually a sufficient period for the aggregates to become large enough to passage without cell differentiation occurring.

3. During the first three passages, the cell aggregates cannot be completely dissociated without lysing the cells. With each passage, the aggregates become easier to dissociate and by passage four or five a suspension of single cells can be obtained.
4. A 10 µL micropipettor (Rainin) can also be used to remove the cell colonies from the dish.
5. Because the GASSI strain of zebrafish lack melanocyte pigmentation on the body, the use of host embryos from this strain makes it possible to use pigment formation as a visual marker of chimerism. However, host embryos obtained from other strains of zebrafish have also been used successfully to generate germline chimeras.

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18

Studying Nuclear Reprogramming with Cell Hybrids

Steve Pells and Jim McWhir

1. Introduction

1.1. Nuclear Reprogramming

Every cell in a mammal, other than odd exceptions (e.g. mature erythrocytes or lymphocytes with rearranged antigen receptors) contains a complete complement of the genetic information required to build another copy of the animal in question. However, the vast majority of adult cells are terminally differentiated with fixed patterns of gene expression, and for a long time it was thought to be impossible to reverse the linear, one-way process of differentiation (with concomitant restriction of gene expression patterns) to create a pluripotent embryonic cell from such a terminally differentiated precursor cell. More recently, the cloning from adult somatic cells of apparently normal individuals of several vertebrate species, most notably the creation of the sheep "Dolly" in 1997 ***(1)***, showed that reversal of this process of increasing specification can be achieved. The cloning of mammalian species is achieved by nuclear transfer (NT); therefore, we conclude that the nucleus of the somatic donor provides all of the genetic information required, and that the mammalian oocyte contains an activity that acts on the donor nucleus to reprogram an embryonic state. This reassignment of a cell's nucleus from a somatic to an embryonic program is generally termed *nuclear reprogramming* (NR) and is a complex process that involves the restructuring of the chromatin in the nucleus, remodeling of DNA modifications (e.g. methylation), and a major change in gene expression patterns. Many events involved in NR are either completely unknown or poorly characterized. As a model system for studying the NR process, NT is not perfect because few cells are reprogrammed at a time, and they must be transferred back into a mother to develop. As measured by the frequency of live

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births, NT has a low efficiency of NR (typically 1–2% of NT embryos). Thus examination of nuclear changes immediately after the NT/NR event is problematic.

1.2. Cell Fusion

Different cell types can fuse at a low rate when cultured together ***(2–5)***. Under appropriate conditions, such as the protocol described in this chapter, the rate of fusion can be dramatically increased. Fusion generates a binucleate cell called a *heterokaryon*, and at a low rate the two nuclei may fuse to generate a hybrid cell with a single, tetraploid nucleus. Cell lines may be easily established from such hybrids. The resulting hybrids' characteristics are not those of an indeterminate phenotype midway between that of the two parental cells, but instead the embryonic, or less-differentiated, cell (usually) appears to reprogram the somatic or more-differentiated partner ***(6–7)***. Because this reprogramming event usually involved the loss of specific differentiated functions in the somatic cell, the phenomenon was traditionally called *extinction*. There is ample evidence from a variety of fusion systems that this reprogramming of one partner in the fusion by the other is not merely a passive, "cytoplasmic dominance" effect, but is an active reprogramming process; activation of embryonic stem (ES)–specific gene expression from alleles derived from the somatic partner has been demonstrated at either the RNA or protein levels in several instances of fusion between ES or embryonic germ (EG) cells and somatic cell types ***(3,5,8,9)***. Examples of embryo-specific genes that may be activated by an NR event include *Oct3/4* and related transgenes such as *OctNeo* or *Oct3/4* promoter-driven GFP, *Foxd3*, and telomerase. In addition to reverse-transcriptase polymerase chain reaction (RT-PCR) and protein expression assays where antibodies are available, some specific assays for NR-induced changes in gene expression include green fluorescent protein (GFP) fluorescence ***(3,8)***, X chromosome reactivation ***(9)***, and the restoration of telomere length after telomerase expression ***(10)***.

Other aspects of NR may be studied in fusion hybrids, but presently much remains to be done. To date, more work has been done on methylation changes to the genome after NR on NT-cloned embryos ***(11–14)***. However, Tada et al. have shown demethylation in both imprinted and nonimprinted genes following fusion of thymocytes with EG cells, but have also shown preservation of the somatic pattern of methylation after reprogramming with ES cells ***(8,9)***. As yet, no studies of chromatin structure changes (e.g. histone modifications) that occur during NR events in fusion hybrids have been published, but good reagents such as antibodies specific for individual histone modifications are now available ***(15)*** and this is likely to become a very exciting area soon.

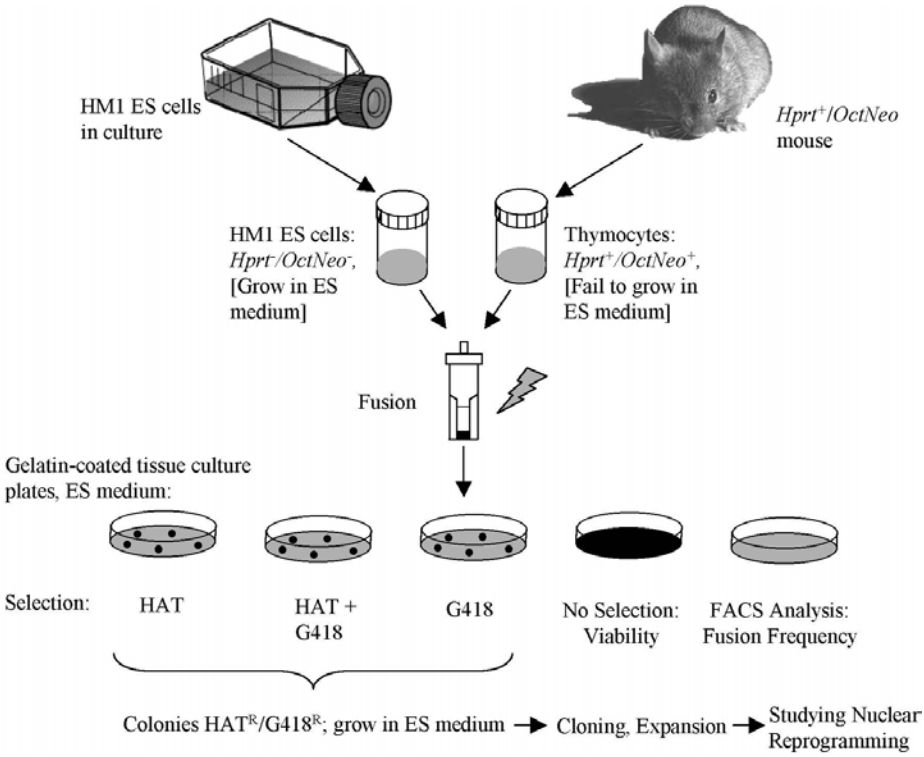

Fig. 1. Overview of the process to generate fusion hybrids from ES cells and adult murine thymocytes.

This chapter describes a method of generating fusion hybrids from ES cells and somatic cells developed in our laboratory from the EG cell–thymocyte system originally described by Tada and colleagues ***(8)***. The protocol is summarised in **Fig. 1**, essentially consisting of electroshock fusion of a reprogramming cell type (HM-1 ES cell) and a somatic cell type (thymocyte), followed by a selection regime that requires fusion to form a hybrid for cell survival. This protocol reliably generates approx 100 fusion hybrid colonies from HM-1 murine ES cells and murine thymocytes after HAT selection. All the colonies are phenotypically ES (**Fig. 2**). Such fusion hybrids generated in vitro provide a very tractable and replicable system for the generation and study of NR events. The phenotypic ES hybrids can easily be cloned and expanded prior to study of the particular aspect of NR of interest to the researcher, such as modification of chromatin structure, patterns of gene expression, DNA methylation patterns, and so on. Therefore, it may be used for generating fusion

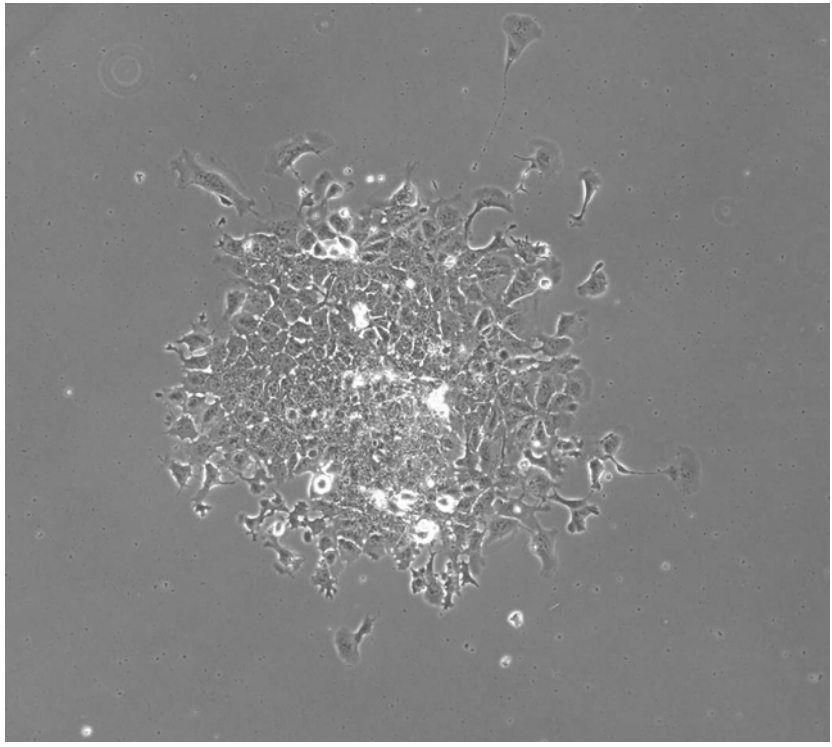

Fig. 2. A murine ES (HM1)-thymocyte fusion colony, after eight days growing in HAT medium. The cells are phenotypically ES, with a modest level of differentiation at the periphery of the colony as is typical for colonies of ES cells. In a few more days, this colony would have expanded further and be ready for picking.

hybrids to study NR events, as the starting point for developing a system to fuse other cell types and also as a positive control in such fusion experiments.

2. Materials

1. Three female mice from a strain wild-type for the *Hprt* gene (e.g. CBA) are required for each fusion. We have also used *OctNeo* mice ***(16)***, which are 94% CBA and also carry the *OctNeo* transgene. The suitability of this strain for fusion experiments studying NR is considered in some more detail in **Note 1**.
2. HM1 murine ES cells (*see* **Note 2**).
3. Thymocytes derived from the adult mice in **item 1** (*see* **Note 3**). Prepared as described in **Subheading 3.**, **steps 1–5**.
4. Standard ES medium: Murine ES cells are routinely cultured in Glasgow's modified Eagle's medium (GMEM, Life Technologies), supplemented with 5% fetal calf serum (FCS), 5% newborn calf serum, 2 m*M* L-glutamine, nonessential amino

acids (0.1 m*M* each of glycine, L-alanine, L-aspartic acid, L-asparagine and L-glutamic acid, 0.2 m*M* each of L-proline and L-serine), 1 m*M* sodium pyruvate, 0.1 m*M* β-mercaptoethanol and 500 U/mL leukemia inhibitory factor.

a. Grow ES cells on tissue culture plastic coated with gelatin. Prepare 0.1% gelatin in water by adding 400 mL Analar water to 0.4 g gelatin in a 0.5 L Duran bottle. Autoclave, allow to cool, and autoclave a second time. Apply the gelatin to the tissue culture plastic a few minutes before the cells are added, and aspirate immediately before adding medium and cells.
b. ES cells are passaged typically every 3 d 1:6 by washing in phosphate buffered saline (PBS) and treating with trypsin–EGTA (TEG) for approx 2 min to release the cells. Neutralize the TEG with standard ES medium prior to recovering the cells by centrifugation for 5 min at 200*g* and plating out as desired.

5. PBS: Dissolve 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, and 0.24 g KH_2PO_4 in 800 mL ddH_2O and adjust the pH to 7.4 with HCl. Make the volume to 1 L with ddH_2O and autoclave.
6. TEG: Dissolve 6.3 g NaCl, 0.12 g Na_2HPO_4, 0.216 g KH_2PO_4, 0.333 g KCl, 0.9 g D-glucose, 2.7 g Tris, and 0.9 mL 1% Phenol red in 800 mL Analar water. Then add 100 mL 2.5 % trypsin, 0.4 g EGTA, and 0.1 g polyvinylalcohol. Adjust the pH to 7.6 and make to 1 L. Filter through a sterile 0.2-μm filter, aliquot, and store at –20°C.
7. 100X HAT: To create HAT-selective medium, standard ES medium is supplemented with hypoxanthine, aminopterin, and thymidine at final concentrations of 0.1 m*M*, 1 μ*M* and 10 μ*M*, respectively. 100X HAT is prepared as follows:
   a. Dissolve 272.2 mg hypoxanthine in 2 mL 1*M* NaOH.
   b. Add 8 mL ddH_2O and mix well.
   c. Add a further 10 mL of ddH_2O and mix until most of the hypoxanthine is dissolved.
   d. Add another 1 mL 1 *M* NaOH, and mix until all the hypoxanthine is dissolved.
   e. Add 75 mL of ddH_2O.
   f. Add 48.4 mg thymidine and mix well.
   g. Add further 100 mL of ddH_2O.
   h. Add 4.0 mL of 5×10^{-3} *M* aminopterin (*see* **item 8**) and mix well.
   i. Filter sterilize and store as 20 mL aliquots at –20°C in foil-wrapped tubes.
8. To prepare 5×10^{-3} *M* aminopterin:
   a. Weigh out approx 100 mg aminopterin.
   b. Dissolve at a concentration of 2.2 mg mL^{-1} in ddH_2O. One milliliter of 1 *M* NaOH may have to be added to make the aminopterin dissolve.
   c. Filter sterilize and store at –20°C in a foil-wrapped tube.
9. 100X HT for weaning cells off HAT selection, is prepared and stored as above but without the aminopterin. Because it is the aminopterin that is light-sensitive, HT aliquots do not have to be kept wrapped in foil.
10. To create G418-selective medium for use of the *OctNeo* transgene, supplement standard ES medium with either 100 μg mL^{-1} or 150 μg mL^{-1} G418 depending on

whether the thymocytes are heterozygous or homozygous for the *OctNeo* transgene, respectively. Prepare G418 stock by dissolving G418 in ddH_2O and make to a final concentration of 100 mg mL^{-1}. Filter-sterilize and store at -20°C in 5 mL aliquots.

2.1 Fusion Reagents

1. 0.3 *M* D-mannitol, 280 mOsmols (*see* **Note 4**).
2. Standard Phosphate-Buffered Saline, sterile.
3. Cell marker dyes (Cambridge Bioscience), 10 µL aliquots at 10 m*M* concentration in dimethyl sulfoxide (DMSO), stored at –70°C in foil-wrapped tubes. Green: 5-chloromethylfluorescein diacetate (CMFDA); and orange: 5-(and-6)-(((4-chloromethyl)benzoyl)amino) tetramethylrhodamine (CMTMR; (*see* **Note 5**).

2.2. Fusion Equipment

1. 10-cm Tissue culture plates.
2. 18-gauge Needles.
3. 10-mL Syringes.
4. 7-mL Sterile bijou tubes.
5. Microscope, inverted phase-contrast.
6. Hemacytometer, Neubauer-improved.
7. Cell incubator. Humidified air, 37°C, 5% CO_2 in air.
8. Bench-top centrifuge and sterile disposable universal tubes (14 mL and 50 mL).
9. Bio-Rad Gene Pulser electroporation apparatus.
10. 2-mm Gene Pulser cuvets.
11. Flow cytometry analyzer (e.g., Beckton-Dickinson FACScan).
12. Osmometer (Advanced Micro-Osmometer model 3MO+, Advanced Instruments, Inc., Norwood, MA).

3. Methods

1. Kill three 6–7 wk-old female mice, CBA strain, *OctNeo* transgenics, or equivalent for each fusion experiment.
2. Isolate the thymi from all three mice, and put them in a bijou tube with 5 mL PBS, supplemented with penicillin and streptomycin.
3. Chop up the thymi with dissecting scissors, then triturate the PBS/tissue mixture with an 18-gauge needle and syringe to generate a cloudy suspension.
4. Allow the crude suspension to settle for 5 min, then remove the middle approx 4 mL of suspension (*see* **Note 6**).
5. While the thymus suspension is settling, isolate ES cells from their flask as normal by aspirating the medium, washing with PBS, then incubate at 37°C for 1–2 min with TEG. Resuspend in ES medium.
6. Count both cell types. Recover the cells by centrifugation at 200*g* for 5 min.
7. Resuspend the cells in the CellTracker/ES medium solutions prepared as in **Note 5** at a final concentration of 10^7 cells mL^{-1}. Thymocytes in CMTMR (orange) and ES cells in CMFDA (green).

8. Incubate the cells in the incubator at 37°C, 5% CO_2 in air for 30 min (*see* **Note 7**).
9. Wash the cells three times by spinning for 5 min at 200*g* and resuspending in PBS.
10. Count the cells.
11. Mix 10^7 ES cells and 5×10^7 thymocytes, centrifuge at 200*g* for 5 min as above and resuspend in 400 µL of 0.3 *M* D-mannitol, 280 mOsmol solution.
12. Transfer the ES cell/thymocyte/mannitol suspension to a 0.2-cm gene pulser cuvet.
13. Centrifuge the cuvet in a 50-mL tube padded with tissue paper for 5 min at 200*g* to pellet the mixture of cells at the bottom of the cuvet. (*see* **Note 8**).
14. Fuse the cells by inserting the cuvet into the Gene Pulser electroporation apparatus and applying a single pulse of 300 V, 25 µF (*see* **Note 9**).
15. Allow the cells to stand at room temperature in the cuvet for 15–20 min after pulsing.
16. Gently resuspend the cells and add to 50 mL ES cell medium. Mix. Add 10 mL to each of 5 gelatinized 10-cm tissue culture plates and place in the incubator.

The following day:

17. Refeed three plates with selective medium 24-h post-fusion (*see* **Note 10** and **Fig. 3**). One further plate remains in nonselective medium as a growth control. The last plate is used to monitor fusion efficiency by fluorescence-activated cell sorting (FACS) analysis.
18. FACS analysis of the fusion experiment and controls of ES cells alone, and ES cells and thymocytes fused alone and then mixed immediately after the electropulse, is used to monitor fusion frequency. Fused cells are positive for both the orange and green dyes, whereas unfused cells are only positive for one color (*see* **Note 11**).

The following days to 2-wk:

19. Re-feed plates with 10 mL selective or unselective medium as appropriate to allow clones to grow (*see* **Note 12** and **Fig. 2**).
20. Resistant colonies are cloned as normal (*see* **Note 13**), then expanded (*see* **Note 14**).

4. Notes

1. The *OctNeo* transgene in *OctNeo* mice ***(16)*** consists of the early embryo-specific *Oct 3/4* promoter ***(17–19)*** driving expression of the neomycin-resistance gene, the product of which provides resistance to the nucleoside analog G418. Because this transgene is not expressed in the thymus of *OctNeo* mice (Pells, S. C., unpublished results), reactivation of *OctNeo* expression and concomitant resistance to G418 provides a functional assay for NR at this transgene locus. The difference in colony frequency between plates subjected to HAT selection and those subjected to both HAT and G418 selection indicates how many phenotypic hybrids are actually reprogrammed, at least at the transgenic *OctNeo* locus.
2. HM-1 is a male ES cell line ***(20)*** with its single copy of the *Hprt* gene carrying the $Hprt^{b\text{-}m3}$ mutation ***(21)***. This mutation is a deletion of exons 1 and 2 of the

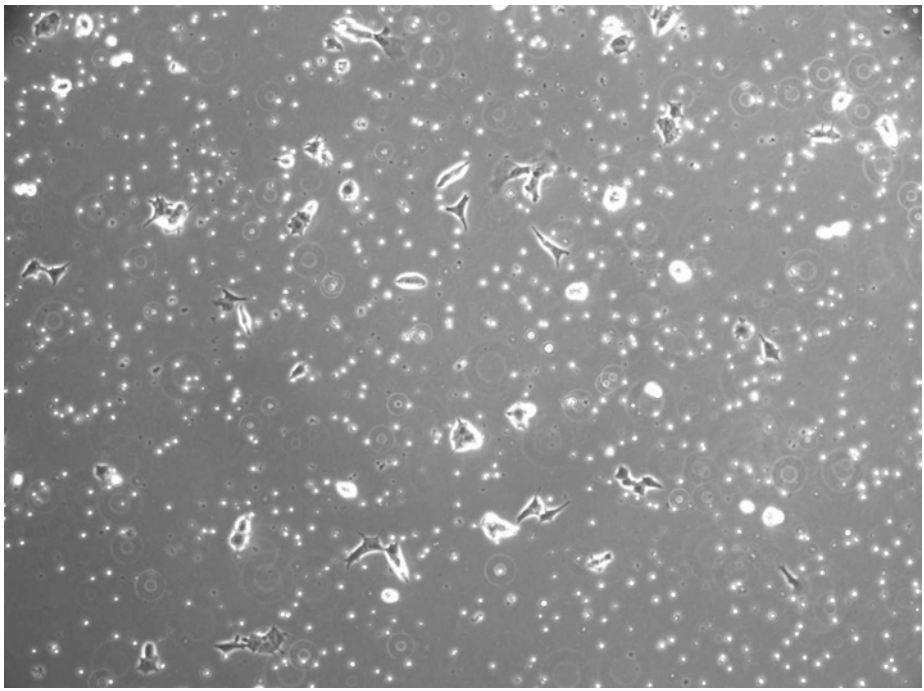

Fig. 3. A field of a murine ES cell-thymocyte fusion experiment tissue culture plate 24 h post-fusion. Two main cell types are visible. The small round, bright cells are dead thymocytes, which have failed to attach themselves to the gelatin/tissue culture plastic substrate. The cells which have adhered to the gelatin substrate are ES cells. Most of these are diploid ES cells and will be killed over subsequent days in selection. A small number represent ES-thymocyte fusions, and will survive in selection to generate a (clonal) colony such as that shown in **Fig.** 2 which can then be picked, expanded and studied in more detail.

gene, and as such reversion events are not observed. Therefore, in HAT selection, *no* colonies should be observed in an ES cell-only control plate, as colony formation is entirely dependent on fusing an ES cell with a somatic cell containing a wild-type X chromosome.

3. Thymocytes are mostly arrested in the G0 phase of the cell cycle, and are dependent on T-cell receptor activation and interleukin stimulation for growth. They do not adhere to the gelatin substrate recommended previously. Thus, ES cell culture conditions do not support thymocyte growth and to survive a thymocyte needs to fuse with an ES cell.
4. The osmolarity of the mannitol solution in which electrofusion is carried out is important to the frequency at which hybrids are obtained. In our laboratory, we make the 0.3 *M* D-mannitol solution freshly prior to use and then correct osmolar-

ity to 280 mOsmols, ± 10 mOsmol, by adding 0.1 m*M* $MgSO_4$ dropwise, mixing, and monitoring the osmolarity of the solution with an Osmometer.

5. Just before use, dilute the CellTracker dyes in ES tissue culture medium. Use a final concentration of either 0.25 μ*M* if the cells are analyzed the same day as the fusion or 2.5 μ*M* if the cells will be examined by FACS on the day following fusion.
6. Avoid any remaining clumps of tissue at the bottom of the tube and any fatty tissue floating at the top.
7. The CellTracker dyes are vital stains, that is, they stain the cell but do not harm it. They are taken up by the cells during the 30-min incubation at 37°C in **step 8**; **Subheading 3.** If FACS is not to be used to monitor fusion efficiency, **steps 6–8** of the protocol (inclusive) may be omitted.
8. This step concentrates all the cells at the bottom of the cuvet, placing them all in close proximity for fusion.
9. When performing experiments that require multiple electroshocks, it is better to leave an interval of a few minutes between pulses. Otherwise, the electroporation equipment appears to deliver less accurate later pulses, which can be seen as a drift in the measured value of the time constant of the pulse with increasing number of pulses. A 300 V, 25 μF pulse typically gives a time constant of 2–2.5 s under the above conditions, and the time constant increases to over 10 s if several pulses are performed in relatively quick succession.
10. Selective media vary depending on the exact combination of cells in use. For thymocytes from wild-type mice and HM1 ES cells, use HAT medium. For thymocytes from *OctNeo* mice and HM1 cells, use either HAT, a combination of HAT and G418, or G418 alone. In a successful experiment, the control plate in nonselective medium quickly reaches confluence, as there should be sufficient viable ES cells to grow over the plate. In selective plates, most cells die but over approx 2 wk following fusion, resistant colonies arise at a rate of approx 20 per plate. This represents a fusion hybrid frequency of approx 100 fusions in a complete fusion experiment, consisting of 10^7 ES and 5×10^7 somatic cells.
11. FACS to monitor fusion frequency is optional, but it is important when quantitative measures of reprogramming events are required. Some modifications to the protocol will give more or fewer hybrid colonies, not because of a change in the NR rate, but because the rate of fusion changes, and to detect this, an assay of fusion efficiency is required.
12. In early selection when there are many dying cells in the plate, plates need to be fed every day, possibly with a gentle wash in PBS first to remove loose dead matter which is toxic to live cells. Once most of the cells are dead, the plate has cleared and colonies are growing up, feeding plates every 2 or 3 d is sufficient.
13. Clones are picked with a sterile 20-μL pipet into a drop of TEG for 2 min to disaggregate the cells and plated individually in a 24-well tissue culture plate.
14. After single-cell-cloning, clones do not have to be maintained in selective medium. If the cells are in HAT medium, note that aminopterin inhibition of dihydrofolate reductase (DHFR), and hence the *de novo* purine pathways, is only

slowly reversed after removal of the aminopterin from the medium because the aminopterin–polyglutamate derivative responsible for enzyme inhibition must be degraded by γ-glutamyl hydrolase first. Therefore, if HAT selection is removed, cells will die even if they are wild-type for *Hprt* unless supplemented with hypoxanthine and thymidine (at the same concentrations as HAT medium) for 3 d following the removal of HAT selection. This transition medium is referred to as HT. After having spent 3 d in HT medium, previously HAT-selected clones may be grown in standard ES medium. G418 may be removed immediately with no ill effects. However, it should be noted that the hybrids are tetraploid cells, and as such may be genetically unstable. In our laboratory, we therefore typically maintain fusion hybrids in selective media permanently to select for at least those somatic chromosomes carrying the selectable markers.

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19

Cytoplasmic Transfer in Mammalian Eggs and Embryos

Henry E. Malter

1. Cytoplasmic Transfer: An Overview

The cytoplasm of oocytes and early embryos is literally the "stuff of life." The mature mammalian oocyte is an enormous cell invested with all the components required for the initiation of development. Through embryonic cloning scenarios, it is now clear that such cytoplasm is a primary requirement—necessary and sufficient (with some limitations) for supporting term development even through the control of a previously quiescent somatic genome ***(1,2)***. However, the components and mechanisms at work in the cytoplasm of eggs and early embryos satisfying this requirement remain mysterious and are the subject of considerable research. Ooplasm harbors the factors responsible for the events of fertilization/activation, maternal and paternal genome remodeling, epigenetic programming, embryonic genome activation and early development. Research into these areas is not only of great importance to our understanding of basic developmental biology, but also has critical implications in animal husbandry and biotechnology ***(3)***, as well as in assisted reproductive medicine ***(4)***. Experimental manipulation of the cytoplasm of oocytes and early embryos has been a significant component of this research. Also, such manipulation has recently been used to directly address ooplasm-specific defects in human reproduction ***(5)***. This chapter presents concepts and techniques for the manipulation of cytoplasm in mammalian eggs and early embryos and provides a brief review of knowledge gained from such research.

For decades, mammalian eggs and embryos have been examined by microsurgical techniques and the majority of procedures are well-established and straightforward. These techniques and procedures are derived from prior work

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Table 1
Research Studies Using Cytoplasmic Transfer

Technique	Species	Research	References
Cytoplast/karyoplast transfer	Mouse/bovine	Creation of hetero plasmic animals	*(3,16–19,21,22)*
	Mouse	Transfer of cytoplasm across developmental stages (MII oocytes to zygotes)	*(23)*
Direct aspiration and injection	Mouse	Transfer of cytoplasm between outbred and hybrid embryos	*(11,24)*
	Primates	Transfer of cytoplasm across developmental stages (MII oocytes to immature oocytes	*(26)*
	Mouse/human	Transfer of mitochondria enriched cytoplasm or suspensions	*(25,31,37)*
	Human	Transfer of cytoplasm between donor and infertile patient oocytes	*(4,5,28–30,32)*

MII, metaphase II.

in amphibians and marine organisms *(6,7)*. Cytoplasmic manipulation, such as the transfer of cytoplasm from one egg to another, has been accomplished by various of means, including cyto/karyoplast creation and fusion, as well as direct aspiration and injection. The choice of particular techniques has been based on cell type, experimental goals, and species-specific factors. Specific techniques for cytoplasmic transfer in experimental animals is discussed followed by a brief discussion of human clinical work. Cytoplasmic transfer research in animal models and humans is outlined in **Table 1**.

1.1. Cytoplasmic Transfer in Experimental Animals

The majority of cytoplasmic manipulation experiments have been carried out in mouse eggs and embryos. Both inbred, outbred, and F1 hybrid mouse strains have been used. F1 hybrid strains are perhaps the most amenable to in vitro embryology. However, in some cases, specific genetic determinants or markers have been examined, requiring the use of defined inbred lines or subspecies. Other experiments have used ruminant and primate material.

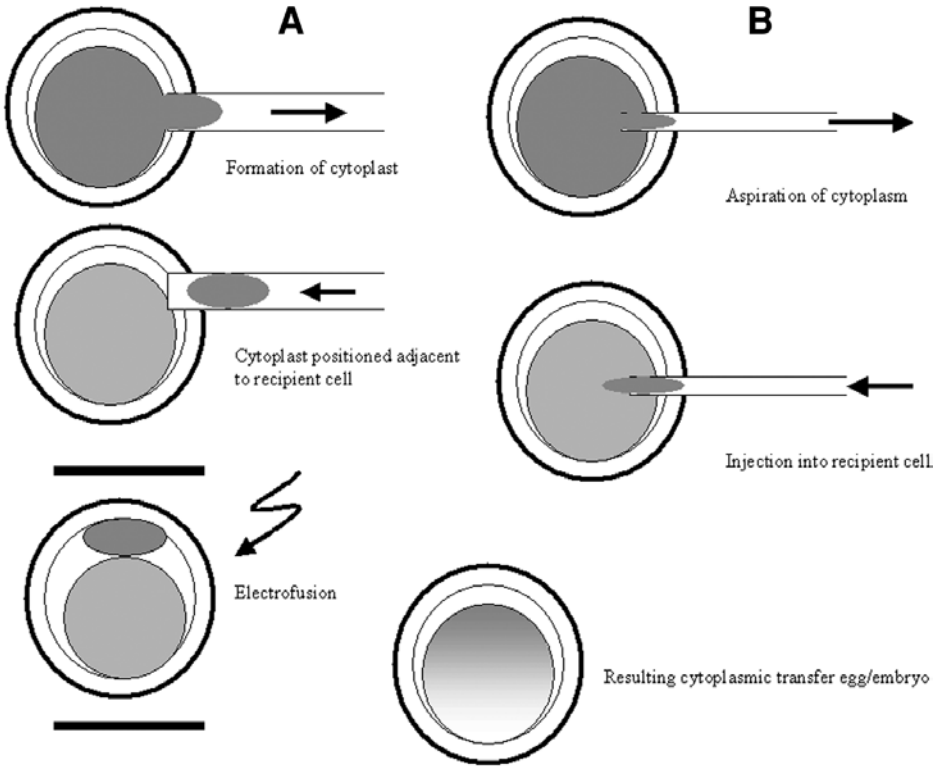

Fig. 1. Diagram of techniques for cytoplasmic transfer. **(A)** Transfer by the formation and electrofusion of cyto/karyoplasts. **(B)** transfer by direct aspiration and injection.

Cytoplasmic transfer experiments have utilized two basic protocols: direct aspiration and injection and cyto/karyoplast transfer and fusion. Both of these techniques can require pretreatment of oocytes and early embryos with chemical agents that relax the cytoskeleton. In the mouse system, the most common form of cytoplasmic manipulation has involved the transfer of cyto/karyoplasts. In this method, a membrane-bound bolus of cytoplasm (cytoplast) or cytoplasm with the cell nucleus (karyoplast) is extracted from the donor cell and then fused with a recipient cell. This technique is diagrammed in **Fig. 1A**. In mammalian embryos, this methodology is based on original experiments published by McGrath and Solter in the early 1980s ***(8,9)***. The technique was further developed (including electrofusion protocols) by Willadsen for use in the nuclear cloning of large animals, and this forms the basis for the vast majority of nuclear transfer (NT) experiments that have been performed in the last 20 yr ***(10)***. This methodology has only recently been partially replaced by direct nuclear extraction and injection ***(2)***.

The second method for manipulating cytoplasm in oocytes and embryos is direct aspiration and injection as diagrammed in **Fig. 1B**. This technique has classically been performed in mouse eggs and embryos using cytoskeletal relaxants such as cytochalasin type B or D ***(11)***. However, work in other species may not require this treatment. Also, new piezoelectric drive micromanipulators can facilitate needle penetration through the plasma membrane of mouse eggs, negating the requirement for relaxant treatment.

1.2. Research Using Cytoplasmic Transfer

In most cases, cyto/karyoplast transfer techniques have been used to manipulate genomic aspects, such as in the creation of embryos harboring only maternal or paternal genomes ***(9,12)*** or embryos with genomes derived from different developmental stages or genetic backgrounds ***(13,14)***. However, other research has used these techniques for manipulating oocyte/embryonic cytoplasm in examining developmental aspects. Many of these experiments have been concerned with studying the behavior of mitochondria during early development, whereas others have been concerned with elucidating developmental determinants.

The maternal mitochondrial genotype(s) present in the oocyte cytoplasm segregate (without replicating) during early development ***(3, 15)***, which is a complex and poorly understood process with important implications in both animal husbandry and human health. Much information about this process has been obtained by manipulating the cytoplasm of early embryos ***(16)***. Mouse strains that harbor informative markers in their mitochondrial DNA (e.g., *Mus brevirostris*-derived New Zealand Black [NZB/BINJ] mouse) allow for experimental protocols that track one strain of mitochondrial DNA ***(17–19)***. In general, heteroplasmic zygotes are created by transferring cytoplasts or karyoplasts between two heterologous strains and followed by mitochondrial DNA analysis during subsequent preimplantation development or in resulting animals ***(19)***. Such experiments have revealed that mitochondrial heteroplasmy is variable during development. Levels of donor cytoplasm-derived mitochondria in embryonic cells and in the resulting offspring vary significantly from undetectable to those surpassing the level of the recipient genotype ***(18)***. Also, variable segregation during early cleavage results in apparently random patterns of mitochondrial distribution in the tissues of mature animals derived from such manipulation ***(18)***. One important observation made from these manipulative experiments is that the proportion of donor mitochondria transferred via karyoplast transfer can markedly increase during development, although this increase was not observed following cytoplast transfer ***(19)***. Perinuclear mitochondria might undergo preferential replication owing to their proximity to nuclear derived factors leading to this apparent amplification ***(20)***.

This phenomenon may have important ramifications for NT-based protocols for manipulating mitochondrial genotype—such as have been proposed for circumventing the inheritance of deleterious mitochondrial mutations in the human. Other experiments have demonstrated specific preferential amplification or loss of particular mitochondrial genotypes following karyoplast transfer in the mouse and bovine ***(21,22)***.

Cytoplast transfer has also been used to examine developmental determinants in oocytes and early embryos. The development of mouse zygotes fused with synchronous and asynchronous cytoplasts derived from germinal vesicle and metaphase II oocytes and zygotes was evaluated ***(23)***. Synchronous transfer of modest (40–45 μm diameter) cytoplasts resulted in development that was identical with that of controls. Transfer of larger cytoplasts and those from aged oocytes resulted in compromised development. However, the transfer of modest metaphase II cytoplasts resulted in implantation rates and d 10 viability rates that were significantly increased over rates of controls.

As with the case for cytoplast transfer, direct cytoplasmic injection has also been used to address both developmental and mitochondrial issues. Early work in the mouse using cytochalasin-facilitated direct transfer of cytoplasm between F1 hybrid and outbred mouse eggs and early embryos revealed that cytoplasmic determinants were involved with the two-cell arrest observed in outbred embryos ***(11,24)***. Transfer of F1 hybrid cytoplasm ameliorated this arrest in outbred recipient embryos. More recently, a similar transfer technique has been used to address mitochondrial issues in early development ***(25)***. Cytoplasts enriched for mitochondria were created by oocytes centrifugation in cytochalasin D media. Small volumes of cytoplasm were aspirated from these cytoplasts and injected into recipient oocytes. Active mitochondria were transferred by this procedure and the net production of adenosine triphosphate (ATP) in recipient eggs was increased over controls.

Direct cytoplasmic transfer has also been reported in cynomolgus monkey oocytes ***(26)***. In these limited experiments, small (20 pL) volumes of metaphase II ooplasm were transferred by aspiration and direction injection into the ooplasm of immature prophase I oocytes. In this case, cytoskeletal relaxant medium was not used. This infusion of mature oocyte cytoplasm seemed to confer developmental competence, and following fertilization and transfer of such oocytes, several monkeys were born. When the extracted ooplasm was subjected to a heating protocol, apparent beneficial effects were lost ***(26)***.

We have recently pursued a model system for direct aspiration/injection cytoplasmic transfer in mature mouse oocytes using a piezoelectric manipulator ***(27)***. Survival following this procedure can be highly satisfactory, and recipient oocytes can undergo in vitro fertilization and development to the blastocyst stage. Transfer of ooplasm has occurred between separate cohorts of

F1 hybrid mouse oocytes and also between NZB mouse oocytes and F1 oocytes for mitochondrial tracking. To date, donor NZB mitochondria have not been detected in cytoplasmic transfer embryos with a detection limit of approx 10% donor.

1.3. Cytoplasmic Transfer in Humans

Recently, direct transfer of ooplasm from one human egg to another has been reported ***(5)***. This protocol attempts to address oocyte-specific deficits evident in a defined population of infertility patients. These patients routinely use oocytes obtained from healthy fertile donors as gametic replacements. Ooplasmic transfer attempts to infuse potentially compromised patient oocytes with a theoretically healthy and whole source of ooplasm. In this way, potential oocyte-related developmental problems could be circumvented while retaining the patient's genetic contribution. Published protocols have utilized either fresh ***(28)*** or cryopreserved ***(29)*** donor oocytes from standard egg donation cycles or polyspermic zygotes ***(30)*** as a source of transferred cytoplasm. Recently, a new procedure has been proposed to inject oocytes with a mitochondrial suspension obtained from the cytoplasm of the patient's own cumulus cells ***(31)***. In this procedure, cumulus cells are homogenized, and a mitochondria-enriched fraction is isolated by differential centrifugation. A small volume of this suspension is then injected into the oocyte, along with the spermatozoa using a standard intracytoplasmic sperm injection (ICSI) needle.

Despite the lack of immediate controls, some studies have strongly suggested, that cytoplasmic transfer can have a strikingly positive effect on implantation and term development in patients where oocyte-specific deficits are suspected ***(28,32)***. These results indicate that positive ooplasmic developmental components could be transferred by the procedure, although knowledge regarding the state of those components in the oocytes of fertile and infertile human subjects is very limited. Donor ooplasmic mitochondria are apparently transferred during the procedure and can persist during development ***(33,34)***. Mitochondrial DNA analysis of informative benign polymorphisms in the noncoding hypervariable region has demonstrated donor heteroplasmy in the peripheral blood in 3 of 17 cytoplasmic transfer offspring ***(34)***. Such hypervariable region heteroplasmy is not uncommon in the human population and has no association with mitochondrial dysfunction or disease ***(35)***. Evidence exists for both an age-related association of mitochondrial mutations in human eggs/embryos ***(36)*** and for positive developmental effects following oocyte mitochondrial infusion ***(25,37)***. Therefore, the mitochondrial component of transferred ooplasm may play a role in any positive effect observed

from the procedure. The underlying cell biology of such a positive effects is an extremely complex issue to address, and further research is needed in this area. Experimental clinical trials of cytoplasmic transfer and related procedures have been halted through a regulatory intervention by the United States Food and Drug Administration ***(38)***. The submission of protocols to address this regulatory issue are in process, and, hopefully, relevant trials will soon be able to proceed.

1.4. Summary

Cytoplasmic transfer has been of great utility in addressing a variety of issues in early mammalian development. New molecular techniques are obviously very powerful in focusing on the role of specific and defined gene products in oocytes and embryos. However, considering the temporal, spatial, and functional complexity of the "molecular soup" that is the early cytoplasm, more general manipulative techniques (e.g., cytoplasmic transfer) will still have importance and utility in elucidating these questions. On the clinical side, although diagnosis and treatment for specific molecular deficits in ooplasmic fertility remain elusive for the immediate future, cytoplasmic transfer theoretically provides a methodology to address such deficits directly by the simple infusion of healthy ooplasm. The application of cytoplasmic transfer techniques in the research and clinical laboratory will potentially continue to provide both answers to basic questions in early development and utilization in the treatment of oocyte-specific deficits.

2. Materials

1. Tissue culture dishes for egg/embryo culture (35 and 60 mm).
2. Shallow tissue culture dishes for micromanipulation. (Falcon 1006).
3. Glass Pasteur or Drummond pipets for embryo handling.
4. 37°C, 5% CO_2 incubator for embryo culture.
5. Stereo microscope for embryo handling.
6. Inverted or fixed-stage microscope for micromanipulation.
7. Embryological joystick-type micromanipulation system.
8. Microtools: holding and transfer/aspiration pipet
9. Electrofusion chamber and power supply (BTX ElectroCell Manipulator)
10. HEPES-buffered culture media for handling and manipulation.
11. Appropriate media for culture (CZB, KSOM).
12. Cytochalasin B for manipulation (1–10 mg/mL in handling media).
13. Mannitol fusion solution: 0.29 *M* δ-Mannitol, 0.05 m*M* magnesium chloride hexahydrate, 0.1 m*M* calcium chloride anhydrous with 0.05% bovine serum albumin (BSA) or .1% polyvinyl pyrolidone (PVP).

3. Methods

3.1. Cyto/karyoplast Aspiration and Transfer

Cyto/karyoplast transfer involves a basic embryological micromanipulation set-up, as well as equipment for performing electrofusion. Despite poor results in humans using such electrofusion-based protocols in the past, a novel procedure for the creation and chemical/mechanical fusion of karyoplasts in the human suggests that this concept may have utility in future protocols ***(39)***.

1. The material to be manipulated is first placed in culture media containing a cytoskeletal relaxant, such as cytochalasin B (*see* **Note 1**). A pre-incubation period of 30 min is usually sufficient after which the material is placed (remaining in cytoskeletal-relaxant media) in an appropriate dish/slide for micromanipulation.
2. The embryo is steadied using a standard holding pipet ,and the zona pellucida is breached to allow the introduction of the transfer pipet (*see* **Note 2**).
3. The tip of the transfer pipet is positioned adjacent to the plasma membrane, and careful suction is applied to draw a membrane-bound bolus of cytoplasm (with the nuclei if desired for karyoplast derivation) into the pipet lumen (*see* **Note 3**). The transfer pipet is operated under the control of an air or oil syringe or, in some cases, via a mouthpiece connection. When the desired volume of cytoplasm has been aspirated, the pipet is pulled away, drawing the membrane carefully out until it "pinches off" and seals—leaving an intact cyto/karyoplast in the pipet. The cytoplast can be expelled into the media or immediately positioned for fusion as desired. Postmanipulation, the material is washed through several drops of fresh media to remove the cytoskeletal relaxant.
4. For fusion, cytoplasts are positioned in close contact with the recipient cell. Depending on the material being used, the zona pellucida can be breached or partially split using a needle to allow for insertion of the transfer pipet and proper positioning of the cytoplast ***(10)***. In general, material for fusion is placed in a nonconductive media (e.g., mannitol) and manually aligned with the axis of fusion parallel to the microelectrodes. A separate alternating current alignment pulse can be applied prior to the direct current pulse that gives rise to membrane poration and subsequent fusion (*see* **Note 4**).

3.2. Direct Aspiration and Injection of Cytoplasm

Direct aspiration and injection is a straightforward and essentially self-explanatory procedure, involving the use of a standard embryological micromanipulation set-up.

As mentioned above, direct aspiration and injection of cytoplasm without the use of cytoskeletal relaxants is possible in mouse oocytes using a piezoelectric drive for the injection pipet as in the mouse ICSI technique ***(40)***. In the

human, cytoplasmic transfer between mature oocytes is performed by a modification of the standard ICSI technique ***(28,41)***.

1. The cytoplasm donor egg is held with a holding pipet while an appropriate aspiration needle is inserted through the zona pellucida (*see* **Note 5**).
2. The plasma membrane is penetrated by the aspiration needle (*see* **Note 6**). Avoiding the area of the metaphase chromosomes, a bolus of cytoplasm is aspirated into the lumen of the pipet (*see* **Note 7**).
3. The aspiration needle is withdrawn and inserted in similar fashion into the cytoplasm of the recipient egg, and the cytoplasm is carefully expelled.

4. Notes

1. The concentration of cytochalasin (either type B or D) used varies between 1 and 10 µg/mL for rodent material depending on developmental stage, timing, and other factors. A 1 µg/mL concentration is usually reported for mouse zygotes. Some authors have also suggested the use of both cytochalasin D and 0.3 µg/mL nocodazole ***(19)***.
2. Several methods of zona opening can be used. Simple dissection using a fine-glass needle can create a slit through which larger tools can be inserted. Alternatively, acidified Tyrode's or other acidic solution (approx pH 2.0) can be used for zona "drilling" ***(42)***. The latest and perhaps simplest methodology for zona opening involves the use of an infra-red diode laser operating through the microscope optics, allowing for the precise ablation of zona material ***(43)***.
3. This pipet lumen can vary in size between 15 and 30 µm depending on the size of the egg or embryo being manipulated and the size of the cyto/karyoplast desired. Karyoplast transfers in which the pronucleus(i) are removed require at least a 20-µm lumen.
4. A detailed description of electrofusion techniques is beyond the scope of this chapter. Technical aspects are well-covered in an excellent review by Zimmerman ***(44)***.
5. Beveled pipets can directly pierce the zona during aspiration and injection, such as in the case of the human ICSI-based procedure. Alternatively, zona opening techniques as discussed above in **Note 2** can be used.
6. Piezo-based systems can be problematic and are sensitive to various issues, including proper mounting, positioning of connecting cables, and even oil viscosity in the manipulation chamber. Our experience is based on the use of the Prime Tech system (Japan model PMM-150FU, Prime Tech Ltd.). Once the piezo system is properly set up, the mouse oocyte membrane can be easily pierced using a single pulse at the lowest power setting available. With primate and some large animal material, bigger microtools can be easily inserted without piezo-based manipulation.
7. Lumen size for the aspiration/injection pipet is in the range of 5–10 µm. For cytotransfer in mature mouse eggs, simple blunt hydrofluoric acid-opened transfer pipets are prepared with a 5–8 µm lumen.

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Conventional Technologies for Cryopreservation of Human Oocytes and Embryos

Michael J. Tucker, Sharon Shipley, and Juergen Liebermann

1. Introduction

1.1. Why Freeze the Human Female Gamete?

In the last few years, there has been a significant resurgence of interest in the potential benefits of human oocyte freezing. Essentially, these benefits are formation of donor "egg banks" to facilitate and lessen the cost of oocyte donation for women unable to produce their own oocytes; provision of egg cryostorage for women who wish to delay their reproductive choices; and convenient cryopreservation of ovarian tissue from women about to undergo therapy deleterious to such tissue, which may threaten their reproductive health.

1.2. Conventional Cryopreservation of the Human Oocyte

The technology for oocyte freezing applied clinically thus far has been based directly on traditional human embryo cryopreservation protocols. These utilize a "slow-freeze/rapid thaw" approach that necessitates the use of a programmable freezer (**Fig. 1**), and these protocols have produced approx 150 offspring worldwide. Fortunately, to date, no abnormalities have been reported from these pregnancies, regardless of the persistent concerns that freezing and thawing of mature oocytes may disrupt the meiotic spindle, thus increasing the potential for aneuploidy in the embryos arising from such eggs. With respect to cryostorage of donated oocytes, several reports have shown some success with this approach ***(1–3)***. Use of frozen donor oocytes postthaw not for whole-egg donation, but for ooplasmic transfer, has also reported a successful delivery of a twin following thawed ooplasmic donation ***(4)***.

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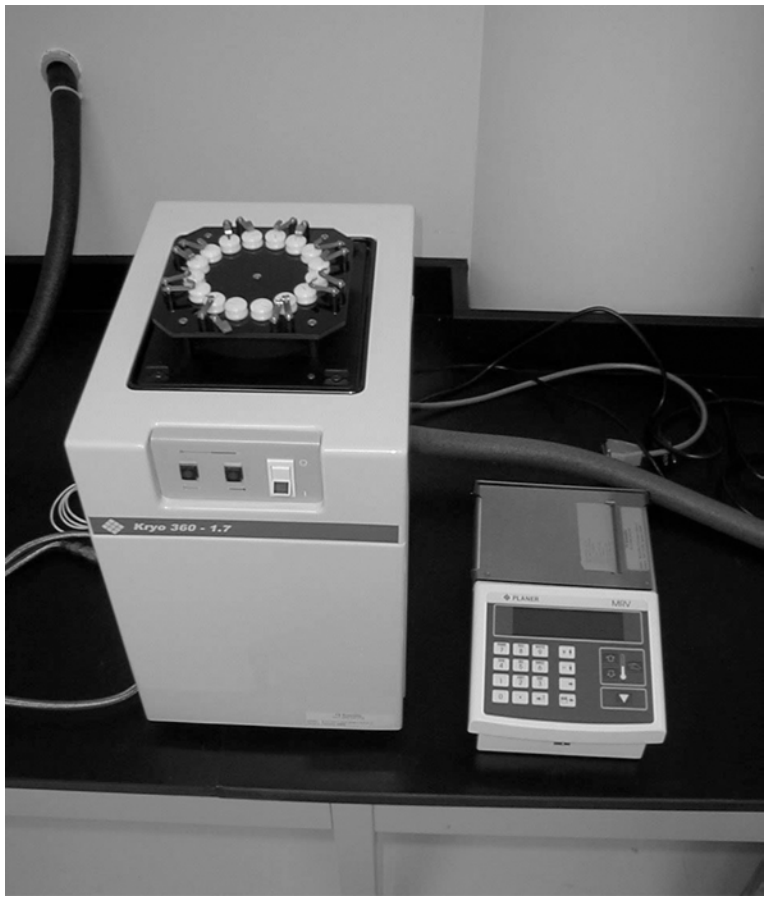

Fig. 1. Conventional slow-freezing programmable freezer, which in this case runs from a continuous liquid nitrogen feed line.

Cryostorage of a women's own oocytes was originally reported in the case of three births over a decade ago by two centers ***(5,6)***. More recently, this success has been reproduced by others ***(3,7–9)***. Generally these pregnancies have arisen from the freezing of oocytes collected for purposes of infertility therapy when couples may have religious or ethical concerns with embryo cryopreservation; when couples have consented to research studies; or when sperm are unexpectedly unavailable after the oocytes have been retrieved. Such circumstances may occur in the last case following an unsuccessful testicular biopsy or when a partner is unable to produce a sperm sample for a variety of unanticipated reasons.

All of these pregnancies arose from frozen–thawed mature oocytes one notable exception: a pregnancy arose from an immature germinal vesicle (GV)-stage egg ***(8)***. Interestingly, this stage of egg development might prove to be a more successful approach for cryopreservation, because its oolemma is more permeable to cryoprotectant, and its chromatin is more conveniently and safely packaged in the nucleus ***(10)***. However, such eggs still have to undergo GV breakdown and maturation to the metaphase II (MII) stage before fertilization, and, therefore, their developmental competency is not as clearly established as with fully mature oocytes that are frozen. The source of the GV eggs, and if they have been exposed to any exogenous gonadotropins, may play a key role in the competency of these eggs ***(11)***.

Whether mature or not, standard cryopreservation technologies appear to have their ultimate limitations, not only in terms of cryosurvival, but more importantly, in their lack of consistency. An adequate overall outcome of cryosurvival may be 50%, but not if it is a statistic arrived at by 90–100% survival in one case and 0–10% in the next. Consequently, radically different types of protocols may provide the answer to increased consistent success. One approach has been to replace sodium as the principal cation in the cryoprotectant with choline in an attempt to shut down the sodium ion pumps in the oocyte membrane during cryoprotectant exposure, which attempts to minimize potentially deleterious "solution effects" during cooling ***(12)***. This has provided significant improvements in murine egg freezing, although it has yet to be applied successfully in the human. Alternatively, traditional slow cooling/rapid thaw protocols might be replaced with vitrification, which again has been successfully applied in the mouse ***(13)***, bovine ***(14)***, and recently in the human ***(9)***. Although the mouse can be a useful model, the murine oocyte is only just over half the volume of a human oocyte; this can have a major impact on permeability and perfusion in the two types of eggs ***(15)***. Intracytoplasmic sperm injection (ICSI) has become the accepted norm for insemination of oocytes postthaw, to avoid any reduction in sperm penetration of the zona with premature cortical granule release ***(16)***.

Potentially, the most plentiful source of oocytes is ovarian tissue itself, containing many thousands of primordial follicles in healthy cortical tissue. Earlier successful work with cryopreservation of rodent ovarian tissue has way to successful cryostorage of both sheep and human tissue ***(16,17)***. Up to 80% survival of follicles has been reported, but the issue is how to handle this tissue following its thaw. For example, tissue that has been removed from a woman about to undergo cancer therapy may contain malignant cells and may therefore not be safely used for autografting into this woman at a later date. The tissue might be screened before or after thawing for the presence of malignant

cells to enable assessment of the safety of this approach, or it may be grown in a host animal (e.g., SCID mouse) until in vitro maturation could be undertaken more effectively. Extended culture of primordial follicles to full oocyte maturity with subsequent embryonic development and birth has only been recorded in the mouse; this was not from cryopreserved tissue ***(18)***. Early studies are being undertaken in the human ***(19)*** and much work remains to be done. Fertility has been restored in sheep in a good model for the human ovary following cryostorage of ovarian cortex and autografting ***(20)***, and this seems the most likely successful clinical model for fertility restoration of women at risk of losing their ovarian function. This may include not only women ready to undergo cancer therapy, but also women with a family history of early menopause, as well as those with nonmalignant diseases, such as thalassemia or certain autoimmune conditions, which may be treated by high-dose chemotherapy. It has been reported that ovarian function was restored by such means in a 29-yr-old patient suffering from hypothalamic amenorrhea subsequent to removal of both ovaries at age 17 yr ***(21)***. Heterotopic transplantation of ovarian tissue in the forearm has also enabled follicular growth to be restored to very convenient oocyte retrieval ***(22)***.

The myriad routes for cryostorage of the female gamete represents a confusing vision of where clinical applications may occur. However, different clinical needs may indeed be met by differing technological approaches, whether incorporating whole-tissue freezing, separate follicle storage, or cryopreservation of mature oocytes.

1.3. Conventional Cryopreservation of the Human Embryo

Although human embryo cryopreservation has become a well-established technology in assisted human reproduction with an estimated 150,000 offspring arisen from this technology worldwide, it is still unclear which stage preimplantation embryos are best cryostored. Indeed, the superiority of blastocyst-stage freezing over one-cell pronucleate-stage freezing in terms of implantation per thawed embryo transferred is countered by the loss of embryos that cannot grow for 5–6 d in vitro ***(23)***. Countering the benefits of freezing cleavage-stage embryos is the partial survival of multicellular embryos ***(24)***, where "partial" embryos may give rise to live births even from one surviving cell, but viability is reduced ***(25)***. However, full cellular survival is preferred, even if there may be a compromise at the level of the *zona pellucida* (**Fig. 2**). Ultimately, there seems to remain some degree of clinical choice of philosophy of approach over when to freeze ***(25)***. However, if it were assumed that the majority of in vitro culture of human embryos might eventually be carried out to the blastocyst stage, then it would seem redundant to freeze embryos at an earlier stage. Selection is the central essence of extended culture, enabling poorer viability

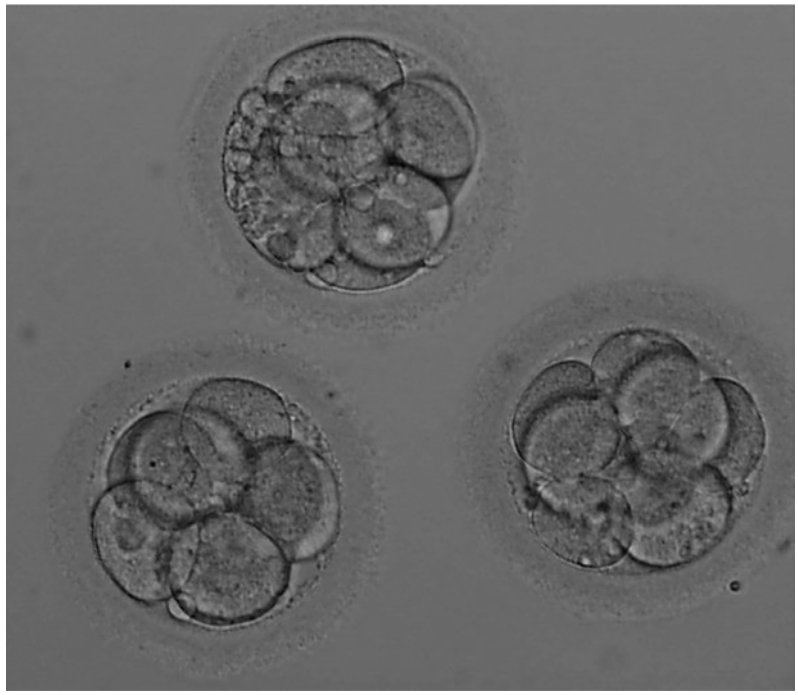

Fig. 2. Three totally surviving frozen-thawed cleavage stage human embryo (d 3 of development); no blastomeres have degenerated during the cryopreservation process; however, the nature of the *zona pellucida* surrounding each embryo appears to have darkened. This is thought to also be associated with actual hardening of the *zona* necessitating assisted hatching in many embryos postthaw.

embryos to arrest in development thus "selecting" themselves as noncandidates for fresh transfer or cryopreservation ***(26)***. Although to some this may seem wasteful of embryos, the net result is likely that chances of pregnancy are more clearly defined and potentially improved, whereas the risks of higher-order multiple implantations is reduced. Additionally, fewer embryos are frozen as blastocysts, reducing storage requirements, and pregnancy expectations from those frozen embryos will be improved. Therefore, overall efficiency will be increased. Nevertheless, given the consistently high rates of cryosurvival of cryopreserved early-stage embryos, there will likely continue to be certain clinical circumstances where early-stage freezing is justified. For example, a woman at risk for ovarian hyperstimulation might appropriately have all of her embryos frozen at the one-cell zygote stage prior to any developmental selection, thus putting her therapy "on ice." If a clinic chooses to move its cryopreservation program principally to the blastocyst stage, a key question

would be what to do with the many early-stage embryos that have already been cryostored? One progressive approach may be to thaw all embryos at these earlier stages and, if possible, grow them to blastocysts. In this manner, fewer embryos will be kept cryostored, and if an excess of embryos for transfer do reach the blastocyst stage, then they may be refrozen for later use.

The first successful reports of human blastocyst cryopreservation came from the culture in a simple salt solution ***(27,28)***. More recently, most cryopreserved blastocysts have arisen from extended culture of supernumerary embryos not transferred fresh on d 2 or 3, usually using coculture ***(29,30)***. However, with increasing confidence in growth stage-sequenced culture media, blastocyst culture for fresh transfer has become increasingly common. More convenient cryopreservation protocols for the blastocyst ***(31)*** have also improved the ease with which this adjustment in a clinic's protocols may be made. Consistently high cryosurvival rates (85–90%) and good postthaw pregnancy rates (>30%) are now being achieved by certain clinics with judicious selection of blastocysts for freezing. How to select potentially viable blastocysts is essential. As culture is extended over a longer period, the rate of development becomes an increasingly important parameter for blastocyst selection ***(32)***. However, a range of selection criteria need to be applied to optimize the choice of blastocysts with the best potential for successful cryopreservation (*see* below).

1.4. Selection Criteria for Human Blastocysts for Cryopreservation

Expanded blastocyst growth rate: d 5 > d 6 > d 7
Overall cell number ≥ 60 cells (depending on the day of development)
Relative cell allocation to trophectoderm/inner cell mass (ICM)
Original quality of early-stage embryo: pronuclear formation, blastomere regularity, and mononucleation, fragmentation

How "early" a blastocyst can be frozen, or if blastocysts partially or totally hatched can be consistently cryopreserved, is yet unknown. The authors' experience suggests that hatching and occasionally fully hatched blastocysts can be cryopreserved, but sometimes with inconsistent outcomes, and early cavitating blastocysts are better cultured to a latter stage of expansion before attempting cryostorage. Much data may exist from mouse and bovine models; however, cell number, overall size, and lipid levels, may have a profound differential impact, thus minimizing the usefulness of such comparative studies. Hence, data will be collected, as has often been the case with human assisted-reproduction technologies (ART) prospectively and used to fine-tune future protocols from clinical hindsight. Most embryo cryopreservation protocols currently use a slow freeze/rapid thaw approach. Slow freeze protocols utilize lower concentrations of cryoprotectant (commonly 1.5 *M*) to avoid the toxicity of such

agents during the initial exposure and slow cooling; higher concentrations of cryoprotectant (approx 4.0 *M*) allow shorter exposure times to the cryoprotectant and rapid freezing.

Despite uncertainties of which cryopreservation protocols will prevail, the future seems to point to increasing success and consistency with embryo cryopreservation. The preparation of the uterus into which the thawed embryos will ultimately be placed seems to be an area of study better resolved. Both natural and hormone replacement cycles seem to provide comparable levels of receptivity in naturally cycling women, although they differ in level of convenience ***(25)***. Indeed, artificially prepared cycles may even effectively dispense with the use of gonadotropin-releasing hormone agonists to lessen cost and improve convenience without loss of success ***(33)***.

1.5. Why Freeze Human Embryos at the Blastocyst Stage?

The very first report of successful cryopreservation of the human embryo was in 1983 ***(34)*** with a pregnancy arising from the freezing in dimethyl sulfoxide (DMSO), thawing and transfer of an eight-cell embryo (*see* **Fig. 2**). The first successes within 1 yr or so appeared of glycerol use to cryopreserve human blastocysts ***(27,28)***. In the same year, Lassalle and colleagues ***(35)*** published a simple, but consistent, protocol using 1,2-propanediol plus sucrose that has become one of the most commonly used approaches for freezing both early-cleavage stage and pronucleate one-cell embryos. Attempts to improve the consistency and convenience of cryopreserving blastocysts reappeared when using Vero cell coculture to enhance extended culture, Menezo and colleagues ***(36)*** explored the use of a combination of glycerol and sucrose as cryoprotectants to freeze "spare" expanded blastocysts. Essentially, all of these protocols employed a slow freeze/rapid thaw approach that requires the use of a programmable freezer for the controlled rate cooling to temperatures between –35°C to –150°C (**Fig. 1**). Variants of these protocols remain the current standards for human embryo cryopreservation.

With an increase in in vitro fertilization-embryo transfer (IVF-ET) cycles being extended to incorporate the fresh transfer of blastocysts, blastocyst cryopreservation is no longer being considered as the last option for "surplus" embryos that develop to this stage. Increasingly, it is being considered as the sole or at least principal stage at which to freeze because if selection of blastocysts is to be optimized, then freezing embryos at an earlier stage would reduce the pool from which to choose fresh blastocysts for transfer. Concerns that embryos are somehow way being "lost" as a result of extended culture, because fewer embryos are being used overall in comparison with previous approaches adopting early-cleavage stage ET, will be allayed by increasing consistency of

Table 1
Progressive Growth of the Conventional Human Blastocyst Cryopreservation Program at Shady Grove Fertility, Maryland

Year	1998	1999	2000	2001	Total
Cryosurvival (%)	51	71	89	82	80
Thaws (#)	9	35	45	140	229
Transfers (#)	7	29	42	131	209
Delivered pregnancy/thaw (%)	11	17	27	38	32
Pregnancy/transfer (%)	14	21	29	41	35
Embryo implantation (%)	5	7.5	18	20	17

extended culture. The central emphasis of blastocyst transfer is to reduce the number of embryos at transfer while maintaining good pregnancy outcomes (**Table 1**).

1.6. Routine Freezing of Blastocysts

Revisiting the original blastocyst cryopreservation protocol, Menezo and Veiga ***(31)*** modified the original protocol to make it more convenient and successful than the earlier protocol ***(36)***. However, clinics have struggled with inconsistent results in the latter protocol, and research variants have been developed to improve the consistency. Much of this has probably been the result of inexperience on the part of many embryologists, both with selecting blastocysts of sufficient quality to freeze, and also understanding the subtleties of cryopreservation, as well as the impact that even the slightest variation, no matter how unintentional, might have on consistency. The most common practice to attempt improved consistency has been to reintroduce one or two glycerol concentration steps in the thaw with one or two extra sucrose dilutions. It is also critical to appreciate that the blastocyst-stage embryo is a more "dynamic" unit than embryos at earlier stages, particularly with regard to the fluid-filled blastocelic cavity that often and preferably collapses during cryopreservation, only to reexpand after thawing (**Fig. 3**).

Embryo cryosurvival may be good, but there can be other factors that may influence outcome, regardless of the blastocyst quality at the time of thaw, with respect to the hormone-replacement protocol. Specifically, changes in the progesterone supplementation seemed to have a differential impact, such that the use of progesterone gel has caused a reduced implantation rate postthaw when compared with the use of intramuscular progesterone (unpublished results). Reports in the literature with the use of vaginal progesterone gels ***(37)***, or suppositories ***(38)*** have been mixed, which does not stimulate lack of cred-

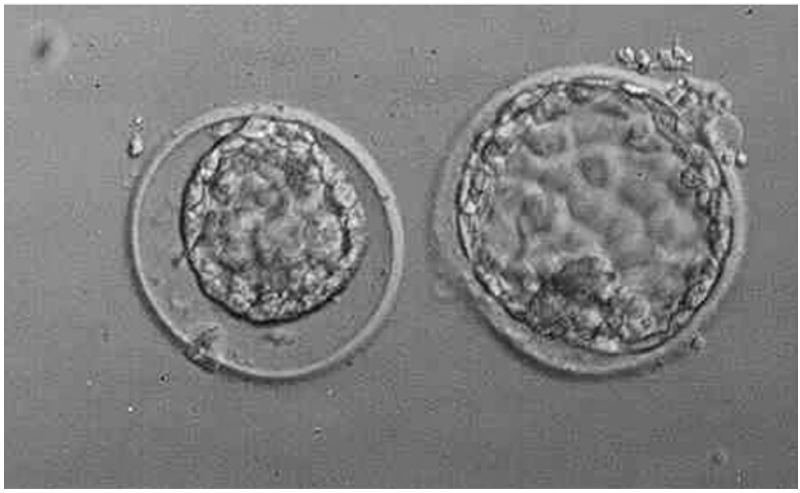

Fig. 3. Two thawed blastocysts with one only partially reexpanded (left); the other (right) being almost fully expanded, and it can be seen to start to hatch from its *zona pellucida*.

ibility in others' results, but underscores the multifactorial nature of assisted reproduction in general, and how the least variation in approaches from clinic to clinic may have a significant effect on outcomes.

The potential benefits of assisted hatching on thawed embryo implantation have not been studied in a full prospectively randomized manner yet. However, it is logical that assisted hatching should be beneficial for thawed blastocysts (**Fig. 4**), given that many have been frozen on d 6, at which stage it appears that assisted hatching is beneficial for fresh blastocysts ***(39)***. Secondly, the *zona pellucida* is thought to undergo problematic hardening during the freeze/thaw procedure ***(40)***. In some cases, holes already exist in the *zona pellucida*, as fractures can be routinely caused by the freeze/thaw procedure ***(24)***, depending on the means of cryopreservation. Embryos with holes already present in the *zona pellucida* can successfully survive cryopreservation and give rise to pregnancies ***(41)***.

Generally, the cryopreservation protocols presented below can be well-defined and controlled from the laboratory perspective. If fluctuations in pregnancy outcomes occur regardless of good cryosurvival, then clinical management problems outside of the laboratory are likely at fault (e.g., inappropriate management of the female cycle during thawed blastocyst transfer). Many simple errors are possible, including calculation of the day of transfer. The easiest way to consider this is to calculate the day of ovulation (whether in

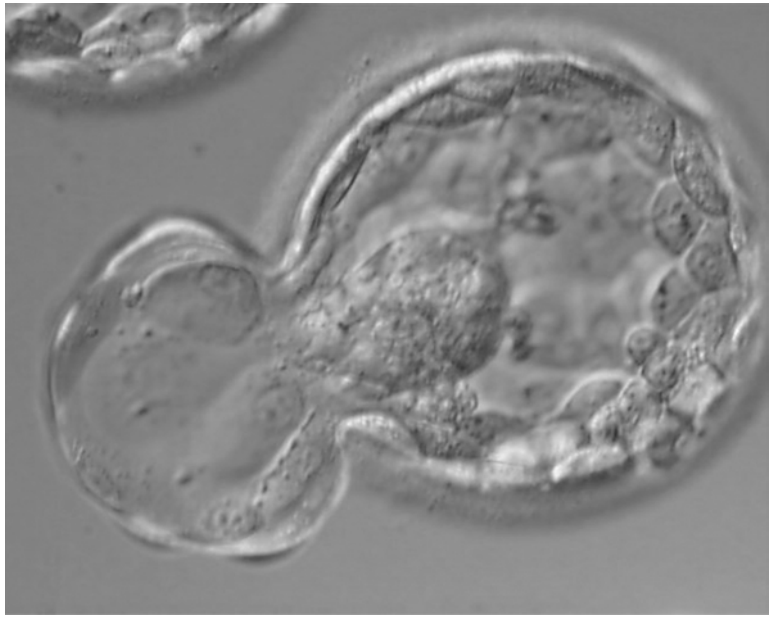

Fig. 4. Thawed blastocyst following assisted hatching exiting the artificially created hole in the *zona pellucida* drilled with a 1.48 μm IR laser.

a natural or artificial transfer cycle), then thaw and transfer all blastocysts on the fifth day of development, counting ovulation day as d 0. This mirrors what would happen normally in an IVF cycle, but where timing manipulation may be needed for practical reasons, it is better to err on the "early" side when thawing the embryos. With regard to the blastocyst day of development, irrespective of the day of cryopreservation, whether d 5, 6, or 7, at thawing, all blastocysts should be treated as if frozen on the fifth day of development.

1.7. Future of Conventional Oocyte and Embryo Cryopreservation

With the production of good quality mid- to fully expanded blastocysts with well-defined ICM and trophectoderm on d 5 or 6, it is possible to settle on consistently successful cryoprotocols using current conventional technology. Even so, as Menezo and Veiga showed ***(31)***, protocols can always be made simpler and more convenient. To this end, it should be noted that vitrification protocols are starting to enter the mainstream of human ART ***(42–44)***. Nevertheless, whatever the approach to cryostorage, the aim of blastocyst cryopreservation particularly will be to maximize the potential viability of each embryo thawed and replaced, such that the number of embryos thawed and transferred may be kept to a minimum. Oocyte cryopreservation will slowly

enter the mainstream of techniques in ART, most likely in the area of oocyte donation. Here, information in terms of clinical success of protocols is generated within months, not years, as would be the case with freezing of oocytes for single women concerned with their future reproductive choices. If it is accepted that human oocyte and embryo cryopreservation is here to stay, it remains of great importance to research the consequences of these therapies carefully to ensure that no harm is done ***(45,46)***.

2. Materials

2.1. General Materials

1. 0.2 μm syringe filter (Gelman Acrodisc).
2. 1-mL, 5-mL, and 10-mL serological pipets (Falcon).
3. 50-mL Tissue culture flasks (Falcon).
4. 60 × 15-mm Tissue culture dishes (Nunc).
5. 12 × 75-mm Tubes (Falcon).
6. Weigh paper (Fisher).
7. Indelible frost-resistant Sharpie pen (Sanford).
8. Cryovials (Nunc).
9. 9-inch Sterile prewashed glass Pasteur pipets (Sigma).
10. 10-mL Syringe (Airtite).

2.2. Reagents

1. 1,2-Propanediol (PROH), sucrose, and glycerol (Sigma).
2. Modified human tubal fluid (HTF-HEPES) (In Vitro Care).

2.3. Equipment

1. Programmable liquid nitrogen freezer (Kryo-360-1.7 System, TS Scientific) or alcohol bath freezer (Bio-Cool, FTS Systems).
2. Timer (Fisher).
3. Easy Pipet (Eppendorf Easypet).
4. Metal spatula.
5. Analytical balance (Mettler).
6. Tube and pipet racks.
7. Liquid nitrogen (LN_2) Storage dewar.
8. Water bath.
9. Thermometer.

3. Methods

3.1. Conventional Oocyte Cryopreservation

Immature (GV, germinal vesicle breakdown/no polar body [GVBD]) or unfertilized mature (MII) oocytes are more difficult to cryopreserve than cleavage-stage embryos, because of the oocyte's surface to volume ratio, its reduced

membrane permeability of the oolemma, its temperature-sensitive metaphase spindle and *zona pellucida*, and its susceptibility to parthenogenetic activation and chillinjury. Conventional slowfreezing of human oocytes is very similar in principle to early embryo freezing, but with several key differences adopted to make outcomes more consistent.

1. After egg collection, maintain all oocytes in culture for >4 h before starting cryopreservation.
2. Strip all oocytes in hyaluronidase within the first 3 h of the 4-h preincubation period.
3. Place all oocytes (GV through MII stage) to be frozen into warm modified HTF, then place onto a warm bench, turn off the bench warmer, and allow them to cool to room temperature (approx. 25°C) for 5 min.
4. Expose to 1.5 *M* 1,2-propanediol (propylene glycol; PROH) in modified HTF with 20% HSA for 15 min.
5. Place into 1.5 *M* PROH + 0.2 *M* sucrose further for 10 min.
6. Rinse cryovial with PROH + sucrose medium and then fill with 0.3 mL of this medium ready to receive the oocytes.
7. Freeze in the following manner: +22°C down to –7.0°C at a rate of –2.0°C/min. Hold 15 min at –7.0°C. "Seed" after 5 min using a cottonbud soaked in LN_2, and ensure that the "seed" has been establishes afterwards. Cool further at –0.3°C/min to –38°C, then plunge into LN_2 for storage.

3.2. Thawing Oocytes

1. Remove cryovial from the LN_2 and place cryovial at room temperature for 1 min; then thaw in a 30°C water bath until ice crystals have gone.
2. Remove contents into a room temperature drop of 1.5 *M* PROH + 0.3 *M* sucrose in modified HTF + 20% HSA, then place into subsequent PROH dilutions for 8 min each of 1.0 *M*, 0.75 *M*, 0.5 *M*, 0.25 *M*, 0.0 *M* all + 0.3 *M* sucrose.
3. Dilute slowly the final 0.3 *M* sucrose drop with modified HTF + 20% HSA, then wash oocytes through two drops of modified HTF, then six drops plain HTF + 20% HSA, and place in the incubator.
4. Undertake ICSI on all mature-thawed oocytes only after 4 h in culture, during which time any cytoskeletal damage that may have occurred during freezing will have an opportunity to repair itself.

3.3. Zygote and Early Cleavage-Stage Embryo Cryopreservation

Make up freezing solutions:

A: 10 mL 20% HSA in modified HTF medium.
B: 1.5 *M* PROH: add 1.24 mL PROH to 8.76 mL of solution **A**. Use a 5-mL pipet and expel several times as PROH is very viscous.

C: 1.5 *M* PROH with 0.1 *M* Sucrose: dissolve 0.171 g Sucrose in 5 mL of solution **B**. Sterile filter PROH and PROH/Sucrose solutions.

1. Ensure that the freezing machine is ready to start: LN_2 vapor freezers need liquid nitrogen, and methanol bath freezers need to be warmed up before cooling down. Ensure machine is programmed correctly.
2. Label cryovials with patient name, identificaton, and date using a frost-resistant marker. Flame-bake the cryovial (carefully) to set the ink.
3. Set up a Nunc culture dish for each group of embryos to be frozen. Use mineral oil with droplets of cryoprotectant at 37°C. Divide the dish into two sections, for PROH, and PROH + sucrose. Use two droplets of each solution.
4. Keep this dish at 37°C to start exposure.
5. Select embryos to be frozen: 2PN or good morphology four- to eight-cell embryos with less than 25% fragmentation.
6. Move selected embryos to the 1.5 *M*PROH droplets for 10 min, and turn off the bench warmer, so that temperature falls to room temperature throughout exposure to the cryoprotectant. Return any other embryos to the incubator.
7. Observe embryos in PROH for contraction and reexpansion.
8. Rinse cryovials with solution **C**, then fill with 0.3 mL of this cryomedium.
9. Move embryos into the 1.5 *M*PROH + 0.1 *M* sucrose droplets for 5 min, and observe for contraction.
10. Transfer embryos to cryovial confirming placement into the vial.
11. Place cryovials in freezing machine and start program.
12. "Seed" cryovials at –7.0°C by touching the cryovial at the meniscus of the medium with a cottonbud soaked in LN_2.
13. Plunge cryovials into LN_2, and store at the end of the cooling program –38(C.

3.3.1. Program for Freezing Machine

Ramp : –2°C/ min to –7.0°C.
Ramp 2: Hold for 15 min at –7°C. (Seeding point: induce ice crystalization after 5 min hold time.)
Ramp 3: –0.3°C/min to –38°C.
Ramp 4: Hold for 30 min (plunging temperature).

3.3.2. Thawing One- to Eight-Cell Embryos

1. Make up thawing solutions:
   20% HSA in modified HTF medium.
   0.2 *M* Sucrose: dissolve 0.68 g sucrose in 10 mL of solution **A**.
   0.2 *M* Sucrose with 1.5 *M* PROH: add 0.6 mL PROH to 4.4 mL of solution **B**.
2. Sterile-filter solutions **B** and **C**.
3. Bring bench to room temperature.
4. Make up dilutions of PROH in 0.2 *M* sucrose. Use small-culture tubes (12 × 75 mm).

Mix	Sucrose/PROH	Sucrose
1.0 *M* PROH	0.4 mL	0.2 mL
0.75 *M* PROH	0.3 mL	0.3 mL
0.5 *M* PROH	0.2 mL	0.4 mL
0.25 *M* PROH	0.1 mL	0.5 mL

5. Set up a 60-mm dish of oil at room temperature with droplets as below:

# PROH	Sucrose
1. 1.5 *M*	0.2 *M*
2. 1.0 *M*	0.2 *M*
3. 0.75 *M*	0.2 *M*
4. 0.5 *M*	0.2 *M*
5. 0.25 *M*	0.2 *M*
6. 0.00 *M*	0.2 *M*

6. Keep this dish at room temperature.
7. Thaw one cryovial at a time, placing at room temperature for 1 min, followed by immersion of the cryovial into a 30°C water bath until no ice crystals remain.
8. Use a Pasteur pipet to remove the contents of the cryovial into a droplet of 1.5 *M* PROH + 0.2 *M* sucrose, pipet up and down fairly vigorously (in case the embryo is stuck to the cryovial). If the embryo is not recovered immediately, wash the cryovial out using the 1.5 *M* PROH + 0.2 *M* sucrose.
9. Immediately after the embryo has been found, move it to a second 1.5 *M* PROH + 0.2 *M* sucrose droplet. Using a clean pipet, move to the 1.0 *M* PROH + 0.2 *M* sucrose droplet.
10. Embryos move through decreasing dilutions of PROH, whereas the sucrose concentration remains constant at 0.2 *M*. Beginning with the 1.0 *M* PROH droplet, embryos remain in each dilution for 8 min.
11. While the embryo is still in PROH solution, make up a culture dish with at least six droplets of HTF + 15% HSA. Keep this dish at room temperature on the bench top but in a 5% CO_2 atmosphere.
12. The embryo spends 2 min in the sucrose (0.2 *M* without PROH), during which time the bench warmer is turned on. After this time, the droplet is diluted with approximately the same volume of solution **A** (to make the solution approx 0.1 *M* sucrose). The embryo remains in this diluted sucrose solution for 1 min.
13. Move the embryo to the dish containing the HTF + 15% HSA medium. Wash through the droplets of medium using a clean pipet for each move. Place this dish onto a heated stage of the microscope, and observe the degree of cryosurvival. Place embryos into the incubator for subsequent culture.

3.4. Conventional Blastocyst Cryopreservation

3.4.1. Blastocyst Freezing Machine Protocol

Ramp 1: –2°C/min to –7.0°C.
Ramp 2: Hold for 15 min. (Seed after 5 min hold time.
Ramp 3: –0.3°C/min to –38°C.
Ramp 4: Hold for 30 min (plunging temperature).

3.4.2. Freezing the D 5–7 Blastocyst

1. Make up freezing solutions:
   A: 4 mL HSA + 16 mL HTF with HEPES (20% HSA in modified HTF).
   B: 10 mL **A** + 1.1 mL glycerol (10% glycerol).
   C: 5 mL **B** + 5 mL A (5% glycerol).
   D: 5 mL **B** + 0.34 g sucrose (0.2 *M* sucrose+10% glycerol)
2. Filter sterilize **C** and **D**.
3. Make up three dishes as follows:
   Dish 1: 1 mL mHTF + 20% HSA ("rinse") in center well and 4-mL rinse in outer well.
   Dish 2: 1 mL **C** (5% glycerol) in center well.
   Dish 3: 1 mL **D** (0.2 *M* sucrose + 10% glycerol).
4. Ensure that the freezing machine is ready to go: nitrogen vapor freezers need liquid nitrogen, and methanol bath freezers should be warmed up before they can cool down. Ensure that the machine is programmed correctly.
5. Label cryovials with patient name, identification, and date using a frost (and methanol)-resistant marker. Flame-bake the cryovial to set the ink.
6. Start the freeze with the bench warmer on. Rinse blastocysts gently in the outer well of dish 1, followed by a second rinse in the center well. This should take up to 2min.
7. Place blastocysts in dish 2 for 10min (**C**: 5% glycerol). At this point, turn the heated stage off.
8. Move into dish 3 (**D**: 10% glycerol + 0.2 *M* sucrose).
9. Load blastocysts into cryovials with a backfilled pipet. Confirm placement of the blastocyst using a stereoscope before fitting the lid.
10. Place cryovial in freezer and start the cooling protocol.

3.4.3. Thawing the D 5–7 Blastocyst

1. Make up thawing solutions:
   A: 4 mL HSA + 16 mL HTF with HEPES (20% HSA in mHTF).
   B: 10 mL **A** + 1.36 g Sucrose (0.4 *M* Sucrose).
   C: 5 mL **B** + 0.55 mL Glycerol (10 % Glycerol + 0.4 *M* Sucrose). Filter-sterilize **B** and **C**.
   D: 1 mL **C** + 1 mL **B** (5% glycerol + 0.4 *M* Sucrose).
   E: 1 mL **D** + 1 mL **B** (2.5% glycerol + 0.4 *M* Sucrose).

F: 1 mL **B** + 1 mL **A** (0.2 *M* sucrose).
G: 1 mL **F** + 1 mL **A** (0.1 *M* sucrose).

2. With the bench and all reagents at room temperature, make a dish with the following droplets: **C**, **D**, **E**, **B**, **F**, **G**, **A**, **A**. Into a second dish, put 1 mL **C** to use to locate the embryos postthaw.
3. Thaw one cryovial at a time, placing it at room temperature for 1 min, followed by immersion of the cryovial in a 30°C water bath until no ice crystals remain. Be careful not to "stir" the cryovial too vigorously, as this tends to disrupt the embryo just as the ice is melting.
4. Remove contents into medium **C** (10% glycerol + 0.4 *M* sucrose), and locate the blastocysts within 30–40 s, then begin dilutions: 3 min each:
   D: 5% glycerol + 0.4 *M* Sucrose.
   E: 2.5% glycerol + 0.4 *M* Sucrose. [Turn on bench warmer at this point].
   B: 0.4 *M* Sucrose.
   F: 0.2 *M* Sucrose.
   G: 0.1 *M* Sucrose.
5. Finally, rinse two to three times in **A** (mHTF + 20% HSA), and perform assisted hatching on the still-collapsed blastocysts either using laser or Acidic Tyrode's medium to drill the *zona pellucida*. Rinse through at least six droplets of warm HTF + 20% HSA in a warm culture dish, and incubate for at least 4 h to observe reexpansion.

4. Notes

1. Because all freezing machines have a different personality, do not believe the temperatures they register. Check temperatures for seeding and plunging regularly using an independent thermocouple.
2. Slow down each step by 1–2 min if blastocyst is still tightly collapsed.
3. Blastocysts should be in **D** for a total of 8–10 min, which includes the time necessary to load vials when they are placed in the freezer and the cooling begins. During this time, rinse labeled cryovials with **D**, then fill (without bubbles) with 0.3 mL of medium **D**.

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21

Vitrifying and Warming of Human Oocytes, Embryos, and Blastocysts

Vitrification Procedures as an Alternative to Conventional Cryopreservation

Juergen Liebermann and Michael J. Tucker

1. Introduction

The important principle of cryopreservation for mammalian embryos is to reduce damage caused by intracellular ice formation. To achieve this goal, two basic approaches were developed: Controlled slow-rate freezing protocols ***(1)***. and Vitrification protocols ***(2)***.

The cryopreservation of human oocytes, zygotes, embryos, and blastocysts has become an integral part of every human in vetro fertilization (IVF) program. However, since the first report of human pregnancy following cryopreservation, thawing, and transfer of an eight-cell embryo ***(3)***, IVF centers have mainly been using traditional slow-rate or equilibrium freezing protocols very successfully. The cryopreservation of biological material includes six steps: initial exposure to cryoprotectant, cooling (slow/rapid) to subzero temperature, storage, thawing/warming, dilution and removal of the cryoprotectant agent, and return to a physiological environment.

The time to complete these freezing procedures for human embryos ranges from 90 min to 5 h. This is because a slow rate of cooling attempts to maintain a very delicate balance between factors, which may result in damage mostly by ice crystallization. Therefore, many studies were undertaken to reduce the time of the freezing procedure and also to try to eliminate the cost of expensive

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Table 1
Primary Benefits of Vitrification

1. Direct contact between cells and liquid nitrogen (LN_2).
2. No ice crystallization.
3. Utilize higher concentration of cryoprotectant (approx 5.0 *M*) that allows shorter exposure times to the cryoprotectant.
4. Rapid vitrify/warm.
5. Small volume used provides a significant increase in the cooling rate.
6. Cooling rates from approx 15,000 to 30,000ºC/min ***(5–7)***.
7. Minimizes osmotic injuries.
8. Reduces the time of the cryopreservation procedure (duration from 2 to 10 min).
9. Very simple protocols.
10. Eliminates the cost of expansive programmable freezing equipment.

programmable freezing equipment. One way to avoid crystallization damage can be achieved by using vitrification protocols. The radical strategy of vitrification has resulted in the total elimination of ice crystal formation, both within the cells being vitrified (intracellular) and in the surrounding solution (extracellular).

1.1. Physical Background

The physical definition of vitrification is the solidification of a solution (water is rapidly cooled and formed into a glassy vitrified state from the liquid phase) at low temperature, not by ice crystallization, but by extreme elevation in viscosity during cooling ***(4)***. In contrast to slow-freezing protocols, there are unique characteristic for rapid freezing protocols (**Table 1**).

There mainly are two ways to achieve the vitrification of water inside cells: increase the speed of temperature conduction and increase the concentration of cryoprotectant.

Therefore, vitrification is a result of high-cooling rates associated with high concentration of cryoprotectant.

1.2. The Importance of Cooling Rates

During cryopreservation, cooling rate is the most important parameter that has an impact on a biological sample cooled from physiological to liquid nitrogen (LN_2) temperature. As cells are immersed into LN_2, it is warmed, and this induces extensive boiling (so that nitrogen gas is produced), evaporation, and a vapor coat will form around the cells. As a result, the vapor surrounding the cells creates insulation that cuts down temperature transfer, which causes a decreased cooling rate. However, to achieve higher cooling rates, it is better to

transfer heat through liquid instead of vapor, because conductive heat transfer in liquid is much faster than in vapor. To improve the chances that the sample is surrounded with liquid and not vapor, the sample size should be minimized so that the duration of any vapor coat is reduced and the cooling rate is increased. Furthermore, to facilitate vitrification by even higher cooling rates, it is also necessary to minimize the volume of the vitrification solution to be as small as possible. Furthermore, to achieve high-cooling rates requires the use of high concentrations of the cryoprotectant solution, which depress ice crystal formation. A negative consequences is that sometimes the cryoprotectant is too toxic. A common practice is to reduce the toxicity of the cryoprotectant, but not its effectiveness is to place the cells first in a cryoprotective solution of lower-strength to partially load the cells with this cryoprotectant before transferring them to the full-strength solution. In addition, often the vitrification solution may contain an almost equimolar combination of ethylene glycol(EG) and dimethyl sulfoxide (DMSO). Also, by reducing the amount of cryoprotectants required, the toxic and osmotic effect of them is also decreased. Furthermore, by further increased cooling and warming rates, it is possible to reduce the cryoprotectant consequent and thus toxicity ***(8)***.

1.3. Container (Carrier System) for Vitrifying Procedures

To minimize the volume of the vitrification solution, special carriers are used during the vitrification process, such as the open-pulled straws (OPS; ***9–13***), or the flexipet denuding pipet (FDP; ***8,14***). In addition, following THE carriers or vessels have also been used to achieve higher cooling rates: microdrops ***(15)***, electron microscopic copper grids (EM; ***16–20***), the Hemi-straw system ***(21–23)***, the nylon mesh ***(24)*** or, the cryoloop ***(8,13,23,25–28)***.

The OPS vitrification method ***(7)*** has been applied successfully to the cryopreservation of matured bovine oocytes, precompaction-and preimplantation-stage bovine embryos ***(9)***, as well as mature mouse and human oocytes ***(10,11)***. More recently successful, pregnancies and deliveries after use of the OPS or cryoloop in vitrification protocols for human oocytes, d-3 embryos, and blastocysts have been reported ***(29–33)***. Furthermore, the efficacy of a rapid cryostorage method using the FDP or OPS for human pronuclear stage (PN) embryos has also been reported ***(14,34,35)***. Also, using EM grids, bovine oocytes, and blastocysts ***(5,17)***, alone with human multipronuclear zygotes, have been vitrified successfully ***(18)***. Interestingly, a new vitrification device called the VitMaster® is able to slightly decrease the temperature of LN_2 to between –205 to –210°C (compared to –196°C) and this is achieved by creating a partial vacuum, thereby significantly increasing the cooling rate by using LN_2 slush. This vitrification device was first introduced by Arav et al. ***(36)*** and

has been very successfully used for bovine, ovine, and human oocyte vitrification *(8,36,37)*.

1.4. Type of Cryoprotectants, Sugars and Macromolecules

The buffered media base used for vitrification is either phosphate-buffered saline (PBS) or HEPES-buffered culture medium, such as HTF.

The most common and accepted cryoprotectant for vitrification procedures is EG (MW 62.02), and it appears to have a low toxic effect on mouse embryos and blastocysts *(38–43)*, along with rapid diffusion and a quick equilibration of EG into the cell through the zona pellucida and the cellular membrane *(42)*. Pregnancies and normal live births achieved with cryopreserved oocytes and embryos in animals *(39,41)* and humans *(29–33)* suggest that this molecule is a good candidate for human embryo vitrification.

Additives with high molecular weights such as disaccharides like sucrose or trehalose, do not penetrate the cell membrane, but can significantly reduce the amount of cryoprotectant required and EG toxicity by decreasing the necessary concentration to achieve a successful cryopreservation of human oocytes and embryos. It is well understood, that solutions of nonpermeating saccharides can serve as an osmotic buffer for the successful recovery of cryopreserved cells *(44)*. Furthermore, the incorporation of nonpermeable compounds in the vitrification solution and the cell incubation in this solution before vitrification helps to withdraw more water from the cells and lessens the exposure time of cells to the toxic effects of the cryoprotectants. The nonpermeating sucrose also acts as an osmotic buffer to reduce the osmotic shock that might otherwise result from dilution of the cryoprotective additive after cryostorage. Additionally, removal of the cryoprotectant agent after warming presents serious problems in reducing toxicity to the cells. During warming, using a high extracellular concentration of sucrose (e.g., 1 *M*) counterbalances the high concentration of cryoprotectant agents in the cell, as it reduces the difference in osmolarity between the intra- and extracellular compartments. The high-sucrose concentration cannot completely prevent the cell from swelling, but it can reduce the speed and magnitude.

Furthermore, the macromolecules polyethylene glycol (PEG; MW 8000), polyvinyl pyrrolidone (PVP; MW 360,000), and Ficoll (MW 70,000 or 400,000) modify the vitrification properties of the solutions. Some studies have evaluated the potential beneficial effects of adding such macromolecular solutes to the vitrification solution to facilitate vitrification of embryos (e.g., in greatly improved viability of oocytes following cryopreservation) and vastly reduced the variability with vitrification solution alone *(45–49)*. These polymers can protect embryos against cryoinjury by mitigating the mechanical

stress occurs during cryopreservation ***(46)***. This is done by modifying the vitrification properties of these solutions by significantly reducing the amount of cryoprotectant required to achieve vitrification, but with little or no effect on the glass transition of the solutions ***(48)***. They also influence the viscosity of the vitrification solution and reduce the toxicity of the cryoprotectant through lower concentration. Furthermore, the polymers may be able to build a viscous matrix for encapsulation of these embryos and also prevent crystallization during cooling and warming ***(45,49)***. Very recently, a study designed and tested cryoprotectant solutions that combined high-polymer concentration with low-penetrating cryoprotectant. These combinations yielded high rates of development of two-cell mouse embryos after rapid cooling and warming ***(49)***.

2. Materials

2.1. Reagents

1. Synthetic serum substitute (Irvine Scientific, Santa Ana, CA).
2. EG (Sigma, St. Louis, MO).
3. DMSO (Sigma).
4. Sucrose (Sigma).
5. PEG (Sigma).
6. Ficoll (Sigma).
7. Mineral oil (SAGE BioPharma, Bedminster, NJ).
8. HTF with HEPES, In Vitro Care).
9. HTF, In-Vitro Care).
10. Zygote and embryo culture media. D 1–3: In Vitro Care; 1 In Vitro Care, San Diego, CA, D 3–5: CCM - Vitrolife, Gothenburg, Sweden).

2.2. Holding and Vitrification Solutions and Culture Media

2.2.1. Vitrifying Solution for Human Oocytes

A. 20% HEPES-HTF: 20 mL HEPES-HTF + 5 mL serum substitute supplement (SSS).
B. 10% EG, 10% DMSO, and 1 mg/mL PEG: 2. 5 mL EG + 2.5 mL DMSO + 0. 025 g PEG, and bring volume to 25 mL with **A**. Mix and filter sterilize into a 50 mL flask.
C. 20% EG, 20% DMSO, 1 mg/mL PEG, 10 mg/mL Ficoll, and 0.65 *M* sucrose: 5 mL EG + 5 mL DMSO + 0.025 g PEG + 0.25 g Ficoll + 5.5 g sucrose, and bring volume to 25 mL with **A**). Mix and filter sterilize into a 50 mL flask.

Prepare the warming solution:

A. 20% HEPES-HTF
B. 1. *M* sucrose: 8.55 g sucrose and bring volume to 25 mL with **A**. Mix and filter sterilize into a 50 mL flask.
C. 0. 5 *M* sucrose: 4.27 g sucrose and bring volume to 25 mL with **A**. Mix and filter sterilize into a 50 mL flask.

D. 0. 25 *M* sucrose: 2.13 g sucrose and bring volume to 25 mL with **A**. Mix and filter sterilize into a 50 mL flask.
E. 0. 125 *M* sucrose: 1.0 g sucrose and bring volume to 25 mL with **A**. Mix and filter sterilize into a 50 mL flask.

2.2.2. Vitrifying Solution for Human Zygotes (Day 1)

A. 20% HEPES-HTF: 20 mL HEPES-HTF + 5 mL serum substitute supplement (SSS).
B. 9% EG: 2.25 mL EG + 22.75 mL **A**). Mix and filter sterilize into a 50 mL flask.
C. 34% EG, and 1.0 *M* sucrose (8.5 mL EG + 8.55 g sucrose and bring volume to 25 mL with **A**). Mix and filter sterilize into a 50 mL flask.

Prepare the warming solution:

A. 20% HEPES-HTF: 20 mL HEPES + 5 mL SSS.
B. 0.5 *M* sucrose: 4.27 g sucrose and bring volume to 25 mL with **A**. Mix and filter sterilize into a 50 mL flask.
C. 0.25 *M* sucrose: 2.13 g sucrose and bring volume to 25 mL with **A**. Mix and filter sterilize into a 50 mL flask.
D. 0.125 *M* sucrose: 1.0 g sucrose and bring volume to 25 mL with **A**. Mix and filter sterilize into a 50 mL flask.

2.2.3. Vitrifying Solution for Human Embryos (Day 3)

A. 20% HEPES-HTF: 20 mL HEPES-HTF + 5 mL SSS.
B. 10% EG and 10% DMSO: 2.5 mL EG + 2.5 mL DMSO + 20 mL **A**. Mix and filter sterilize into a 50 mL flask.
C. 20% EG, 20% DMSO, and 0.40 *M* sucrose: 5 mL EG + 5 mL DMSO + 3.4 g sucrose and bring volume to 25 mL with **A**. Mix and filter sterilize into a 50 mL flask.

Prepare the warming solution:

A. 20% HEPES-HTF: 20 mL HEPES-HTF + 5 mL SSS.
B. 1.0 *M* sucrose; 8.55 g sucrose and bring volume to 25 mL with **A**. Mix and filter sterilize into a 50 mL flask.
C. 0.25 *M* sucrose: 2.13 g sucrose and bring volume to 25 mL with **A**. Mix and filter sterilize into a 50 mL flask.
D. 0.125 *M* sucrose: 1.0 g sucrose and bring volume to 25 mL with **A**. Mix and filter sterilize into a 50 mL flask.

2.2.4. Vitrifying Solution for Human Blastocysts (Days 6 and 7)

A. 20% HEPES-HTF: 20 mL HEPES-HTF + 5 mL serum substitute supplement (SSS).
B. 10% EG: 2.5 mL EG + 22.5 mL **A**. Mix and filter sterilize into a 50 mL flask.
C. 40% EG, and 0.40 *M* sucrose: 10 mL EG + 3. 4 g sucrose and bring volume to 25 mL with **A**. Mix and filter sterilize into a 50-mL flask.

Prepare the warming solution:

A. 20% HEPES-HTF: 20 mL HEPES-HTF + 5 mL SSS
B. 0. 125 *M* sucrose (1.0 g sucrose and bring the volume to 25 mL with **A**. Mix and filter sterilize into a 50 mL flask.

2.2.5. Prepare the Vitrifying Solution for Mouse Blastocyst (Day 4)

A. 20% HEPES-HTF (20 mL HEPES-HTF + 5 mL serum substitute supplement (SSS).
B. 0. 25 *M* sucrose: 2.1 g sucrose and bring volume to 25 mL with **A**. Mix and filter sterilize into a 50 mL flask.
C. 10% EG: 2.5 mL EG + 22.5 mL **A**. Mix and filter sterilize into a 50-mL flask.
D. 40% EG, and 0.4 *M* sucrose: 10-mL EG + 3.4 g sucrose and bring volume to 25 mL with **A**. Mix and filter sterilize into a 50-mL flask.

Prepare the warming solution:

A. 20% HEPES-HTF. 20 mL HEPES-HTF + 5 mL SSS. Mix and filter sterilize into a 50-mL flask.
B. 0.8 *M* Sucrose 36.85 g sucrose, and bring volume to 25 mL with **A**. Mix and filter-sterilize into a 50-mL flask.
C. 0.5 *M* Sucrose. 4.3 g sucrose and bring volume to 25 mL with **A**. Mix and filter sterilize into a 50-mL flask.
D. 0. 25 *M* Sucrose. 2.14 g sucrose and bring volume to 25 mL with **A**. Mix and filter sterilize into a 50-mL flask.
E. 0. 125 *M* Sucrose. 1.0 g sucrose and bring volume to 25 mL with **A**. Mix and filter sterilize into a 50-mL flask.

2.3. Vitrification and Culture Dishes

1. Culture dish for vitrification procedure (Nalge Nunc International, cat. no. 150326, Denmark).
2. Six-well tissue culture plate for warming procedure (Genesis Instruments, Elmwood, WI).

2.4. Carriers for Vitrification Procedures

1. Cryoloop (Hampton Research, Laguna Niguel, CA).
2. FDP (Cook IVF, Spencer, IN).
3. Cryostraw for Hemi-straw system (HSS; IVM, L'Aigle, France).
4. 0. 5-mL Cryo straw with plastic color print (Cryo Bio Systems, France).
5. Pulled glass Pasteur pipet.

3. Methods

3.1. Using the Carriers (see *Note 1)*

3.1.1. The Cryoloop

The vitrification method using the cryoloop was based on the method described by Lane and colleagues ***(25,26)***. In brief, oocytes were exposed to

the HM for 10 min at room temperature, then placed in vitrification solution 1 (VS1), and finally transferred into VS2. During this time, the nylon loop was dipped into VS2 to create a thin filmy layer of the VS2, by surface tension, on the nylon loop. After short exposure to VS2, the oocytes were placed onto the nylon loop preloaded with the thin film of VS2 using the FDP (end hole 140-μm) powered by the forces of surface tension. The loaded nylon loop was plunged into LN_2, then screwed using the stainless rod into the cryovial that had previously been submerged under LN_2. Standard canes were used for storage in LN_2.

3.1.2. FDP

The FDP vitrification procedure is undertaken in a similar fashion to the OPS vitrification method, which was reported by Vajta et al. ***(9)*** as follows. Briefly, the oocytes were pretreated with VS1. After precooling, the oocytes were then placed in VS2, after which they were transferred with a minimum amount of VS2 into a 20 μL-droplet and were loaded into the FDP in approx 1–2 μL of cryoprotectant by placing at a 30° angle and allowing the oocytes to be drawn up by capillary action. The tip of the pipet was then plunged at a 10° angle directly into LN_2, and placed into a prelabeled 0. 25-mL cryo straw held under LN_2. This was then plugged with a plastic color print-coded plug prior to cryo storage.

3.1.3. HSS

The HSS vitrification procedure was first reported by Vandervorst and colleagues ***(22)***. The 0. 25-mL straw was prepared in the following manner. Cut one end of the 0.25 mL straw with a sharp scalpel so that the end is open (approx 1 cm), and it is now easy to pipet a small droplet (<1.0 μL) onto the open inner face. The oocytes or embryos were briefly pretreated with VS1. After precooling, the oocytes or embryos were then placed in VS2, after which they were transferred with a minimum amount of VS2 into the 20-μL droplet, and were loaded in a small volume (<1.0 μL) on the inner surface of the open edge of a 0.25 mL straw (HSS). The HSS was then plunged directly into LN_2 (vertically) and placed into a prelabeled 0.5-mL cryo straw or into a 5-mL cryovial held under LN_2. The straw was then plugged with a plastic color print-coded plug prior to cryo storage.

3.2. Oocyte Vitrification Using Cryoloop and HSS

3.2.1. Stepwise Vitrifying Procedure

Fill LN_2 dewar.

1. At room temperature, add 0.5 mL **A** to one well of a four-well plate.
2. Prepare a culture dish with droplets of 0.1 mL of **B**, **C**, and an additional 20-μL droplet of **C**.

3. Preequilibrate oocytes in **A** for 10 min.
4. Empty pipet on a clean space, load with solution **B**, and pick up the oocytes from **A**.
5. Transfer the oocytes in the culture dish and expose to **B** for 60 s (*see* **Note 2**). Empty pipet and load with solution **C**.
6. Transfer to **C** for 20 s, then in the 20-µL-droplet of **C** (*see* **Note 3**).
7. While the oocytes were incubated in **C**, dip the cryoloop into **C** to create a thin filmly layer of this solution on the nylon loop.
8. Pick up the oocytes and load four or five oocytes into the loop powered by the force of surface tension (*see* **Note 4**). Examine to check if all the oocytes are in the loop (*see* **Note 5**).
9. Plunge the loaded nylon loop into LN_2 (*see* **Note 6**), then screw tight using a stainless rod into the cryovial previously submerged under LN_2.
10. Place on the cane and move into the storage tanks.
11. If there are more oocytes to vitrify, repeat **steps 1–10**.

When using HSS as carrier, follow **steps 1–6**.

7. Then place the oocytes in a small volume as possible on the open edge of the hemi-straw (*see* **Notes 4** and **5**).
8. Plunge loaded hemi-straw directly in LN_2 (vertically) (*see* **Note 6**).
9. Place the hemi-straw in a 0.5-mL straw, and plug with a plastic color print-coded plug.
10. Store on a cane in the nitrogen dewar tank.

3.2.2. Stepwise Warming Procedure

1. At 37°C prepare a six-well dish with 1.0 mL of **E**, **D**, **C**, **B**, then **A**.
2. Remove the nylon loop or HSS from LN_2 and place the loop or hemi-straw directly in **B** (*see* **Note 7**). Stay for 2 min.
3. Transfer the oocytes to **C** for 3 min (*see* **Note 8**).
4. Transfer the oocytes to **D** and **E** for 5 min each.
5. Transfer the oocytes in **A** and leave for 5 min.
6. Rinse through oocyte rinse dish, then incubate for culture (approx 4 h before performing intracytoplasmic sperm injection [ICSI]).

3.2.3. Results of Oocyte Vitrification

Table 2 shows the oocyte postwarming survival rates after using different carriers, such as the cryoloop or the HSS technique. A total of 928 aged human oocytes that failed to fertilize were subjected to vitrification. The oocyte survival rate following 24 h of culture was lower in the cryoloop group than that of the oocytes in the HSS group (80.6% vs 85.4%), but the difference was not significant (χ^2; $p = 0.061$). Overall, the percentage of surviving oocytes was 83.0% (771/928).

Table 2 Postwarming Survival of Human Failed-Fertilized Oocytes after 24-h Culture Relative to the Different Carriers Used

Vitrification technique	Cryoloop	Hemi-straw system
No. of oocytes	448	480
Oocyte survival (%)	361 (80.6%)[a]	410 (85.4%)[a]
Overall survival (%)	738/890 (82.9%)	

[a] $p = 0.061$, according to χ_2 test.

3.3. Zygote Vitrification Using the FDP

3.3.1. Stepwise Vitrifying Procedures

Fill LN_2 dewar.

1. At room temperature, prepare a culture dish with 0.1-mL droplets of **B**, **C** and an additional 20 µL-droplet of **C**.
2. Move zygotes in **B** and hold for 5 min (*see* **Note 2**).
3. Empty pipet on a clean space, load with solution **B** and pick up the oocytes from **A**.
4. Move the zygotes into **C** in the smallest volume possible. After 20 s move zygotes quickly by pipeting to the 20-µL-droplet of **C** (*see* **Note 3**).
5. Loading of four or five zygotes by capillary action is performing by placing the narrow end of the straw into the solution **C** (*see* **Notes 4** and **5**). Take the FDP at shallow angle (approx 30°).
6. Plunge the loaded FDP into LN_2 (approx 10° angle; *see* **Note 6**), and then place into a prelabeled 0.25-mL cryo straw held under LN_2.
7. Then plug this with a plastic color print-coded plug.
8. Place on the cane and store vertically in the storage tanks.

If there are more zygotes to vitrify, repeat above from **steps 1–8**.

3.3.2. Stepwise Warming Procedure

1. At 37°C prepare a six-well dish with 1.0 mL of **D**, **C**, **B**, then **A**.
2. Remove the FDP from LN_2, and plunge directly in to **B**, the zygotes expel (*see* **Note 9**). Hold for 2 min.
3. Move the zygotes to **C** for 2 min (*see* **Note 8**).
4. Place zygotes in **D** and leave for 2 min.
5. Move zygotes to **A** for 1 min (rinse).
6. Transfer the zygotes to equilibrated HTF + 20% SSS dish and incubate for culture.

3.3.3. Results of Zygote Vitrification

A total number of 65 of 1 PN and 152 of 3 PN were vitrified after warming 82% (53/65) of 1 PN and 90% (137/152) of 3 PN survived (**Table 3**). **Table 3**

Table 3
Human Pronuclear Stage Embryos Survival and Cleavage After Being Vitrified with 34% EG Using the FDP As Carrier

	Control group		Vitrified group	
	1 PN	3 PN	1 PN	3 PN
No. of zygotes	45	91	65	152
PN survival (%)	–	–	53 (82%)	137 (90%)
Cleavage on d 2 (%)	36 (80%)[a]	79 (87%)	35 (66%)[a]	112 (82%)
Overall cleavage on d 2 (%)	115/136 (85%)		47/190 (77%)	

[a] $p = 0.001$, according to χ^2 test.

Table 4
Human Embryos Cleaved on D 3, and Blastocyst Formation on D 5 Developed from Human PN Embryos Vitrified with 34% EG

	Control group		Vitrified group	
	1 PN	3 PN	1 PN	3 PN
Cleavage on d 3 (% of ≥ four-cell stage)	27/36 (75%)	62/79 (78.5%)	28/35 (80%)	81/112 (72%)
Overall cleavage on d 3 (%)	89/115 (77%)		109/147 (74%)	
Blastocyst formation on d 5 (%)	12/36 (33%)	26/79 (33%)	12/35 (34%)	34/112 (30%)
Overall blastocyst Formation on d 5 (%)	38/115 (33%)		46/147 (31%)	

No statistically significant differences were observed between the two groups according to χ_2 test.

shows also that the overall percentage of warmed zygotes (1 PN and 3 PN) that cleaved and reached 2-cell stage did not differ (χ^2; $p = 0.32$) from the control groups (77%; 147/190 85%; 115/136). In addition, when we examined the cleavage behavior on d 3 for ≥ four-cell stage, no significant differences (χ^2; $p = 0.95$) were observed between the vitrified group and the unvitrified control groups (74%; 109/147 vs. 77%; 89/115; **Table 4**). Comparing the developmental potential up to cavitation and blastocyst formation on d 5, the overall outcome of the vitrified PN was 31% compared to 33% for the controls (χ^2; $p = 0.76$; **Table 4**).

3.4. D-3 Embryo Vitrification Using the Cryoloop and HSS

3.4.1. Stepwise Vitrification Procedures

Fill LN_2 dewar.

1. At room temperature, prepare a culture dish with droplets of 0.1 mL of **B**, **C**, and an additional 20-µL droplet of **C**.
2. Place embryos expose to **B** for 4 min at room temperature (*see* **Note 2**).
3. Empty pipet on a clean space, load with solution **C**, and pick up the embryos from **B**.
4. Place into **C** for further 20–30 s, then in the 20-µL droplet of **C** (*see* **Note 3**).
5. While the embryos were incubated in **C**, dip the cryoloop into **C** to create a thin filmly layer of this solution on the nylon loop.
6. Pick up the embryos and load four or five embryos onto the loop powered by the force of surface tension (*see* **Note 4**). Examine to check if all the embryos are in the loop (*see* **Note 5**).
7. Plunge the loaded nylon loop into LN_2 (*see* **Note 6**) then screw tight using a stainless rod into the cryovial previously submerged under LN_2.
8. Place on the cane and move into the storage tanks.

If there are more embryos to vitrify, repeat above **steps 1–8**.
When using the HSS as carrier, follow **steps 1–4**.

5. Then place the embryos in a small volume as possible on the open edge of the hemi-straw (*see* **Note 4** and **5**).
6. Plunge loaded hemi-straw directly in LN_2 (vertically) (*see* **Note 6**).
7. Place the hemi-straw in a 0.5-mL straw and plug with a plastic color print-coded plug.
8. Store on a cane in the nitrogen dewar tank.

3.4.2. Stepwise Warming Procedure

1. At 37°C prepare a six-well dish with 1.0 mL of **D**, **C**, **B** then **A**.
2. Remove the nylon loop or HSS from LN_2, and place the loop or hemi-straw directly in **B** (*see* **Note 7**). Stay for 5 min.
3. Transfer the embryos to **C** for 2 min (*see* **Note 8**).
4. Move the embryos to **D** for 2 min.
5. Place the embryos in **A** and leave for 5 min.
6. Transfer the embryos to equilibrated CCM + 20% SSS dish, rinse through 3–4 droplets, and incubate for culture.

3.4.3. Results of Embryo Vitrification

The results are shown in **Table 5**. The postwarming survival rate of d 3 embryos with more than half of intact blastomeres was slightly lower in the cryoloop group (84.0%) when compared to the survival in the HSS group (89.6%), but the difference was not significant (χ^2; $p = 0.07$). Generally, the

Table 5 Post-Warming Survival of Human D 3 Embryos After 2 H Culture and Cleavage Behavior after 24 H as a Result of Different Carriers Used

Vitrification technique	Cryoloop	Hemi-straw system
No. of embryos	144	144
Survival (>50% of intact blastomeres)	121 (84.0%)[a]	129 (89.6%)[a]
Overall embryo survival (>50% of intact blastomeres)	250/288 (86.8%)	
D 4 (≥ 8 blastomeres and stage of compaction in %)	38 (31.4%)[b]	50 (38.8%)[b]
Overall compaction rate (%)	88/250 (35.2%)	

[a] $p = 0.07$, according to χ_2 test.
[b] P = 0. 002, according to χ_2 test.

survival rate of vitrified embryos after 2 h of culture was 86. 8% (250/288). As shown in **Table 5**, reasonable further cleavage and compaction rate occurred in the cryoloop group (31.4%; 38/121), which was significantly different from the HSS group (38.8%; 50/129, (χ^2; $p = 0.002$). For the overall compaction rate, 88/250 of the d 3-embryos became compacted (35.2%).

3.5. Human Blastocyst Vitrification Using the HSS

3.5.1. Stepwise Vitrifying Procedures

Fill LN_2 dewar.

1. At room temperature, prepare a culture dish with droplets of 0.1 mL of **B**, **C**, and an additional 20-µL droplet of **C**.
2. Place blastocysts expose to **B** for 5 min at room temperature (*see* **Note 2**).
3. Empty pipet on a clean space, load with solution **C** and pick up the blastocysts from **B**.
4. Place into **C** for further 20–30 s, then in the 20-µL droplet of **C** (*see* **Note 3**).
5. Then place the embryos in a small volume as possible on the open edge of the hemi-straw (*see* **Notes 4** and **5**).
6. Plunge loaded hemi-straw directly in LN_2 (vertically) (*see* **Note 6**).
7. Place the hemi-straw in a 0.5-mL straw, and plug with a plastic color print-coded plug.
8. Store on a cane in the nitrogen dewar tank.

If there are more blastocysts to vitrify, repeat **steps 1–8**.

3.5.2. Stepwise Warming Procedure

1. At 37°C prepare a six-well dish with 1. 0 mL of **B**, then **A**.
2. Remove the HSS from LN_2, and place the loop or hemi-straw directly in **B** (*see* **Note 7**). Stay for 5 min.

3. Transfer the blastocysts to equilibrated CCM + 20% SSS dish, rinse through 3–4 droplets, and incubate for culture.

3.5.3. Results of Blastocyst Vitrification

The postwarming survival rate of d-6 or d-7 blastocysts was 72. 4% (21/29), and the reexpansion rate 2 h after warming was 71.4% (15/21). The partial and hatched rate of blastocysts after 24–48 h was 52. 3% (11/21).

3.6. Mouse Blastocyst Vitrification Using the HSS

3. 6.1. Stepwise Vitrifying Procedures

Fill LN_2 dewar.

1. At room temperature, prepare a culture dish with 0. 1-mL droplets of **B**, **C**, **D** and an additional 20-µL droplet of **D**.
2. Use a large pulled pipet to move the blastocysts to **B** and hold for 3–4 min. They will collapse in solution **B**.
3. Transfer to **C** for 3 min (*see* **Note 2**).
4. Empty pipet on a clean space, load with solution **D**, and pick up the blastocysts.
5. Move the blastocysts into **D** in the smallest volume possible. After 20 s move blastocysts quickly by pipeting to the 20-µL droplet of **D** (*see* **Note 3**).
6. Load the blastocysts (4–5) into the tip of the pipet and on the open edge of the cutting straw (*see* **Note 4**). Examine to check if all the blastocysts are on the edge of the straw (*see* **Note 5**).
7. Plunge the loaded hemi-straw into LN_2 (*see* **Note 6**), then place into a prelabeled 0.5 mL cryo straw with a plastic color print-coded plug held under LN_2.
8. Place on the cane and store vertically in the storage tanks.

If there are more blastocysts to vitrify, repeat **steps 1–8**.

3.6.2. Stepwise Warming Procedure

The **D** before the warming procedure prepare and incubate a blastocyst rinse dish and a growth dish (using HTF or CCM media).

1. At room temperature prepare a six-well dish with 1.0 mL of **E**, **D**, **C**, then **A**.
2. Remove the hemi-straw from LN_2, and plunge directly in to **B**, the blastocysts expel (*see* **Note 7**). Hold for 3 min.
3. Transfer to **C** for 4 min (*see* **Note 8**).
4. Move to **D** and **E** and stay for 4 min in each.
5. Rinse through blastocyst rinse dish, and perform assisted hatching.
6. Then transfer to growth dish and incubate for culture.

3.6.3. Results of Mouse Blastocyst Vitrification Using the HSS

Table 6 shows the mouse blastocyst postwarming survival rate after using the HSS technique. A total of 268 mouse blastocysts were subjected to vitrifi-

Table 6
Postwarming Survival and Hatching Rate of Mouse Blastocysts After 24–48 h Culture

Vitrification technique	Hemi-straw system
No. of vitrified blastocysts	268
Blastocyst survival (%)	230 (85.8%)
Partial hatched (%)	131 (66.8%)
Fully hatched (%)	65 (33.0%)
Overall hatching rate (%)	196/230 (85.2%)

Table 7
Variables of Vitrification that Can Profoundly Influence its Effectiveness

1. Type and concentrations of cryoprotectant (almost all cryoprotectants are toxic).
2. The media used as base media ("holding media").
3. The temperature of the vitrification solution at exposure.
4. The length of time cells are exposed to the final cryoprotectant before plunging into LN_2.
5. The variability in the volume of the cryoprotectant solution surrounding the cells.
6. Device that is used for vitrification (size of the vapor coat and cooling rate!).
7. Technical proficiency of the embryologist.
8. The quality of the tested oocytes and embryos, and their developmental stage.
9. Direct contact of the LN_2 and the vitrification solution containing the biological material can be a source of contamination ***(52–55)***.

cation. Overall, the postvitrification hatching rate after 24–48 h culture was 85.2% (196/230).

3.7. Conclusions

In conclusion, increasing the thermal conduction speed and decreasing the concentration of cryoprotectants is an ideal strategy for cryostoring cells with vitrification methods. There are two main ways to achieve the vitrification of water inside cells efficiently: increase the temperature difference between the samples and vitrification medium and find materials with fast-heat conduction. However, the actual rate during vitrification procedures may extremely vary depending on the device used, technical proficiency, and also the specific movement at immersion (**Table 7**). Additionally, it is very important to mention that every cell has its own optimal cooling rate, i.e., oocytes are more prone to chilling injury than other developmental stages, such as cleavage stage

embryos or blastocysts. To date, the "universal" vitrification protocol has yet to be defined. However, vitrification as a cryopreservation method has had very little practical impact on human-assisted reproduction, and human preimplantation embryo vitrification is still largely experimental. Besides the inconsistent survival rates that have been reported, another explanation could be that such a variety of different carriers or vessels have been used for vitrification and that so many different vitrification solutions have been formulated, not helping to focus efforts on perfecting a single approach. On the other hand, the reports of successfully completed pregnancies following vitrification is encouraging for further research, but clearly, attention needs to be paid to improve the inconsistent survival rates following vitrification ***(50,51)***.

4. Notes

1. Special care must be given to the selection of carriers. It is necessary to use types of carrier or vessel material with rapid-heat transfer that support the process of uniform heat exchange to achieve higher cooling rates.
2. To minimize the toxicity of the cryoprotectant, a stepwise exposure of cells to precooled concentrated solutions is recommended.
3. Utilize higher concentration of cryoprotectant that allows shorter exposure times to the cryoprotectant be careful—the toxicity of the cryoprotectants will arise with their concentration. Because almost all cryoprotectants are toxic, it is important to watch the duration of exposure to the final cryoprotectant before plunging into liquid nitrogen very carefully.
4. To facilitate vitrification by even higher cooling rates, it is also necessary to minimize the volume of the vitrification solution as much as practical. From this point of view, it is very important to use a small pulled pipet. Furthermore, by collecting the cells on one place and loading not more than four-cells at the same time in the pipet it is possible to keep the volume small.
5. To ensure that the cells were loaded on the carrier, perform the loading process under a light microscope. Check the number of loaded cells.
6. Submerge the carrier loaded with the cells directly in LN_2 by passing rapidly through the vapor phase (nitrogen gas).
7. Before moving the carrier quickly from the nitrogen in the warming solution, pull a glass pipet. Fill the pipet with a small amount of the first warming solution. In case of using the HSS as carrier, rinse the open edge of straw after placing in the warming solution (the vitrification solution containing the cells is still viscous). This way it is possible to make sure that the cells drop from the straw in the warming solution.
8. Even when switching the cells between different concentrations of warming solutions, fill up the pipet with the next lower concentration of warming solution before picking up the cells for moving in the following concentration.
9. To make sure that the cells expelled from the pipet, blow the pipet empty by using a mouth piece with connecting rubber tubing.

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22

Microfluidic Removal of Cumulus Cells from Mammalian Zygotes

Henry C. Zeringue and David J. Beebe

Introduction

Micron-sized fluid channels have been used for biology-related procedures, such as single-cell manipulation ***(1–3)***, cell deformation ***(4,5)***, cell assays ***(6)***, and subcellular molecule positioning ***(7)***. Owing to laminar fluid flow on this scale, small volumes of fluid can be precisely controlled ***(8,9)***. Microfluidic tools allow for the control and manipulation on the size scale of a single embryo ***(10)***. Precise microfluidic manipulation can be employed to perform multiple IVP-processing steps ***(11–13)***.

This chapter presents a method of cumulus cell removal using a microfluidic technique. We present an alternative to traditional cumulus removal techniques (e.g., vortexing and pipetting). Using this technique, cumulus can be removed from approximately one to four zygotes at a time. Cumulus removal from bovine zygotes at 24 h post-fertilization typically takes 15–30 min for 12 cumulus–oocyte complexes (COCs), depending on proficiency of the operator (**Note 1**). COCs are introduced to the device and moved to the cumulus removal region (**Fig. 1**). Fluid pulsed through the removal ports allows COC rotation and facilitates cumulus removal.

Because this is not yet a standard process, a nonstandard piece of equipment is required. A microfluidic cumulus removal device from Vitae, LLC (Madison, WI) is used (*see* **Note 2**).

1.1. Bubbles in the Device

Bubbles can be a serious problem in microfluidic systems. **Figure 2** shows typical locations where bubbles tend to reside. Most bubbles in the device will

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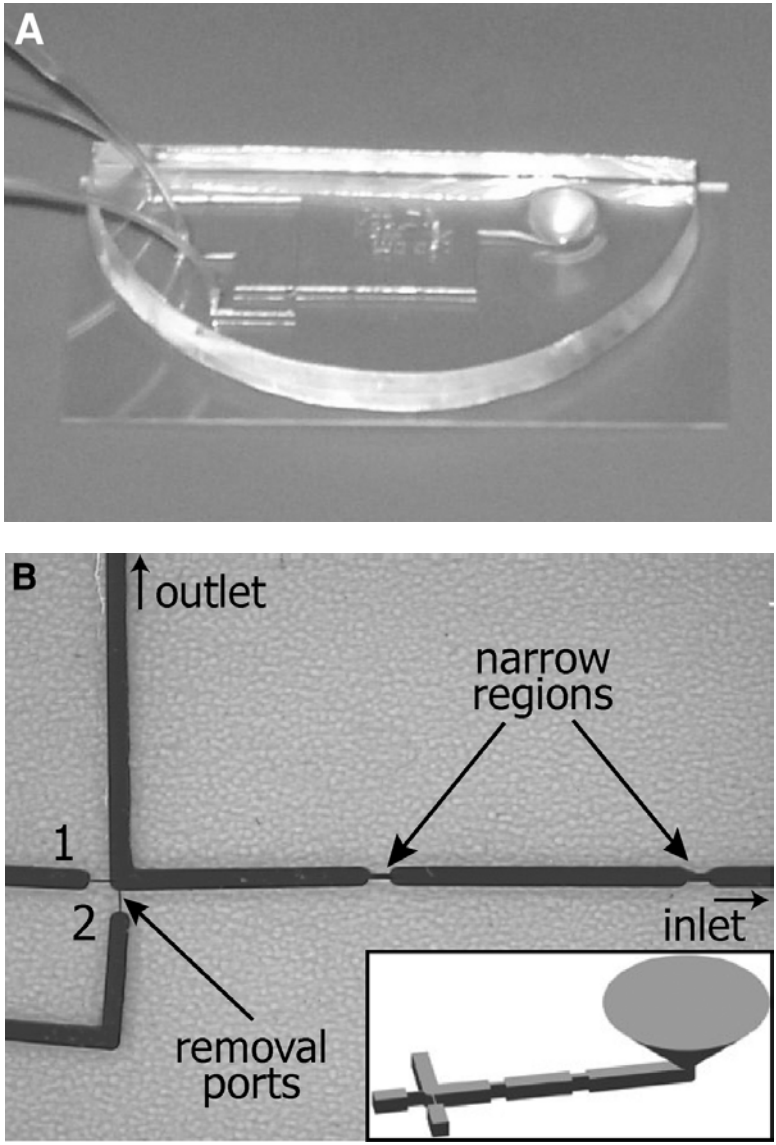

Fig 1. The cumulus removal device. **(A)** Device set-up. The tubing (right) connects the device to syringes (not shown) for flow control. **(B)** Close-up of channel network. Fluid flows between the inlet and outlet to position the COC near the removal ports. The narrowed regions regulate COC movement into the removal region. At the removal ports, cumulus is removed using pulsed fluid-flow. Clean zygotes are flushed back into the inlet for retrieval. (Inset) Schematic of the channel network showing the inlet funnel well.

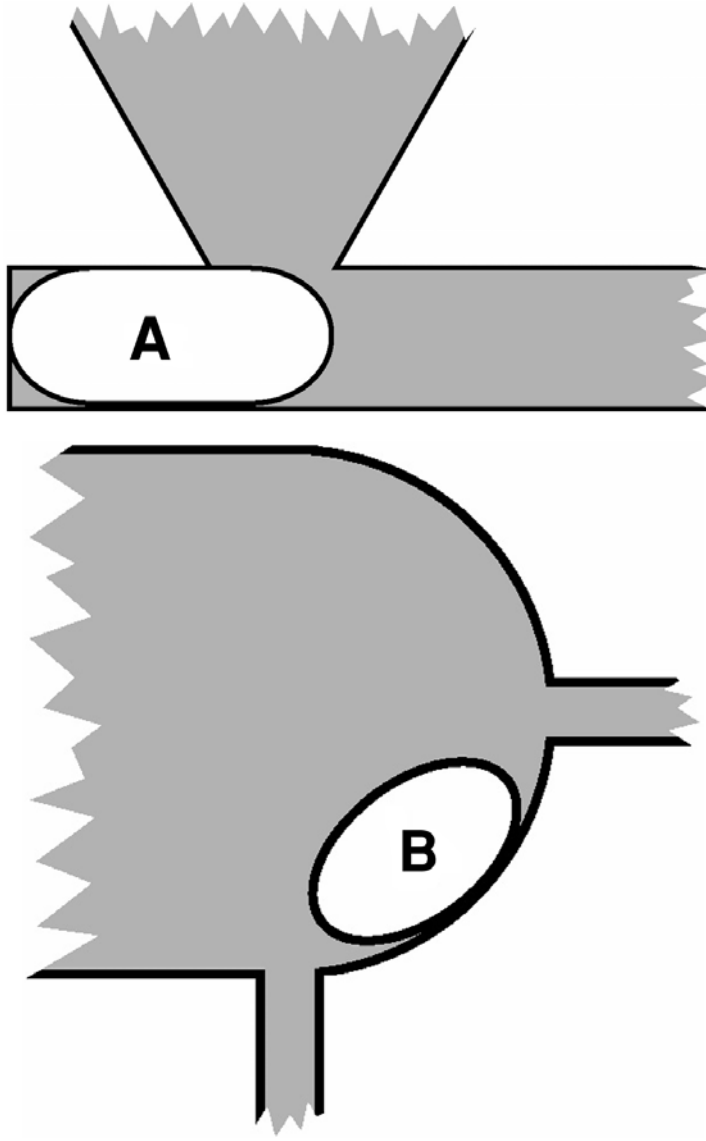

Fig. 2. Typical bubble locations: **(A)** the funnel/channel junction at the inlet of the device and **(B)** between the two removal ports.

not affect device operation. The chief location to inhibit device operation is the funnel/channel junction (**Fig. 2A**). Other locations that bubbles typically reside have a minimal effect on device performance (**Fig. 2B**). Methods to

minimize (*see* **Note 3**) and remove (*see* **Subheading 3.3.**) bubbles are discussed.

2. Materials

All equipment is purchased from Fischer Scientific (Itasca, IL) unless otherwise stated.

1. Tubing, 0.06-inch od, 0.02-inch id (Cole-Parmer, cat. no. E-06418-02, Vernon Hills, IL).
2. 1-cc Syringes.
3. Syringe needles, 23G, 1 inch
4. Scissors (to cut tubing).
5. Cumulus removal device (CRD; Vitae, Madison, WI, *see* **Note 2**)
6. Pipets: P1000, P200, and P20.
7. Sterile tips: 20 µL, 200 µL, and 1000 µL.
8. Sterilization bags (cat. no. 01-812-50).
9. Autoclave machine (cat. no. 02-964-1).
10. TL-HEPES wash media (Whitaker Biosciences).
11. 60 mm × 15-mm, polyethylene Petri dishes (cat. no. 08-757-100B).
12. Slide warmer (cat. no. 12-594).

3. Methods

3.1. Inspection and Sterilization

1. Inspect device to ensure there are no obvious obstructions, debris, or other problems.
2. Place device in sterilization bag and seal.
3. Autoclave device at 121°C for 30 min, and then dry for 20 min.

3.2. Device Preparation and Use

1. The CRD should be warmed for 30 min at 39°C for bovine zygotes prior to use (*see* **Note 3**).
2. Fill two petri dishes with 2 ml warm TL-HEPES each, and place on slide warmer: first dish for COCs and the second for clean zygotes.
3. Make fluidic connections. Syringe needles should be placed on two 1-cc syringes. Tubing should be cut to approx 12–15 inches (*see* **Note 4**). One needle should be threaded into one end of each piece of tubing. The other end of each piece of tubing should then be press-fit into an outlet hole in the CRD.
4. Fill the CRD with TL-HEPES. The medium can be placed in the inlet well with the P1000 pipet and should be drawn through the device into the tubing, at least to the syringe tip (*see* **Note 5**).
5. Place a COC into the well of the CRD using a P20 pipet. Allow it to sink near the channel opening.

6. Pull on the syringes to draw the COC down to the removal ports (*see* **Note 6**).
7. Once the COC is at the removal port, adjust the pressure to an appropriate base level for cumulus removal without causing undue stress to the zygote (*see* **Note 7**).
8. When cumulus is no longer drawn into the port, tap on the syringe to aid with cumulus removal and allow rotation of the COC (*see* **Note 8**).
9. Once cumulus has been stripped from the zygote, reverse the flow to move the zygote into the well.
10. Using a P20 pipet, retrieve the zygote from the CRD.
11. Place the zygote into the clean holding dish (*see* **Note 9**).
12. Repeat **steps 3-11** for each zygote (*see* **Note 10**).

3.3. Handling Bubbles in the CRD

Note size and location of bubbles, and determine those that may interfere with device function (*see* **Note 11**).

3.3.1. Bubble Occluding the Channel Entry at Bottom of Funnel Well

Use a P20 pressed to the bottom of the funnel to extract the bubble.

3.3.2. Bubble in Between the Removal Ports

1. If the bubble is occluding the removal ports, the bubble can usually be made smaller by sucking a portion of the bubble through the removal port.
2. If the zygote is caught at the bubble surface, it can usually be dislodged with a quick pulse of fluid from one or both of the removal ports.

4. Notes

1. The operator should practice cumulus removal with the device multiple times prior to using experimental embryos. The operator should pay special attention to **Notes** 4–8 throughout the learning period. The operator should also focus on the relationship between syringe movement (both the plunger movement and vertical movement of the syringe) and fluid flow along with how it affects zygote movement. One should be able to repeatedly perform cumulus removal after approx 5–6 40-min training sessions. Remove cumulus from 12 zygotes in 25–30 min. After 15–20 h (or approx 1 mo) of normal device use, the operator will become proficient enough to safely remove cumulus from 10–12 zygotes in approx 15 min.
2. Currently, the devices are not in mass production. Please contact Vitae LLC (608-222-1908 or http://www.vitaellc.com) or Penetrating Inovations (608-845-3270, http:wwww.po-ivt.com) for availability of this product.
3. The devices should be warmed to the physiological temperature of the embryos being processed. Warming should be performed on a slide warmer or hot plate. Warming of the device is not strictly for environmental maintenance of the zygote, but also to control bubbles. Raising the temperature of the device prior to media introduction greatly reduces the number and size of bubbles formed in the

device during filling. Increasing CO_2 concentration in the device also reduces bubbles, but TL-HEPES is not designed to buffer a high CO_2 environment. If TL-HEPES is used when the device was equilibrated in a high CO_2 environment, the reader should be aware that the pH of the medium would likely be lowered during device use.

4. Tube length is partly determined by operator hand-position during cumulus removal. The tubing should be long enough to allow free movement of syringes, but excess tubing dampens the pressure fluctuations necessary for efficient cumulus removal (*see* **Note 7**). If the tubing is too short, syringe movement can shift the device position under the stereomicroscope and possibly cause exaggerated pressure fluctuations on the zygote. The tubing is shipped in a coil. A good rule of thumb for the length is that the tubing should remain curved during use, but there should be no excess coils.
5. When filling the device, media should be drawn through the tubing at least to the syringe tip because of two reasons. First, if there is too much air in the tubing, pressure fluctuations required for efficient cumulus removal (*see* **Note 7**) will be greatly dampened. Second, it will be necessary to flow the media in both directions during processing. If there is not enough fluid reserve in the tubing and syringe, air will be forced into the device. Bubbles in the device will impede cumulus removal and are difficult to remove (*see* **Note 2**).
6. The operator should focus on fluid flow through the two removal ports. If fluid is not flowing in the same direction in both ports, the flow at one port can be much higher than expected. This can become a problem when moving COCs to the removal ports. When a COC gets close to the removal ports, ensure fluid is stationary in one port. Controlling the flow with one syringe is easier than trying to control both syringes at once.
7. Before using the device, the operator should become familiar with the appropriate pressure levels to use during embryo processing. Because of the small volumes in the device, it is very easy to cause high pressure with modest changes in syringe volume. To become familiar with pressures, the operator should first use traditional methods to remove the cumulus from a group of zygotes. Follow the protocol using these bare zygotes in the place of COCs. When the zygote is at the removal port, the operator should pay special attention to the zona region in contact with the removal port (**Fig. 3A**). If the zona begin to "pinch" or "v-in," the pressure is too great (**Fig. 3B**). With the zygote against the removal port, adjust the pressure and observe the zona transform from a normal curve at lower pressures to more of a "v" or pinched area at the port with higher pressure. One may find it helpful to raise and lower the syringe to achieve a proper base pressure. During cumulus removal, the operator should not create high enough pressure to achieve pinching of the zona. A pressure that retains the zygote at the port without causing a pinch should be a good starting point for a base pressure during cumulus removal.
8. A constant pressure is inefficient to achieve cumulus removal due to the high pressure required. High pressures will likely be detrimental to the zygote. A more

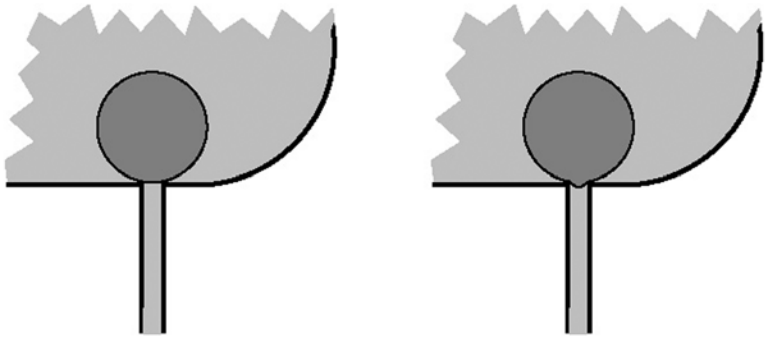

Fig. 3. When performing cumulus removal, the pressure should not be great enough to cause "pinching" of the zona. **(Left)** A good base pressure retains the embryo without deformation. **(Right)** When the base pressure becomes too high, the zona begins to deform as it is pulled into the removal port

efficient method is to use pressure fluctuations during cumulus removal. One should first draw slightly on the syringe to create a proper base pressure to hold the COC at the removal port (*see* **Note 5**). With the negative-base pressure holding the COC at the removal port, tap on the syringe to achieve pressure fluctuations necessary to continue cumulus removal. The authors recommend holding the syringe lightly between the thumb and forefinger near the rear end of the syringe casing. The operator should quickly tap on the forward end of the syringe casing alternately with the first and second fingers of the other hand. The pressure fluctuations can be adjusted by the firmness of the grip on the syringe, the force of tapping, and the area of tapping (e.g., where the syringe is tapped along the casing). Bulk cumulus can typically be removed with light tapping, whereas slightly harder tapping may be required for cumulus nearer the zygote (depending on timing of cumulus removal).

To achieve cumulus removal completely around the zygote, it is necessary to rotate the zygote. Rotation can be achieved by a harder tap, which allows the zygote to be briefly released from the removal port. When the zygote flows back, it will typically rotate slightly, allowing cumulus to be removed from another area of the zygote.

9. When processing the zygotes, it may be harmful to keep zygotes in holding media too long. The authors have typically transferred the cumulus-free zygotes into culture drops every 30 min.
10. As the operator becomes more efficient at cumulus removal, multiple COCs can be processed at one time. The authors find it useful to place 2–3 COCs in the device and process them at once. Once the cumulus is removed from one zygote, that zygote can be held at one port while the other port is used to continue cumulus removal. Alternatively, cumulus can be removed from multiple zygotes using a single port. By tapping more firmly on the syringe, larger pressure fluctuations

Fig. 4. Cumulus can be removed from multiple zygotes at a single port. Firm tapping allows one zygote to be released and a second zygote rotated to the removal port.

can be created that allow multiple zygotes to take turns at a single removal port (**Fig. 4**). This occurs when one zygote rotates away from the port while another zygote moves toward the port.

11. Multiple bubbles in the device at the outset are to be expected. It is likely that no bubbles will initially inhibit device performance. During use, bubbles will grow. If the device is used longer than 20 min, bubbles will likely have to be removed for continued device use. The operator will probably first encounter this problem near the channel entrance at the bottom of the funnel well (**Fig. 2A**). A second problem area could be between the two removal ports (**Fig 2B**). Bubbles here typically take 1 h or more to develop and can be partially sucked into the removal port when they get too large.

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23

Mammalian Embryo Culture in a Microfluidic Device

Eric M. Walters, Sherrie G. Clark, David J. Beebe, and Matthew B. Wheeler

1. Introduction

Over the last decade, the use of in vitro production of mammalian embryos and the utilization of assisted reproductive technologies (ART), such as nonsurgical embryo transfer, cryopreservation, and intracytoplasmic sperm injection (ICSI), has increased. However, the efficiencies of ART remain low. Currently, millions of couples in the United States are affected by infertility and seek treatment. More than 16,000 babies in the United States, each year are born using ART such as in vitro fertilization (IVF), assisted hatching, and ICSI ***(1)***. A critical area for ART is culturing embryos and evaluating their morphology. There have been some advances made in embryo culture medium; however, the efficiency of embryo culture remains low. Additionally, embryo-handling techniques and tools for culture and evaluation have not changed for several years. The growth of miniaturization technologies toward miniature mechanical and fluidic systems has created opportunities for fresh examination of basic embryo physiology. There are many existing limitations in mammalian embryo-handling procedures that may be addressed with these new miniaturization technologies.

A new miniaturized technology called the Micro Embryo Culture Chip (MECC), which has been shown to improve in vitro production of mammalian embryos, was recently developed ***(2–7)***. The MECC represents a significant paradigm shift from the traditional microdrop culture systems. There are several advantages to using the MECC for embryo culture. First, the MECC provides a microenvironment that is more in vivo-like. If the embryo-to-medium

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volume ratio is compared in the traditional microdrop system, the ratio is larger when contrasted to the estimated ratio in the oviduct. In the MECC, the embryo-to-volume ratio more closely mimics the oviductal environment. Also, the MECC reduces stress to the embryo by reducing handling. During the traditional in vitro production, mammalian embryos can be exposed to more than 20 different washes and culture drops. Each wash can impose stress on the embryos by temperature changes, pH, osmolarity, and chemical composition, as well as potentially exposing the embryos to human error. In the MECC, the embryo remains stationary, whereas the medium or chemical composition can be gradually changed—a situation that more closely mimics the conditions the embryo experiences in vivo.

The MECC (**Fig. 1**) is designed with a single channel (250 × 1000 μm) with a constriction region in the mid-point of the channel. The constriction region allows the embryos to "park" in this region while retaining the ability to flow medium past the embryos to a collection or outlet well. The MECC contains a funnel feature that facilitates loading and unloading of the embryos.

In summary, the MECC is a new tool that may lead to improvements in the understanding of basic embryo physiology as well as improved efficiencies of ART.

1.1. Structures of MECC

1. Inlet well: Serves as a reservoir for medium and is the port through which the embryos and medium are loaded into the device.
2. Inlet funnel: Guides embryos directly into the microchannel for easy loading and unloading.
3. Inlet channel: Embryos travel down the inlet channel until they come to rest at the constriction region.
4. Constriction region: a narrowing of the channel that holds embryos/oocytes in place while still allowing media to flow though to the outlet.
5. Outlet channel: only medium is present in this part of the device, which leads to the outlet reservoir.
6. Outlet luer: The Luer serves a dual function, providing a reservoir for downstream medium, and also to being an airtight Luer connector for a syringe when suction/pressure is needed in the channel.
7. PDMS: Polydimethylsiloxane (PDMS): is material from which the top of the device is made.

2. Materials

All chemicals are purchased from Sigma Chemical Company (St. Louis, MO) unless otherwise stated.

1. MECC (Vitae LLC, Madison, WI). Currently, the devices are not in commercial production. Please contact Vitae LLC (608-222-1908 or http://www.vitaellc.com) or Penetrating Innovations (608-845-3270 or http://wwww.pi.ivf.com) for availability of this product.

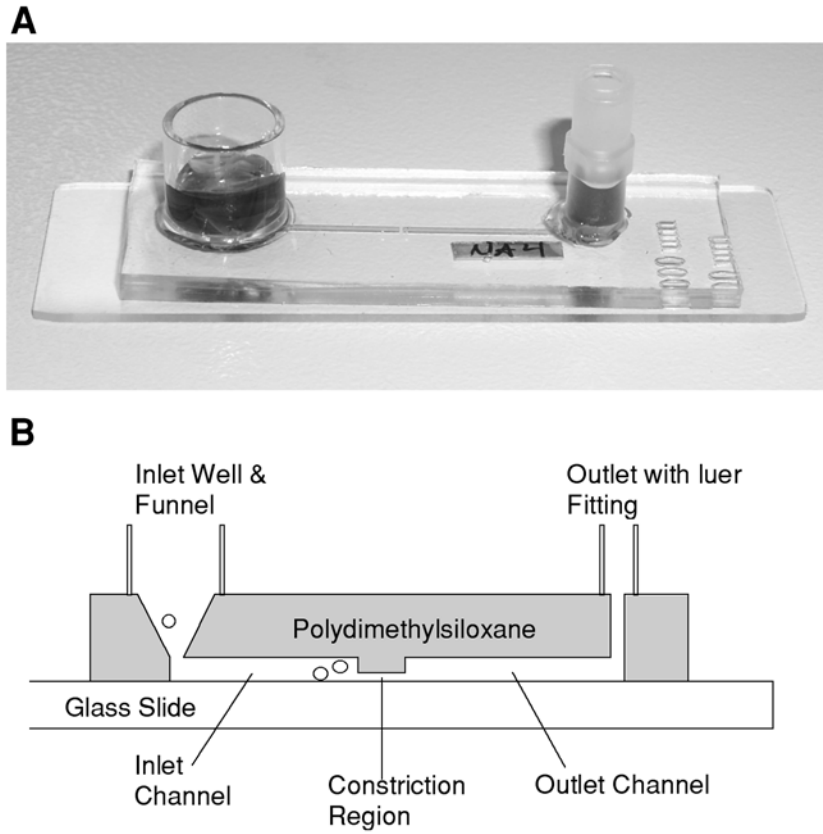

Fig. 1. The physical characteristics of the MECC. **(A)** A photograph of MECC filled with medium. **(B)**. A schematic diagram illustrating the various structures of the MECC. (Illustration courtesy of Kathryn Haubert, Vitae, LLC, Madison, WI.)

2. Incubator capable of producing 5% carbon dioxide (CO_2) in atmosphere (Fisher Scientific Company, Pittsburgh, PA).
3. Embryo-handling pipet
4. 100-mm Falcon 3003 dishes (Fisher Scientific Company).
5. 1 × 3-inch Microscope slides (Fisher Scientific Company, Pittsburgh, PA).
6. 1-cc syringe (Fisher Scientific Company).
7. M2 medium supplemented with 0.4% BSA (fraction V) and M16 medium supplemented with 0.8% bovine serum albumin (BSA) (fraction V) used for mouse embryo culture *(7)*.
8. Synthetic oviductal fluid (SOF *[8]*), Specialty Media, Phillipsburg, NJ) used for bovine embryo culture.
9. North Carolina State University-23 medium *(9)* used for porcine embryo culture. This can be purchased from Cook Veterinary Supplies, Queensland, Australia.
10. Dual Peel sterilizing film (Fisher Scientific Company).

11. Mineral oil (embryo tested)
12. P1000 pipetman and sterile P1000 tips (Rainin, Woburn, MA).
13. Stereomicroscope (Nikon Corporation, Tokyo, Japan).
14. 15-mL conical tube (Fisher Scientific Company).

3. Methods

3.1. Inspection and Sterilization

1. Inspect the MECC microchannel to make sure there are no obvious obstructions, debris, or other problems.
2. Place individual microchannel into Dual Peel, heat-sealed at both ends (Fisher Scientific), and autoclave at 121°C for 30 min, and then dry for 20 min.

3.2. Preparation of the MECC

1. Transfer-sterilize the MECC into the lid of a Falcon 3003 Tissue Culture Dish (Fisher Scientific), covering it with the bottom of the dish.
2. Place the MECC into a CO_2 incubator for a minimum of 1.5–2 h before filling with medium. In addition, the medium to be used must be equilibrated to the gas (5% CO_2 in air typically) and temperature (37°C typically but dependent on species) by incubation in a loosely capped 15-mL conical tube for a similar amount of time before it is loaded into the channels. This preheating and gassing of the channel and medium dramatically reduces bubble formation when the medium is loaded (*see* **Note 1**).
3. After the equilibration period, pipet approx 500 µL of warm medium into the loading well of the MECC. Attach a 1-mL syringe to the outlet Luer, and slowly draw the medium through the channel until the height of the medium in the inlet and outlet ends is approximately equal. If large bubbles (more than one-fourth the width of the channel) are visible, try to displace them by pushing and pulling the medium back and forth in the channel. Tiny bubbles will likely be absorbed, so temporarily ignore this. Disconnect the syringe, and put the filled channel back into the incubator. Let it equilibrate for a minimum of 1.5–2 h before loading embryos (*see* **Note 1**)
4. After the medium has equilibrated in the channel, it is very important to check for bubbles before loading embryos.

3.3. Handling of Bubbles in the MECC

1. Determine the size and location of the bubble(s):
   a. If the bubble is small (**Fig. 2**), determine if it will interfere with movement of the cells placed into the channels (*see* **Note 2**).
   b. If the bubble is large (**Fig. 2**), determine the mobility of the bubble (i.e., can it be pushed or pulled out of its current position?)

3.3.1. Bubble(S) Occluding the Funnel Entry to the Channel and Bubble-Embryo Interactions

1. Use a long micropipet or pulled pipet tips and remove the bubble.

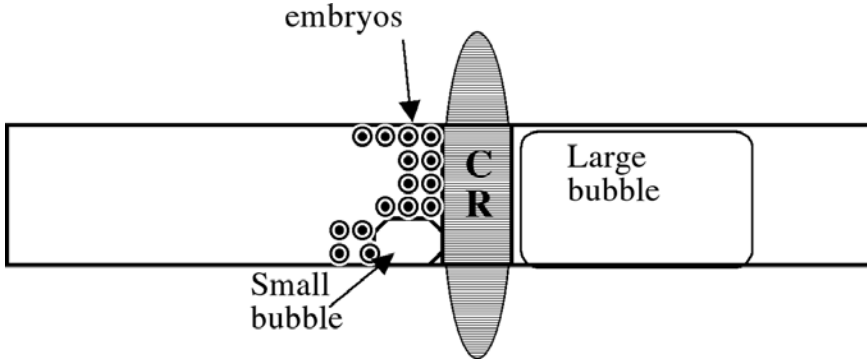

Fig. 2. Embryos near the constriction region (CR) with a bubble anterior and posterior to the CR.

2. Longer pipettes allow pressure to be applied, which should move or pull the bubble out of the funnel opening without contamination of the equipment owing to excess medium being pulled into the micropipet.
3. Pulled pipets with smaller diameter allow the pipet tip to be placed along side of the bubble in the bottom of the funnel and the bubble "plucked" from its occluding position. The bubble will then be allowed to float to the surface of the medium in the funnel and removed by the micropipet.
4. Use a push–pull method to agitate the embryos off of their attachment/contact site (**Fig. 2**, *see* **Note 3**).

3.4. Loading the MECC

1. Using traditional embryo-handling techniques, load the embryos approximately halfway down into the funnel. In general, slightly elevating the funnel end of the device is sufficient for embryos to pass through the funnel, into the channel, and toward the constriction region. Gentle tapping may also be necessary (*see* **Note 4**).
2. Loaded channels must be kept at a slight incline (raise the funnel end of the MECC 4–5 mm higher than the other; this can be accomplished by resting one end on a stack of 4–5 microscope slides) to prevent embryos from migrating back toward the funnel during incubation.
3. Cover the medium in the inlet and outlet wells with enough mineral oil to prevent evaporation.

3.5. Unloading the MECC

1. On the day of recovery, tilt the outlet well above the inlet well. The embryos will migrate back to the funnel region into the reservoir of medium. This should be done under a stereomicroscope to observe when the embryos reach the funnel region.
2. Once in the funnel region, lay the MECC flat. Using traditional micropipetting, pick up the embryos before they settle back into the channel (*see* **Note 5**).

4. Notes

1. Humidity in the incubator must be close to 100% to prevent bubble formation and minimize growth of existing bubbles. Bubbles are not problematic if channels and medium are properly preequilibrated and the humidity level is correct. This requires checking the water level in the incubator on a regular basis. If problems with bubbles persist, an additional small-humidified chamber may be required to incubate the MECC.
2. Checking the microchannel for bubbles prior to embryo loading allows the bubble to be extracted in an easier manner.
3. The push–pull method use a 1-mL syringe attached to the Luer fitting. Depending on the location of the bubble before (push method) or after (pull method) the CR determines which method is used for extraction of the bubbles. The push-pull method needs to be done with care if the microchannel is loaded with embryos. Checking the microchannel prior to loading of embryos allows for easier bubble extraction from the microchannel.
4. Preparation, loading, and unloading are the same for murine, porcine, and bovine. The only difference between species is the culture medium; e.g., M16 for mouse, NCSU-23 for porcine, and SOF for bovine. Gravity will help position the embryos in the desired location during loading and unloading.
5. Patience is needed to acquire each and every embryo, whether it is one or more at once depending on the number that appear in the funnel area for retrieval.

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Application of Alginate Gels to the Study of Mammalian Development

Richard L. Gardner

1. Introduction

Alginic acid is a linear polysaccharide of approx 240 kDa present in the cell walls of the fronds of various seaweeds, including the giant brown kelp (*Macrocystis pyrifera*), horsetail kelp (*Laminaria digitalis*), and sugar kelp (*L. saccharina*). It is a hydrophilic colloidal polyuronic acid consisting of β-(1→4)-D-mannuronic acid and α-(1→4)-L-guluronic acid residues with composition varying according to both the age and species of alga. It is capable of absorbing 200–300 times its weight of water in which it has only very limited solubility. The sodium salt of alginic acid, referred to as sodium alginate, is a cream-colored powder that dissolves in aqueous media to form a viscous colloidal solution. Upon addition of calcium, the saccharide chains of sodium alginate crosslink to form a stable gel through the displacement of two monovalent sodium ions by this divalent cation. Aside from its many industrial applications, including giving ice cream a creamy texture and preventing crystalization, it has also been employed widely both clinically in the dressing and repair of wounds, and in basic biomedical research. Encapsulation of cells or tissue for transplantation and achieving controlled release of drugs, growth factors, and other molecules are prominent among the ways it has been exploited in these latter contexts.

1.1. Advantages of Alginate Gels

Sodium alginate has two obvious advantages over agar as a gelling medium. First, its gelation is not temperature-dependent. Because agar gelates at 35–36°C after obligatory heating to at least 85°C , there is only a small margin

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between failure of satisfactory encapsulation and overheating the cells. The second advantage is that its gelated state can be reversed simply by the use of calcium chelators such as sodium citrate or EDTA. Since, for reversing its gelation, agar needs to be heated nearly to boiling temperature, the only practical way of releasing live material from gels is by physical dissection ***(1)***. A further virtue of alginate gels is that they degrade spontaneously in vivo at a rate that can be controlled by manipulating the concentration of sodium alginate and calcium ions ***(2)***.

However, encapsulation in sodium alginate does entail exposing conceptuses for several minutes to much higher than normal calcium concentrations. Commonly, calcium chloride is present in the gelating solution at 1.5% which, depending on the hydration state of its source, may be as much as 60-fold higher than the normal extracellular calcium concentration ***(3)***. Yet, no adverse effect on development to the blastocyst-stage of mouse two-cell conceptuses encapsulated in alginate using 1.5% calcium chloride was found, regardless of whether the *Zona pellucida* (ZP) was left on or removed ***(2,4,5)***. Moreover, eight-cell stage conceptuses encapsulated using 3% high viscosity sodium alginate yielded fertile adult mice at a similar frequency to non-encapsulated controls ***(4)***. Parenthetically, the very poor pregnancy rates reported in this study are likely attributable to superovulation of the recipients and their having been the same postcoital age as the donors, rather than 1 d behind them. Given the very large differential between intra- and extracellular concentration of calcium that obtains normally ***(3)***, it seems unlikely that a further marked increase of relatively short duration would have lasting consequences. Early studies showed that *Amoeba proteus* could survive continuous immersion in 100 m*M* calcium chloride for more than 1 d ***(6,7)***.

1.2. Terminology

The starting material from which gels are made is normally sodium alginate. Being a variable mixture of both sodium and calcium salts of alginic acid, the gels are referred to as "alginate" gels.

1.3. Embryological Applications

So far, alginate gels seem to have been used in both amphibian and mammalian embryology simply to protect embryos from damage following disruption to, or removal of, their investing membranes ***(2,4,5,8-11)***. A specific problem in mammals is that precompaction cleavage stages will not develop in the oviduct if the ZP is absent or has obviously been damaged (reviewed in **ref. *12***). This poses particular difficulty for testing the developmental potential of manipulated early cleavage-stage conceptuses or isolated blastomeres under optimal conditions in vivo. An early solution to this problem was to encapsulate

the conceptuses or blastomeres in gelated agar ***(13)*** or, in one instance, in gelatin ***(14)***. Later, alginate was introduced as an alternative ***(4)***. Alginate has also been used instead of agar for the double-embedding of very small or delicate objects for histology ***(15)*** or electron microscopy ***(16)***, further ways in which it could be of value embryologically.

Rather than simply making good damage to the ZP or its loss, alginate gels can aid the study of mammalian development more generally. For example, they enable conceptuses to be cultured through cleavage in a fixed orientation. This is particularly the case with denuded two-cell stages because the presence of a single deep interblastomeric groove into which the sodium alginate can readily penetrate means that conceptuses can be held very effectively once gelation has been induced. Moreover, so long as attention is paid to the concentration of the sodium alginate, the gel can provide an enduring imprint of the first-cleavage plane without obviously impairing morphogenesis to the blastocyst stage (***17*** and **Fig. 1**).

Contrary to expectation, the mouse conceptus has been found to undergo little or no rotation within its ZP during development in culture from the two-cell to the blastocyst stage ***(17)***. This has been exploited to achieve strictly non-invasive marking of features of the early conceptus by injecting small drops of mineral oil into the core of the ZP immediately overlying them. This strategy has revealed the hitherto unsuspected existence of a consistent topographical relationship between the blastocyst and two-cell stage in normal mouse development ***(17)***. However, between the early zygote stage and completion of first-cleavage, substantial net rotation within the ZP is the rule rather than the exception (Davies, T. J. and Gardner, R. L., unpublished observations). Thus, by the early two-cell stage the 2nd polar body is often remote from oil drops in the ZP that were used to mark its location in the early zygote, notwithstanding evidence that this body remains tethered to the conceptus for as long as it survives ***(18)***. The postfertilization ZP has been found to be freely permeable to linear polymers of up to 110 kDa in the mouse ***(19)***. Therefore, providing it is of relatively low viscosity (**Table 1**), sodium alginate can readily penetrate through the intact ZP. This penetration allows gelation of the perivitelline space, thus preventing the conceptus from rotating in the ZP, which can then be used for oil-drop marking. This approach has been used both to mark the site of sperm entry to check its relationship to the plane of first cleavage ***(20)***, and to prevent blastomere rotation during second cleavage ***(21)***. Encapsulation in alginate has also been used recently to facilitate the mass production of embryoid bodies from embryonic stem (ES) cells ***(22)***.

Therefore, the ways in which alginate gels have been used thus far for studying aspects of early mammalian development are rather limited. However, their applications in this field can be expected to increase once their value becomes

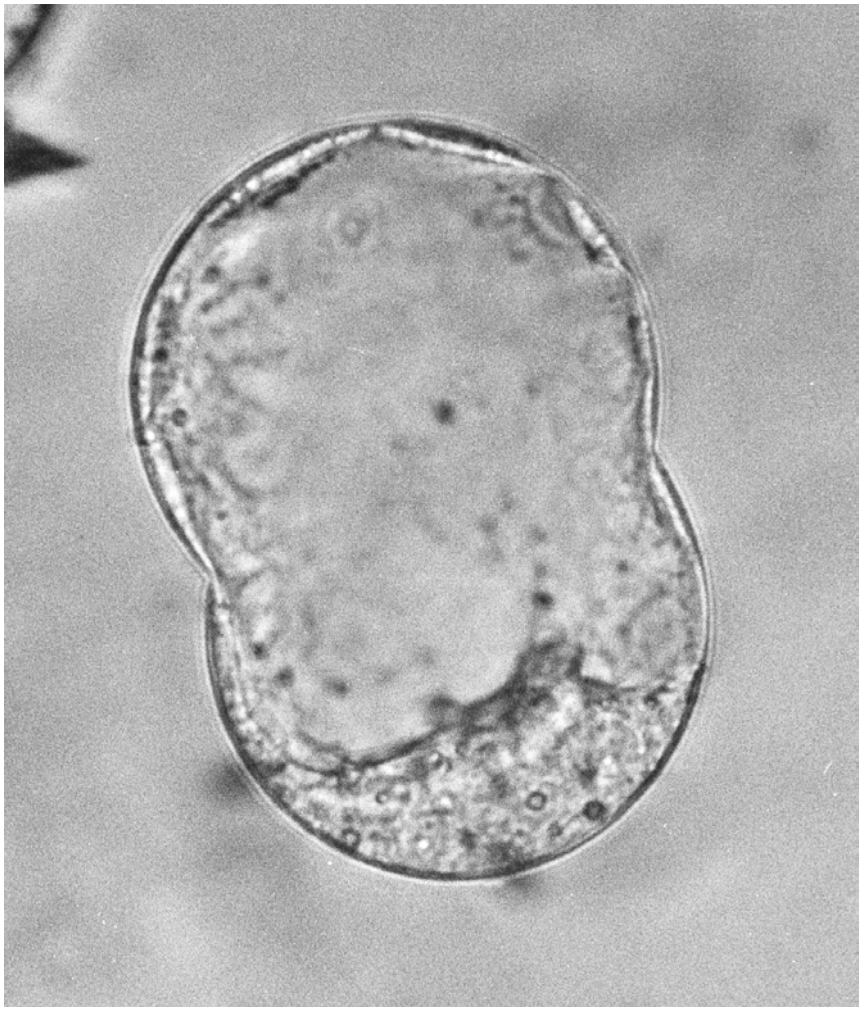

Fig. 1. Blastocyst developed from conceptus that was embedded in alginate at the two-cell stage with a persisting slight constriction marking the plane of first cleavage.

more widely appreciated. Details are provided regarding the use of these gels for two specific purposes. The first is to enable conceptuses to be cultured in fixed orientation following their denudation, and the second is to prevent their rotation within an intact ZP. It is important to note that these approaches were devised without prior experience of alginate gels, so there is unquestionably scope for refining and extending the protocols. It is to be hoped that this will be aided by others choosing to adopt alginate gels in their embryological studies.

Table 1
Details of the Three Types of Sodium Salt of Alginic Acid Available from Sigma-Aldrich

Cat. no.	Description	Approximate viscosity in centipoises	Molecular weight range*
A7128	High viscosity	14,000	120,000–190,000
A2033	Medium viscosity	3,500	80,000–120,000
A2158	Low viscosity	250	12,000–80,000

*Information provided by Sigma technical services

2. Materials

1. Sodium alginate powder: (Sigma-Aldrich) in three different viscosities, the details of which are presented in **Table 1**. (*see* **Note** 1).
2. Culture medium: CO_2-buffered medium that supports complete preimplantation development of mouse conceptuses.
3. Handling medium: a HEPES- or phosphate-buffered variant of culture medium for recovery and handling of conceptuses at room temperature.
4. Acidified Tyrode's saline: standard Tyrode's saline containing 10 kDa polyvinyl-pyrrolidone (PVP) at 400 mg/100 mL, which is acidified before use by titration against 1 *N* HCl until the pH falls below 3.0. (approx 25 µL HCl/10 mL saline).
5. Gelating solution: 0.9% (w/v) NaCl plus 1.5% $CaCl_2{\cdot}2H_2O$ made up in analar-grade water. For more gentle gelation, dilute 1/9 with handling medium.
6. Solating solution: Dulbecco A PBS containing 10 kDa PVP at 4 mg/mL and 1 mg/mL EDTA.
7. Sterile 60- or 30-mm disposable bacteriological dishes.
8. Non-toxic light mineral oil for overlaying culture drops.
9. Sterile-plugged Pasteur pipets.
10. Disposable syringes and corresponding 0.22-µm filters.

3. Methods

3.1. Embedding of Denuded Conceptuses for Culture in Fixed Orientation

1. Add low-viscosity sodium alginate to culture medium to achieve a final concentration of 0.6–0.7% (w/v).
2. Incubate the medium with agitation, preferably at 37°C, until the sodium alginate is fully dissolved (*see* **Note 2**).
3. Draw the solution into a syringe, and sterilize by passing through a 0.22-µm disposable filter.
4. Dispense the sterilized sodium alginate-containing medium as approx 15 µL drops in plastic bacteriological grade culture dishes, and immediately cover with light mineral oil.

5. Incubate the dishes at 37°C in an atmosphere of 5% CO_2 in air for equilibration before transferring zygotes or conceptuses to the drops.
6. Expose zygotes or conceptuses to warmed acidified Tyrode's saline and, when the ZP has almost disappeared, quickly transfer to handling medium.
7. Transfer zygotes or conceptuses sequentially through two drops of the equilibrated sodium alginate-containing culture medium before placing them individually in a third drop for culture.
8. Culture for 30–60 min before flooding the drops with gelating solution.
9. After exposing the drops to gelating solution for up to 3 min, remove as much of the fluid as possible from around them, and replace with sodium alginate-free culture medium (*see* **Note 3**).
10. Rinse drops three more times by addition and removal of sodium alginate-free culture medium before returning the dish to the incubator for further culture.

3.2. Gelation of the Perivitelline Space

1. Add low-viscosity sodium alginate to culture medium to achieve a final concentration of 1.0–1.1% (w/v).
2. Incubate the medium with agitation, preferably at 37°C, until the sodium alginate is fully dissolved (*see* **Note 2**)
3. Draw the solution into a syringe and sterilise by passing through a 0.22-µm disposable filter.
4. Dispense the sterilized sodium alginate-containing medium as approx 50-µL drops in plastic bacteriological-grade culture dishes, and immediately cover with light mineral oil.
5. Incubate the dishes at 37°C in an atmosphere of 5% CO_2 in air for equilibration before transferring zygotes or conceptuses to the drops.
6. Transfer ZP-intact zygotes or conceptuses sequentially through two drops of the equilibrated sodium alginate-containing culture medium before placing them in a third drop in groups of 5–6 for culture.
7. Culture for 75–90 min.
8. Remove zygotes or conceptuses from culture, and rinse them free of external sodium alginate by repeated sedimentation for up to 1 min in handling medium before transferring them to gelating solution.
9. Leave in gelating solution for 20–25 min.
10. Use gentle pipetting to free any zygotes or conceptuses that are enmeshed in gel (*see* **Note 4**).
11. Rinse in handling medium to remove excess calcium before further manipulation or incubation in sodium alginate-free culture medium.

3.3. Solation of Gel (see Note 5)

1. Incubate conceptuses for 15 min at room temperature in solating solution.

3.4. Conclusions

Alginate offers a versatile non-toxic way of embedding or encapsulating entire living conceptuses or parts thereof for studying developmental processes under conditions in which normal three-dimensional architecture is maintained. Therefore, it is surprising that it has so far not been used more widely in developmental biology, particularly in view of its obvious advantages over agar, and the fact that many applications have been found for alginate in other fields of research.

4. Notes

1. In the study in which live young were obtained from encapsulated eight-cell mouse conceptuses, the gel was prepared from a 3% solution of high-viscosity sodium alginate ***(2)***. However, encapsulation was done with the ZP intact, and not only were the conceptuses exposed only briefly to the solution before it was gelated, but the molecular weight range of sodium alginate of this viscosity exceeds the permeability limit of the ZP (*see* **Table 1**; ***19***). Therefore, no constraint should have been imposed on the shape of conceptuses through alteration of the perivitelline space. However, for culturing denuded conceptuses in fixed orientation and preventing their rotation in the ZP by gelating the perivitelline space, the gel has to be in direct contact with the surface of the conceptus. Consequently, in both cases it is important to ensure that it is sufficiently malleable to allow changes in shape of the conceptus as it develops. Hence, only medium and low viscosity sodium alginate were tested. They were invariably added to KSOM, a medium that was formulated specifically for culturing preimplantation mouse conceptuses from the zygote to blastocyst stage ***(23)***. The handling medium used throughout these studies was a HEPES-buffered variant of KSOM ***(24)***.
   The concentrations of sodium alginate tested ranged from 0.3% to 1.5% (w/v). The low-viscosity preparation was found to be suitable for both the culture of denuded conceptuses and, at somewhat higher concentration, for gelating the pervitelline space.
   The importance of testing a range of sodium alginate concentrations is highlighted by recent experience with encapsulation of ES cells for the mass production of embryo bodies ***(22)***. Gels made from a 1.6% solution of alginate (viscosity not stated) supported development of compact lens-shaped colonies that did not display either obvious cavitation or further differentiation. In contrast, ES cells in gels made from a 1.1% solution could become cystic and exhibit spontaneous contraction.
2. If the sodium alginate powder is simply placed as a lump at the bottom of a tube and the medium poured on top, it will dissolve extremely slowly, even when agitated sufficiently vigorously to make the culture medium froth and thereby

promote protein denaturation. Therefore, to ensure that it dissolves completely and does not do so unduly slowly, it is vital to ensure that the powder is well dispersed before the culture medium is added. Then, with gentle agitation, preferably in conjunction with warming to 37°C it should, depending on concentration, dissolve completely within 2–3 h.

3. Following use of this standard gelating solution ***(4,5)***, a single conceptus lies at the bottom of each gelated drop ***(17)***. However, fixation of the orientation of the conceptus throughout the period of culture is not guaranteed using this procedure. Being immediately adjacent to the lower surface of the gel, conceptuses can escape either partially or completely beneath it. Moreover, pipetting such a high concentration of calcium onto the drop can cause it to develop so deep a concavity during gelation that embedding of the conceptuses can be barely sufficient from above as well as below. In addition, conceptuses will lie in their preferred orientation, which may well differ from the desired one. These difficulties can be circumvented by introducing several modifications to the basic protocol. The first, and most important, is to slow the rate of gelation by diluting the NaCl/$CaCl_2$ solution 10-fold with handling medium. More uniform gelation of the drops can be obtained by preparing cylindrical 3% agar gels tailored to their diameter, which are then thoroughly soaked in the diluted gelation solution before being cut into thin discs. One disc is then held horizontally on top of each drop with forceps until gelation has progressed sufficiently to support its weight. Using the lower calcium concentration, gelation should be allowed to proceed for at least 15 min before rinsing with sodium alginate-free culture medium. If pipetted into the drop near its upper surface shortly before gelation is initiated, the conceptus can be immobilized before it falls to the floor. Although its orientation cannot be readily controlled at this stage, there is scope for doing so after gelation has occurred. Thus, once the gel has fully set, possibly aided via a switch from diluted to full-strength gelating solution, it can be trimmed with a small sterile scalpel blade so that, following repeated rinsing in sodium alginate-free culture medium, the conceptus is oriented appropriately during subsequent culture. One further problem is that the gelated drops can occasionally rotate during subsequent culture ***(17)***. This rotation can be countered by making the original drops of sodium alginate in culture medium oval rather than circular in profile.
4. Immediately on transfer to the elevated calcium solution, conceptuses often become enmeshed in threads of gel as a result of carryover of residual sodium alginate. To avoid damaging these conceptuses, it is important to resist the temptation to free them by pipetting until the process of gelation has been allowed to proceed for at least 20 min can be checked. That gelation of the perivitelline space has been effective in preventing subsequent rotation of the conceptus within its ZP by marking the latter immediately over the second Pb with a drop of mineral oil.
5. If it proves necessary to fix or permeabilize conceptuses, presence of the investing gel can cause them to become wrinkled or otherwise distorted. Therefore, whether dealing with denuded conceptuses embedded in gel or ZP-intact specimens with

only the immediately surrounding perivitelline space gelated, it is advisable to depolymerize the gel before subjecting them to any further treatment. Without PVP in the solating solution, conceptuses tend to be very sticky and thus difficult to handle without losses. Others have depolymerized alginate gels with either isotonic sodium citrate at pH 7.4 ***(25)*** or a buffer consisting of 50 m*M* Na-citrate, 77 m*M* NaCl, and 10 m*M* MOPS, also at pH 7.4 ***(21)***.

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Using TUNEL in Combination with an Active Caspase-3 Immunoassay to Identify Cells Undergoing Apoptosis in Preimplantation Mammalian Embryos

Steven F. Mullen and John K. Critser

1. Introduction

1.1. Overview of Apoptosis

The term *apoptosis* was coined in a now classic paper by Kerr et al. in 1972 ***(1)***, which summarized the morphological features observed in electron micrographs of cells dying by a process that had been previously termed *shrinkage necrosis*. It was noted that these features, such as cell shrinkage, nuclear condensation, and fragmentation, along with cellular blebbing and fragmentation, were seen in various tissues and under physiologically normal circumstances. Some features, such as the maintenance of intact organelles, an apparent healthy cytoplasmic membrane, lack of localized inflammation, and frequent phagocytosis of the cell fragments by neighboring cells, suggested that this phenomenon is a normal part of cell turnover and unassociated with large-scale cell damage. The common features of this phenomenon suggested that it was a process under genetic control. The recognition of cells undergoing removal from tissues during normal mammalian embryo development, and cells known to be deleted during the normal development of the nematode *Caenorhaditis elegans* has confirmed this hypothesis ***(2)***.

There have been several recent reviews on the history of our understanding of cell death ***(3,4)***, and perhaps, not surprisingly, some classic features of apoptosis were described over a century ago. Walther Flemming is one of the

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pioneers of cell biology, and during the late 19th century, he made important contributions to the knowledge of cell division and mitosis ***(5)***. In one of his illustrations beautifully reproduced by Lockshin and Zakeri ***(4)***, the cells of a rabbit ovarian follicle are diagrammed. Several cumulus granulosa cells are drawn with nuclear morphology now known to be a characteristic of apoptosis. After reading these reviews, it becomes clear that cell death is much more complicated than a simple dichotomy of apoptosis vs necrosis, as is often portrayed. However, it is clear that apoptosis is a very common phenomenon not only across cell types, but also across species.

The biochemistry and molecular biology of apoptosis has been studied extensively over the past 15 yr, and many mechanisms responsible for dismantling the cell have been elucidated ***(6)***. The first biochemical hallmark of apoptosis identified was cleavage of DNA that gave a specific ladder-like pattern when run on an agarose gel ***(7,8)***. The endonuclease responsible for this pattern and the mechanism of activation during apoptosis have recently been determined ***(9,10)***. This enzyme cuts the DNA between nucleosomes, which are roughly 180 basepairs apart; the bands on the gel thus being multiples of 180 basepairs. The translocation of phosphatidylserine from the inner leaflet of the cytoplasmic membrane to the outer leaflet is another event that occurs in apoptotic cells ***(11)***. It is generally accepted that this event identifies apoptotic cells and apoptotic bodies to surrounding cells, leading to their engulfment ***(12)***.

The signaling mechanisms for the induction and execution of apoptosis have recently been reviewed ***(13)***. The initiation of an apoptotic response can come from two primary sources: the cell membrane or mitochondria. In the former, membrane-bound receptors of the tumor necrosis factor (TNF) family act to initiate the death response ***(14)***. The activation of these receptors causes self-trimerization and the consequent association of their intracellular death domains (DD). This association recruits intracellular molecules with their own death domains (i.e., Fas-associating protein with death domain [FADD]), that interact with the receptor DD. These molecules propagate the signal downstream, some by additional domains known as death effector domains (DED).

This signal eventually triggers the activation of the principal effectors of apoptotic death—the caspases ***(15)***. Caspases are a family of proteins whose name derives from the presence of a cysteine residue at the active site of the enzyme, and because they are proteases that cleave their substrates specifically after aspartate residues. Caspases are constitutively expressed in cells in the form of zymogens and are activated during the response to apoptotic stimuli. Caspase 8 and 10 are 2 of the 14 caspases described to date and are the principal caspases activated by the death receptors described above. Their activation

eventually leads to the activation of the effector caspases (i.e., Caspase 3) and cleavage of cellular proteins producing the characteristics of apoptotic death ***(16,17)***.

Apoptosis can also be triggered by changes at the mitochondria within cells ***(18,19)***. Stimuli (e.g., oxidants and irradiation) can affect the mitochondria directly without having to interact with surface receptors. The mitochondria responds to this stimuli by releasing cytochrome *c*, which, combined with a protein called APAF-1, dATP, and procaspase 9, forms a complex called the apoptosome ***(20)***. This results in the activation of caspase 9, which then activates caspase 3 and other effector caspases, initiating a cascade of caspase activation. Members of the Bcl-2 family of proteins regulate the release of cytochrome *c* and other apoptosis factors from the mitochondria ***(21)***. Although their precise actions are still controversial, it is clear is that some members of the family are promoters of apoptosis, whereas others are inhibitors.

The final outcome of the caspase cascade is the cleavage of many intracellular proteins, such as pro-IL1β, poly(ADP) ribose polymerase, lamin, actin, as well as other caspases ***(22)***. This protease activity causes the morphological changes described by Kerr et al. and his predecessors.

1.2. Apoptosis in Preimplantation Mammalian Embryos

Investigations of apoptosis in preimplantation mammalian embryos have revealed similarities and surprising anomalies. Results from several studies ***(23–25)*** have shown that insults to other cell types causing extensive apoptosis are less effective in triggering apoptosis in cleavage-stage embryos.

When oocytes and early embryos degenerate, they commonly undergo fragmentation, which is characterized by cytoplasmic blebbing similar to the formation of cellular fragments seen in apoptosis. Some investigators have argued that this is a telltale sign of apoptosis. However, when other markers of apoptosis, such as phosphatidylserine (PS) redistribution and TUNEL labeling, were utilized in one study ***(26)***, the later markers were absent in nearly all of the fragmented embryos. It is possible that the PS transition fails to occur in these cells ***(27)***, which could explain this discrepancy. In another report investigating fragmented cleavage-stage human embryos, Jurisciova et al. (1996) ***(28)*** showed inconclusive evidence linking fragmentation to apoptosis. Of 203 fragmented embryos studied, 24 showed neither nuclear morphology or biochemical evidence for apoptosis. Only 42 of these embryos showed both morphological and biochemical evidence, suggesting that using the morphological criterion of cell fragmentation as an assessor of apoptosis in early embryos may be inappropriate.

Compelling evidence that apoptosis with all of the classic features occurs in blastocyst-stage embryos is frequently seen in in vivo produced blastocysts (reviewed in **refs. *29*,*30***). Nuclear condensation, fragmentation, TUNEL labeling, and even phagocytosis by other embryonic cells have all been shown in these cell stages ***(23*,*31*,*32)***. The in vivo percentage of naturally occurring apoptosis in blastocyst cells is estimated at 2–3%.

Numerous recent publications have shown that several proteins in the caspase and Bcl-2 family are expressed in all stages of preimplantation embryos of the mouse ***(33–35)***. Possibly all the machinery to conduct apoptosis is present from the zygote stage, and there are strong inhibitors preventing the program from being carried out. The results from a very recent report have provided evidence for this hypothesis ***(36)***. By using immunofluorescence, the presence of active caspases was not seen until the embryos reached the morula stage, indicating that the activation of caspases is strongly inhibited in early-stage preimplantation embryos. During the development of this protocol, we made similar observations and have only seen apoptotic cells in morula and balstocyst-stage mouse embryos using these methods.

These apparent anomalies make this a fascinating field of study, and new insights into the control of apoptosis can be potentially gleaned by using preimplantation embryos as model cells.

The assay described in this chapter on embryos uses TUNEL labeling ***(37)*** and immunocytochemistry to detect DNA fragmentation and the active form of caspase 3 to identify cells undergoing apoptosis. Owing to the challenges associated with identifying apoptotic cells as previously described, particularly in preimplantation embryos, using multiple characteristics of apoptosis to identify cells dying through this process seems prudent. Because DNA fragmentation through caspase-activated DNase (CAD) relies on caspase-3 activation ***(9*,*10*, *38)***; only cells undergoing apoptosis should score positive for both tests. Coupling these two assays will provide greater confidence in scoring apoptotic cells.

2. Materials

2.1. Embryo Fixation

1. 35-mm Petri dishes.
2. 4% Formalin fixative solution in PBS, pH 7.4. Make fresh and store at 4°C protected from light until use. This material is very toxic; handle with care. To prepare this solution, dissolve 4 g paraformaldehyde in 100 mL PBS in a 250-mL beaker. Have the solution on a hot plate during preparation with the temperature near 60°C to facilitate dissolution. Once the paraformaldehyde has dissolved, place the beaker in ice and allow it to cool to room temperature. Adjust the pH of the solution to 7.4.

3. PBS with 1% Triton X-100. Add 1 mL Triton X-100 to 99 mL PBS in a glass bottle. Stir until Triton X-100 has dissolved.
4. TL-HEPES medium: 114 m*M* NaCl, 3.2 m*M* KCl, 2.0 m*M* $NaHCO_3$, 0.4 m*M* NaH_2PO_4, 10.0 m*M* Na-lactate, 10.0 m*M* HEPES, 0.1 m*M* Na-pyruvate, 50,000 U penicillin/L, 50 mg streptomycin/L, 2.0 m*M* $CaCl_2$, 0.5 m*M* $MgCl_2$, 1 mL 0.5% phenol red solution (*see* **Note 1**).
5. Embryo manipulation pipets.

2.2. TUNEL Labeling

1. Death Detection Kit-Fluoroscein (Roche, cat. no. 1684795; *see* **Note 2**).
2. TUNEL termination buffer: 300m*M* NaCl, 30 m*M* Na-citrate.

2.3. Active Caspase-3 Immunochemistry

1. Immunochemistry blocking buffer: PBS + 0.2% (v/v) Triton X-100, 3 m*M* sodium azide, 0.1% saponin (w/v), 130 m*M* glycine, 2% bovine serum albumin (BSA) (w/v), and 5% horse or donkey serum (v/v) (*see* **Note 3**).
2. Affinity-purified rabbit IgG monoclonal antibody to active caspase 3 R&D systems, cat. no. AF835 (*see* **Notes 4** and **5**).
3. Anti-rabbit secondary antibody labeled with fluorophore of choice. We have had good experience with donkey-anti-rabbit with Texas Red ® (Jackson Immunoresearch; *see* **Notes 5** and **6**).

2.4. Mounting Embryos on Slides

1. Microscope slides.
2. Microscope slide coverslips no. 1.5 (*see* **Note 7**).
3. DAPI DNA stain (*see* **Note 8**).
4. ProLong antifade reagent (Molecular Probes, cat. no. P7418; *see* **Note 9**).
5. Waxy material for mounting coverslips on slides to prevent the coverslips from squishing the cells upon mounting: 50% petroleum jelly and 50% paraffin wax (*see* **Note 10**).
6. Clear nail polish.

3. Methods

3.1. Embryo Fixation

All treatments described here are performed at room temperature unless otherwise noted.

1. Pipet approx 2 mL 4% formalin into a 35-mm Petri dish. Fix the cells by placing them into the formalin solution with a transfer pipet for 60 min and cover the dish with foil to protect it from light (*see* **Note 11**).
2. Evacuate the transfer pipet of all solution (*see* **Note 12**).
3. After the fixation, transfer the embryos to approx 2 mL PBS plus 1% Triton X-100 in a 35-mm dish and hold for at least 1 h at room temperature or overnight at 4°C. (*see* **Note 13**).

4. Transfer the cells to TL-HEPES at 4°C for extended periods if desired (*see* **Note 14**).

3.2. TUNEL Labeling

1. Make up 50 µL of TUNEL reaction mix from the death detection kit per treatment group. Remove one vial of tube 1 and tube 2 from the kit and place them on ice. Allow the solutions to thaw. For each treatment, remove 45 µL from tube 2 (labeling solution), and put this into a centrifuge tube and return to the ice. To this, add 5 µL of the enzyme solution (tube 1) and mix well. Put the tube into a 37°C water bath to bring to the reaction temperature (1 min should suffice). Transfer the 50-µL volume to a 35-mm Petri dish and transfer the cells to this drop. Put the dishes into a humidified incubator at 37°C for 1 h (*see* **Note 15**).
2. Remove the dishes from the incubator and transfer the cells to 2 mL TUNEL termination buffer at room temperature in a 35-mm Petri dish and incubate for 15 min. Protect the cells from light as much as possible from here through the end of the procedure (*see* **Note 16**).
3. Transfer the cells to 2 mL immunochemistry blocking buffer in a 35-mm Petri dish and hold for at least 3 h or longer if desired. If longer storage is required, keep the cells at 4°C and periodically check to ensure that the fluid does not completely evaporate.

3.3 Active Caspase-3 Immunochemistry

1. Dilute the primary antibody aliquot with 1.5 mL blocking buffer for the final working concentration. Always keep the stock solution of antibody on ice. Pipet this solution into a 35-mm Petri dish (*see* **Notes 4** and **5**).
2. Transfer the cells to this dish.
3. Maintain the cells in this solution for 1 h at room temperature under foil. If desired, the cells may be held in this solution overnight at 4°C (*see* **Note** 5).
4. Transfer the cells to a 35-mm dish containing 1.5–2 mL of blocking buffer as a wash. They should be held in this solution for at least 1 h under foil. They may be washed for a few hours or overnight at 4°C if desired.
5. Dilute the secondary antibody stock solution 1:1500 in blocking buffer. Remove 1 µL from the stock solution and add to 1.5 mL blocking buffer in a microcentrifuge tube, mix, and add this solution to a 35-mm Petri dish (*see* **Note 6**).
6. Transfer the cells to this solution and maintain the cells in this solution for 1 h at room temperature. Remember to keep the dish covered with foil.
7. Transfer the cells to a 35-mm dish containing 1.5–2 mL of blocking buffer as a wash. Wash for 30 min at room temperature or overnight at 4°C (*see* **Note 6**).

3.4. Mounting Embryos on Slides

After the final wash, prepare to mount the embryos on microscope slides.

1. Gather the microscope slides, no. 1.5 coverslips (*see* **Note 7**), and the "waxy material."

2. Get one each of the two types of tubes from the ProLong Antifade reagent box. The liquid in the clear tube needs to be thawed (*see* **Note 9**), which can be done at room temperature or in a warm water bath. *Carefully* transfer 200 µL of the clear solution to the amber tube. There is a crystalline substance in the bottom of the amber tube. Upon transfer, use the pipet tip to suspend the crystals in the liquid (with the action of a mortar and pestle). Once the crystals are suspended, let the solution sit for at least 15 min to allow the crystals to dissolve. If DNA counterstaining is desired, add 0.75 µL of the DAPI stock solution to the remainder of the liquid in the clear tube. After 15 min, draw the solution in the amber tube into a clear pipet to ensure all of the crystals have dissolved. Return the 200 µL solution from the amber tube to the clear tube. Mix thoroughly but *carefully* with a 1-mL pipet. This solution is very viscous and foams easily. If excessive foaming occurs, centrifuge the tube at maximum speed in a small microcentrifuge for 30 s. Keep this solution capped when not being used.
3. Label the slides with your sample information.
4. Draw up 20 µL antifade reagent into a pipet and set the pipet down on the bench next to you. Transfer the embryos to the microscope slide on a dissecting microscope with as little liquid as possible. Suck up as much of the liquid as possible once the embryos are on the slide. Grab the pipet and watch the remaining solution on the slide evaporate. When it has nearly all evaporated (this will facilitate the cells sticking to the glass), quickly, but carefully, add the 20 µL of the antifade reagent to the top of the embryos. By adding this carefully, the embryos should stay stuck to the glass. Adding more just makes a mess, whereas adding less may create air bubbles in the solution as it dries. Once the cells are covered with the drop, add a small amount of the waxy substance to the corners of a coverslip, and carefully place the coverslip onto the slide (waxy side down) at an angle so two of the corners touch the slide initially. Allow the coverslip to slowly fall onto the drop of antifade reagent. Press down on the corners of the coverslip so that the waxy material flattens out, antifade reagent spreads out evenly under the coverslip, and observe the embryos being compressed slightly. The coverslip will rebound so that the embryos do not remain compressed. Place the slides off to the side, and allow them to dry. Within 15 min, the antifade reagent will be dry enough to observe carefully under a fluorescence microscope. It is recommended to let them dry for at least 1 h before extensive manipulation. We keep them under foil on the bench top during the drying period just to be safe (*see* **Note 17**). Seal the edges of the coverslip with clear nail polish after at least 1 h of drying time.

3.5. Analysis

For cells that are apoptotic, there should be bright green TUNEL staining in the nuclei, and the nuclei are usually broken into small fragments. When the red fluorescence is observed, the cells with the TUNEL staining should also be seen fluorescing red from the caspase-3 immunostaining (*see* **Fig. 1**). It is possible caspase-3 positive cells can be TUNEL-negative, although the probabil-

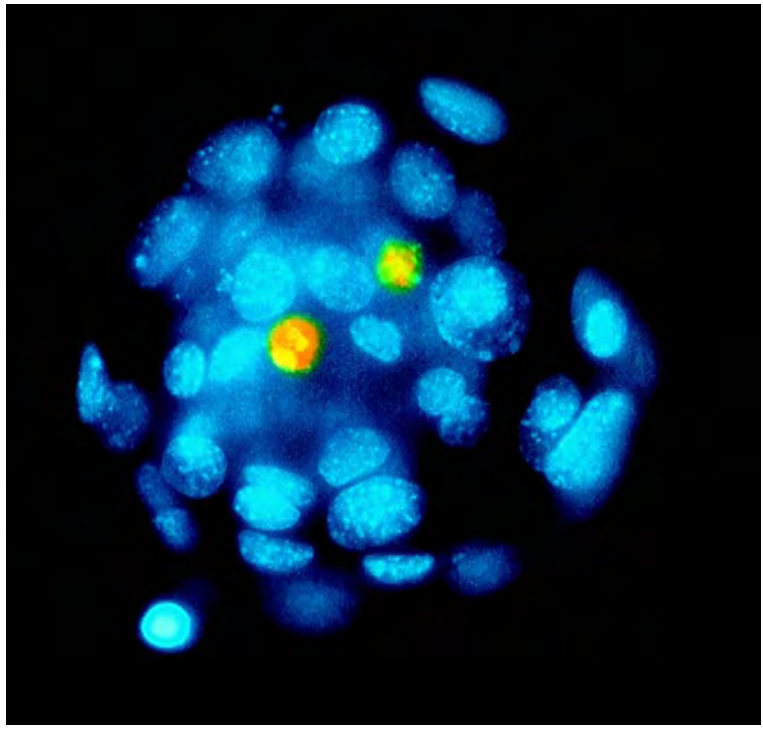

Fig. 1. A mouse embryo to which the apoptosis assay has been applied. Notice the two cells near the center of the embryo showing green TUNEL staining, along with red active caspase-3 staining. Also observe the condensed and fragmented nature of the nuclei these cells. We would score these cells as positive for apoptosis, and the remaining cells would be scored negative.

ity is low. TUNEL positive cells shouldn't be observed with a lack of Caspase-3 staining. However, if the TUNEL-stained nuclei are obviously fragmented, but caspase-3 staining is absent, it is likely that those cells are still undergoing *apoptosis*. Scoring apoptotic cells can often be subjective. The combination of positive TUNEL and caspase-3 staining allows the judgment to be more objective, but each investigator should be aware of the potential pitfalls in making this determination and use their best judgment.

4. Notes

1. TL-HEPES is a commonly used medium for manipulating preimplantation embryos under atmospheric CO_2. After dissolving the components in water, adjust the pH to 7.4 and check the osmolality. It should be near 280 milliosmolal. Sterile-filterize the solution by passing it through a 0.2-µm filter. It is recom-

mended that this solution be stored at 4°C. for no more than 2 wk. To make a working solution, add BSA at a concentration of 150 mg/50 mL and filter this solution as before.

2 There are many TUNEL kits commercially available. We have found the kit from Roche to be easy and robust, but are not suggesting others available cannot perform just as well.

3. Serum from a species closely related to the host of the secondary antibody is important during the blocking step. We use horse serum because we are using a donkey antibody. Donkey serum is available from Jackson Immunoresearch, and either should work well for this application. The blocking buffer we use was derived empirically. Because of the presence of sodium azide, this material is considered toxic and should be appropriately handled and disposed. This buffer should be stored at 4°C. Periodic filtration through a 0.45 μm filter is usually performed owing to protein precipitation over time. We make 1-L vol if we will be using all of it within 1 mo. If longer storage is expected, we make up 1-Lvol of the solutions without the BSA and serum, and then make 200-mL vol working solutions containing BSA and serum from this stock solution.
4. It is best to store the antibodies in frozen aliquots if long-term storage is expected, as multiple freeze-thaw cycles will destroy their activity. Reconstitute the antibody stock solution according to the manufacturer's recommendation of 50 μg in 100 μL. We aliquot ours in 1 μL vol in 1.5-mL microcentrifuge tubes and store them in a –80°C freezer. We resuspend these aliquots in 1.5 mL blocking buffer for the working solution. Although we know of at least two manufacturers that sell antibodies directed at the active form of caspase 3, the antibody from R&D systems has worked very well in our experience.
5. Antibody dilutions and holding times are determined empirically. The concentrations here work well, but different concentrations may work just as well for others. It is important to note that these concentrations were derived for the specific antibodies that we use. If antibodies are obtained from other manufacturers, the required dilutions will likely differ than those discussed here. The holding times are not strict; typically there is a minimum holding time and washing time necessary to obtain saturation of the antigen as well as dilution of any unbound antibody. In our experience, longer washes work better up to a point. With antibodies that have high affinities, overnight washes often maintain a high level of specific staining with minimal nonspecific staining. We have found that overnight incubations also maximize the binding, but 1 h at room temperature is often sufficient. Washes for 1 h may be sufficient for a highly specific antibody when the concentration has been optimized. Essentially, developing immunochemical procedures is more of an art than a science.
6. The secondary antibody comes in the form of a lyophilized powder, and we use the manufacturer's recommendations for its resuspension. Add 400 μL of quality room temperature water (DI or milli-Q) to the powder and gently mix by pipetting. After approx 1 h, draw the solution into a clear pipet to ensure complete dissolution. We either maintain this solution in a refrigerator if expected it

to be used within 2 mo or we freeze aliquots of 50% in glycerol. To do this, add 400 µL glycerol to this solution and aliquot into 0.5-mL tubes in volumes of 10 µL and store at –80. To use this solution, thaw one tube and add the entire contents to 7.5 mL blocking buffer. Add 1.5 to 2 mL of this solution to a 35-mm Petri dish for the final working solution. We use glycerol with these antibodies for long-term storage, but not for the primary antibody, only because that is what the manufacturer recommends. Either way likely works for both antibodies, but assume that the manufacturers have recommended the methods that work best for their products. It may be found that the final wash is being performed at the end of the work day. Washing overnight has been successful in our expeience; putting the dishes in the refrigerator overnight and performing the remaining steps the following day is acceptable. Washing longer than overnight will likely reduce the signal, so we do not recommend performing this assay if at least two full days can not be dedicated to the procedure.

7. Most high power microscope objectives are designed to work with coverslips with a thickness of 0.17 mm, which is equivalent to a no. 1.5. Check the objectives on your microscope to verify this before mounting the cells. Although this sounds trivial, it is important for getting good images. We use 18-mm square coverslips. If larger coverslips are used, use a proportionately larger volume of antifade reagent when mounting the cells.
8. DAPI often comes as a crystalline substance. Dissolve the crystals to make a stock solution at a final concentration of 1 mg per milliliter in water. Remove a 0.75-µL volume and add this to the antifade reagent, mixing thoroughly prior to mounting the embryos.
9. The Prolong Antifade reagent has proven to be an exceptional antifade reagent. We have received positive comments from multiple core facilities on its photostable properties. The kit comes with multiple amber tubes, each containing a crystalline precipitate and two bottles of a transparent solution. We thaw this bottle and aliquot this solution into 1-mL volumes in transparent 1.5-mL microcentrifuge tubes. A tube can then be thawed for each procedure instead of having to repeatedly thaw and freeze the entire solution in the bottle. We use transparent tubes because it is easier to see when the solution has been thoroughly mixed.
10. The "waxy material" is a homemade concoction of half petroleum jelly ("Vaseline") and half paraffin wax. To make the waxy material, take a 500-mL beaker and put it on a hot plate. Add approx 100 mL parrafin wax and approximately an equal volume of petroleum jelly. The wax and jelly can be purchased at any large grocery store. When the wax has fully melted, stir the two together to mix well, and draw the solution into 30 milliliter syringes (without needles). The material will solidify as it cools. The precise ratio of the two materials is not critical, as long as it is solid, but flows when pressure is put on the syringe plunger.
11. Formalin may present a health hazard, particulary when working with embryos where in vitro manipulations are usually performed under a dissecting microscope. If a downdraft fume hood is available, it should be used. Avoid mouth

pipetting during this step. Placing a drop of formalin under mineral oil and transferring the embryos to this drop may work and will prevent exposure to the hazardous vapors of formalin during treatment. However, our laboratory has not tried this procedure.

12. Owing to the hazadous nature of formalin, it is best to ensure that no residual liquid remains in the pipet.
13. As a result of the small volume transferred along with the proteins in the TL-HEPES, extensive washing of the cells after fixation is unnecessary.
14. The fixed embryos can be stored in the TL-HEPES solution at 4°C. We do not keep the dishes in a humidified chamber. Instead, we check on the volume of the liquid and add additional medium if necessary to prevent the all of the medium from evaporating. The holding time can be for several days (if desired). Be sure to check on the volume of the liquid to avoid complete evaporation. The dishes can also be sealed with parafilm and a good precaution if extended storage is expected.
15. The steps for using the Death Detection kit are outlined on the product information sheet. Although one could cover the drop with oil to prevent evaporation, we find this unnecessary. However, be careful to keep the drop small and dome-shaped. If the solution spreads out, it may evaporate. We perform the reaction in our culture incubator with an atmosphere of 5% CO_2 and find that this has no appreciable effect on the reaction. Transfer as little medium to the reaction drop with the embryos as possible. If a large number of embryos are being assayed for each reaction, it may be necessary to increase the reaction volume to accommodate the additional volume of medium transferred with the embryos. This decision is based solely on economics and success of the reaction. It is important to use fluorophores that have different emission spectra for the TUNEL and caspase-3 assays, so the two results can be distinguished. The TUNEL reaction buffer is toxic; thus, standard and safe laboratory practices are required during its handling and disposal.
16. Although this step may not be necessary, we do it as a precaution. After the TUNEL step, the cells should be protected from light as much as practical to prevent photobleaching of the fluorophores.
17. This step requires practice. It may be frustrating initially, but with experience, it will become routine. However, performing it properly, will result in high-quality imaging and less frustration in the long run. We have found that within 2 d the fluorescence signal begins to diminish, especially if the coverslips are not sealed with nail polish. Therefore, we recommend imaging within 2 d. Although we do not store them in a freezer, reports in the literature suggest that this prevents loss of the signal. We recommend imaging with fluorescence microscopy as soon as possible.

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