Chapter 7

Observing Meiosis in Filamentous Fungi: *Sordaria* and *Neurospora*

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Abstract

The filamentous fungi *Neurospora crassa* and *Sordaria macrospora* are materials of choice for recombination studies because each of the DNA strands involved in meiosis can be visually analyzed using spore-color mutants. Well-advanced molecular genetic methodologies have been developed for each of these fungi, and several mutants defective in recombination and/or pairing are available. Moreover, the complete genome sequence of *N. crassa* has made it possible to clone virtually any gene involved in their life cycle. Both fungi provide also a particularly attractive experimental system for cytological analysis of meiosis: stages can be determined independently of chromosomal morphology and their seven chromosomes are easily identified. The techniques for light, immunofluorescence and electron microscopy presented here have been used, with success, for monitoring of chromosome behavior during both meiotic and sporulation processes. They have also proved useful for the analysis of mitochondria and peroxisomes as well as cytoskeleton and spindle pole-body components. Moreover, all techniques of this chapter can be easily applied to other filamentous ascomycetes, including other *Sordaria* and *Neurospora* species as well as *Podospora, Ascobolus, Ascophanus, Fusarium, Neotiella,* and *Aspergillus* species.

Key words: Meiosis, homologous pairing, methods for light and electron microscopy, immunofluorescence, synatonemal complex, fungi, *Neurospora*, *Sordaria*.

1. Introduction

Mycelial fungi like *Sordaria macrospora* and *Neurospora crassa* are particularly well suited to meiosis and genetic studies. First, both have a brief life cycle during which several hundred meiocytes (asci) and the resulting gametes (ascospores) can be analyzed: a Petri dish contains over 1,000 fruiting bodies, each of which contains 150–200 asci. Also, samples can be removed (e.g. for drug treatment) at different times from the culture, in which fruiting bodies develop synchronously, without any apparent

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effect on development of the remaining fruiting bodies. Second, the four products of a single meiosis are held together in a large cell (ascus). Moreover, the four resulting haploid nuclei are arranged in a linear order, which reflects the preceding nuclear/spindle position: this allows easy detection of pre- or post-segregation of allelic pairs (**Fig. 7.1A**). Third, meiosis is followed by a post-meiotic mitosis giving a linear series of eight haploid nuclei that allow determination of the genetic constitution of each of the DNA strands involved in meiosis by visual analysis of the 8-spored asci (including post-meiotic segregations, e.g. 1, 2). The ability to discern the genetic information of each DNA strand at a particular locus has provided considerable insight into recombination mechanisms (reviewed in *1–3)*. Moreover, the eight ripe ascospores remain mostly associated when ejected from fruiting bodies on agar plates. Ascospore-color mutations can be used to recognize recombination events (**Fig. 7.1A**) and to score (and map) the different types of chromosome rearrangements (e.g., *4–6* for respectively *Neurospora* and *Sordaria*). Fourth, all linkage groups are mapped genetically and assigned to cytologically distinguished chromosomes in both species *(5–7)*. Finally, since mutations that

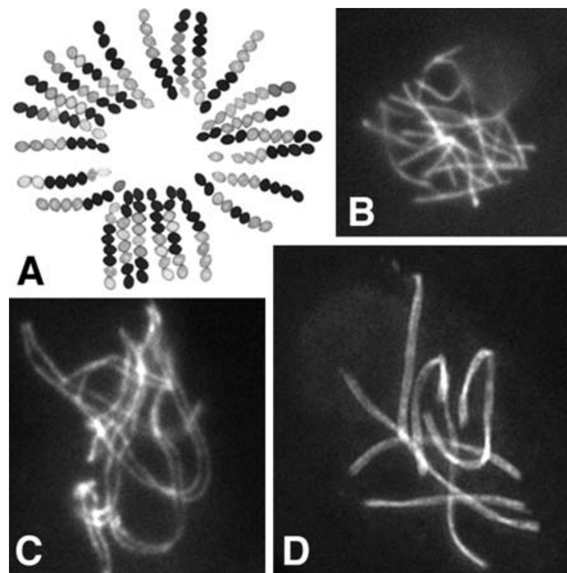

Fig. 7.1. Recombination and meiosis of *Sordaria*. (**A**) Rosette of 8-spored asci showing the 4:4 segregation of black (B) and white (W) ascospores. Asci with 4B and 4 W ascospores imply segregation at first meiotic division without a crossover having occurred between the centromere and the spore-color marker. Crossover between the white gene locus and the centromere results in 2 W:2B:2 W:2B or 2 W:4B:2 W spored-asci (and reciprocal segregations), depending on how homologs segregated to the poles of the spindles during the second meiotic division. (**B**–**D**) Meiotic prophase: chromosome axes are stained with the cohesin-associated protein Spo76/Pds5 tagged with GFP. At early leptotene (**B**) chromosomes are not paired while they are all aligned at late leptotene (**C**), and finally synapsed (**D**).

affect meiosis lead to defects in sporulation due to aneuploid meiotic products (e.g. 8), the presence of abnormal ascospores (generally white when compared to the black spores in wild type) or absence of 8-spored asci on the Petri-dish lid provides an easy screen for potential meiotic mutants (after mutagenizing strains or protoplasts and inducing them to fruit).

S. macrospora is self-fertile (homothallic) and thus able, by self-mating, to form a diploid nucleus homozygous for any induced mutation. Homothallism not only guarantees isogenic genomes but allows one to generate and screen directly for recessive (and dominant) mutants affecting meiosis and to isolate molecularly transformed or tagged genes: the homokaryotic mycelium established from a single haploid nucleus has the potentiality to progress through the dikaryotic stage, meiosis and ascospore formation. For easy genetic analyses, selfing can be prevented by the use of nonallelic self-sterile but cross-fertile mutants (e.g. *6–8)*. *N. crassa*, in contrast, is self-incompatible (heterothallic): mating between homokaryotic mycelia of mating-type *a* and mating-type *A* is required to complete the sexual cycle *(4, 5, 9)*.

Also, *Neurospora* has developed several ways of controlling the integrity of its genome (e.g., inactivation of duplications created by transformation) during both premeiosis [RIP for Repeat-Induced point Mutation (reviewed in *9)*] and meiotic prophase [Silencing by Unpaired DNA or MSUD *(10, 11)*]. *Sordaria*, in contrast, does not exhibit such inactivation, allowing easy introduction of modified or GFP-tagged sequences *(8, 12)*. On the other hand, as RIP extensively mutates endogenous genes with G-C to A-T transitions when a second copy of the gene is introduced to the *Neurospora* genome and the duplication strain is subsequently crossed, RIP provides an interesting tool for in vivo mutagenesis of specific genes of interest, available from related organisms (reviewed in *9)*. Finally, the genome of *N. crassa* (estimated size of 41 Mb) is completely sequenced (www.genome.wi.mit.edu). The genome of *S. macrospora* is not, but the two species being very close, availability of the *Neurospora* genome allows easy primer design to clone *Sordaria* genes of interest (e.g. *8, 12)*.

Both fungi also provide several particularly attractive features for examination of meiotic pairing (**Fig. 7.1B and C**) and synapsis (**Fig. 7.1D**). First, their chromosome number is low (n=7) and chromosomes can be recognized by length and centromere position in electron microscopy (EM) *(6, 7, 12, 13, 18* for *Sordaria*; *14–17, 19* for *Neurospora*). Second, the progression of nuclei through the various stages of meiosis can be monitored independently of chromosome status, by progressive increase in ascus size (from 10 μm just after karyogamy to 60 μm at zygotene, 100 μm at mid-pachytene and 150 μm at diplotene), thus permitting a clear establishment of event time lines in mutant situations in comparison to wild-type meiosis *(*e.g., *8, 12, 18)*. Third, chromosome axes seen by cohesins (e.g. tagged with GFP, **Fig. 7.1B–D**) or

synaptonemal complex (SC) axial elements emerge concomitant with DNA replication and occur all along the lengths of the chromosomes at early prophase, just before appearance of foci of the RecA homolog Rad51, thus very soon after double-strand break formation, which initiates meiotic recombination. This allows an accurate analysis of the alignment and chromosome location and movements during the recognition and juxtaposition processes (*see 7, 12, 13, 18, 20* for *S. macrospora* and *14, 15, 16, 17, 19* for *N. crassa*). Fourth, the volume of the nucleus increases steadily from leptotene to late pachytene; consequently the seven bivalents are widely spaced within the nucleus, allowing easy observation of alignment and synapsis (**Fig. 7.1B–D**). Fifth, contrary to most eukaryotes, the two sets of homologous chromosomes are in separated nuclei before meiosis starts: progression of homologue juxtaposition can therefore be conveniently described with respect to two major landmarks: karyogamy and SC formation. These advantages have, for example, permitted elucidation of the mechanism of presynaptic coalignment *(12)*. Finally, *Sordaria* and *Neurospora* meiotic mutants do not arrest when either recombination or synapsis is defective: although defective for segregation at anaphase I and/or II, they form ascospores around nuclei whatever the number of chromosomes present in the nucleus (e.g. six or only two, instead of the seven expected, as seen in the *spo11* mutant of *Sordaria,* **Fig. 7.2B**).

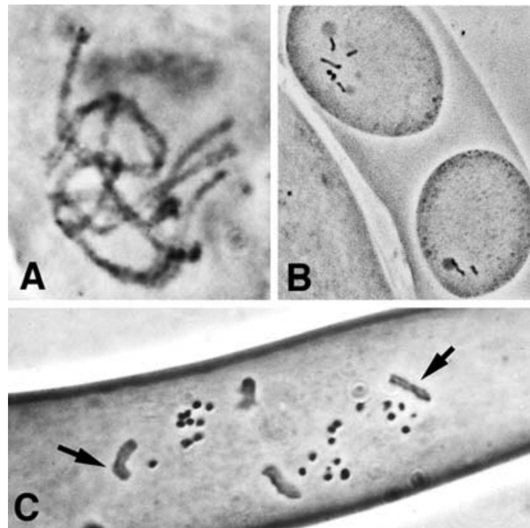

Fig. 7.2. Hematoxylin staining (*Sordaria*). (**A**) Pachytene nucleus with bright chromosome axes. (**B**) Post-meiotic mitosis in two ascospores of the *spo11* mutant (no recombination is initiated) with respectively six (*upper*) and two chromosomes (*lower* spore). Hematoxylin stains both prometaphase condensed chromosomes and nucleolus (round dark ball attached to a chromosome), indicating that both spores contain a nucleolar organizer chromosome. (**C**) Anaphase of second meiotic division: chromosomes segregate along the spindles indicated by the spindle-pole bodies (*arrows*) that are also stained by this procedure.

2. Materials

2.1. Strains and Culture Media

1. *N. crassa* and *S. macrospora* laboratory strains: Fungal Genetics Stock Center (Department of Microbiology, University of Kansas Medical Center, Kansas City, KS). Strains can also be collected from nature (*see* **Note 1**).
2. *Sordaria* growth and fruiting medium: To 1 L of distilled water add successively: 10 g glucose, 5 mL TKP (TKP: 5 g KH_2PO_4, 6 g K_2HPO_4 in 100 mL H_2O), 5 mL 5% $MgSO_4$, 5 mL 10% urea, 5 mL of 12 mg/mL biotin, 0.1 mL mineral concentrate (5 g ascorbic acid·$1H_2O$, 5 g $ZnSO_4 \cdot 7H_2O$, 1 g $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, 0.25 g $CuSO_4 \cdot 5H_2O$, 0.05 g $MnSO_4 \cdot 1H_2O$, 0.05 g H_3BO_4, 0.05 g $Na_2MoO_4 \cdot 2H_2O$ for 100 mL). Store concentrate at room temperature. Solidify with 15 g agar. Autoclave 20 min at 120°C. For ascospore germination, replace urea with 0.44% ammonium acetate in minimal medium.
3. *Neurospora* synthetic cross medium: To 1 L of distilled water add 3 g KNO_3, 1.4 g K_2HPO_4, 1 g KH_2PO_4, 1 g $MgSO_4 \cdot 7H_2O$, 0.2 g NaCl, 0.2 g $CaCl_2 \cdot 2H_2O$, 0.1 mL biotin (5 mg in 100 mL of 50% ethanol, stored at −20°C), 0.2 mL mineral concentrate (same as for *Sordaria*) and 1% sucrose. Solidify with 2% agar. (*21,* and *see* **Note 2**).

2.2. Strain Storage Medium

1. *Sordaria*: 216 g saccharose in 1 L of minimal medium. Store agar strips with mycelium in 1.5 mL aliquots at −80°C.
2. *Neurospora*: 5 mg biotin in 100 mL of 50% ethanol. Store in 2.5 mL aliquots at −20°C. Conidia are kept on minimal media in slants at −20°C or on anhydrous silica gel *(21)*.

2.3. Iron-Hematoxylin Staining

1. Iron mordant: prepare stock solution with 10 g ferric acetate in 100 mL of 50% aqueous propionic acid (glass bottle). Working solution: 0.5–1 mL in 50 mL 50% aqueous propionic acid. Keep in dark at room temperature.
2. Hematoxylin: 2% (Gurr, London or Sigma) in 50% aqueous propionic acid. Keep in dark at room temperature. Better when 1–2 months old.
3. Fixative for *Neurospora*: 9 vol of 95% ethanol, 6 vol of propionic acid, and 2 vol of 10% aqueous chromic acid.
4. Fixative for *Sordaria* and *Podospora*: 9 vol of butanol, 6 vol of glacial acetic acid, and 2 vol of 10% aqueous chromic acid.
5. Hydrolysis solution for *Neurospora* 1:1 (v/v) 12 N HCl and 95% ethanol.
6. Hydrolysis solution for *Sordaria* and *Podospora*: 1 N HCl.
7. Glycerin: 1 vol glycerin plus 1 vol of 45% acetic acid.

2.4. Acriflavine Staining

1. Hydrolysis solution: 4 N HCl.
2. Acriflavine (Sigma): 100–200 μg/mL in $K_2S_2O_5$ (5 mg/mL in 0.1 N HCl).
3. Washing solution: 2 vol HCl in 98 vol 70% ethanol.

2.5. Immuno-fluorescence: Antibodies and GFP/RFP

1. Siliconized slides. Wash slides in 50% ethanol and dry. Under fume hood, dip each slide quickly into dimethyldichlorosilane (2%, Merk 103014.0500) and dry on pipette. Store in slide box to avoid dust. Both slides and dimethyldichlorosilane (filter if necessary) can be used two to three times.
2. Polylysine (Sigma): add 24.875 mL distilled water and 125 μL of 10% Triton to a 25 g polylysine tube (fractioning is difficult). Filter and distribute as 500 μL samples in Eppendorf tubes. Store at −20°C. Do not refreeze more than twice.
3. Polylysine-coated coverslips. Clean coverslips with 95% ethanol, let dry and place them into a large Petri dish (14 cm diameter, allows easy transfer) on parafilm. With a pipette tip, cover each coverslip with 10 μL polylysine. Let dry for 10 min (cover the dish with lid to avoid dust) and wash coverslips one by one in distilled water (hold edge with thin tweezers). Air dry (dab the last drop of liquid with Kimwipes paper) and place coverslips with polylysine side up into a second large Petri dish also covered with parafilm. Prepare coverslips one night before (room temperature) or at least 4 h before staining (dry at 37°C) and keep in dark.
4. PEM buffer: In 600 μL distilled water add 9 mL 100 mM Pipes, 200 μL 500 mM EGTA, 200 μL 500 mM $MgCl_2$. Store PEM, as well as stock solutions of PIPES, EGTA, and $MgCl_2$at 4°C. The pH of the Pipes stock solution should be adjusted to 6.9 with 10 N HCl.
5. Phosphate-buffered saline (PBS) (10x stock solution): 50 mL 1.6 M Na_2HPO_4, 50 mL 0.4 M NaH_2PO_4 and 0.5 g NaN_3(Aldrich); store at room temperature, as well as stock solutions of Na_2HPO_4(place 1 h at 37°C before use if crystals are formed) and NaH_2PO_4. Working solution: 1 vol in 9 vol distilled water.
6. PBS + Triton: 44.75 mL distilled water, 5 mL 10x PBS, 250 μL 10% Triton.
7. Paraformaldehyde: Dissolve 1.85 g in 23.5 mL of PEM plus 1.5 mL of distilled water (7.4%), on stirring hot plate (60°C) in a chemical hood (toxic) until the solution becomes clear. Filter if slightly opaque. Cool to room temperature and store at 4°C for 14–20 d.
8. Post-extraction: 2 mg/mL myristoyl lysolecithin (Sigma) in 90 mM Pipes, 10 mM EGTA, 10 mM $MgSO_4$. Store at 4°C. Working solution: 30 μL stock solution in 970 μL PEM.

9. DAPI. Stock solution: 5 mg/mL in distilled water. Store in 40 μL aliquots at −20°C in Eppendorf tubes in the dark. Working stain: 0.5 μg/mL in PBS + Triton (keep in dark).
10. Antifade: Vectashield H100 (Biovalley or Vector laboratories) or DABCO: 10% w/v 1,4-diazobicyclo(2,2,2)octane in 90% gycerol, 10% 100 mM PBS, pH 8.7. Keep in dark at 4°C.
11. Tested antibodies: anti-β-tubulin (1:1,200, Amersham), anti-α-tubulin (1:500–1:1,200, Amersham), anti-actin (1:3,000–1:5,000, Amersham), MPM2, a mitotic phosphoprotein, which recognizes spindle-pole bodies and synaptonemal complex (1:300–1:750); anti-Rad51 (1:400; Oncogene); anti-GFP (1:500–1:250, Roche). For HA-tagged genes: anti-HA 3F10 (Boehringer Mannheim) at 1:4,000. When diluted, antibodies are conserved in small volumes at 4°C (2–6 months) and at −20°C (1–3 years). Do not refreeze, but keep at 4°C.

2.6. Drugs

1. Nocodazole (Aldrich). Stock solution: 5 mg/mL in DMSO. Working solution: dilute to 10 μg/mL in distilled water.
2. Cytochalasin D (CD, Sigma). Stock solution: 4 mM in 95% ethanol; working solution is 10 μM.
3. Latrunculin B (Calbiochem): 0.1–0.5 μg/mL in 0.5% DMSO.
4. 2,3-Butanedione monoxime (BDM, Sigma): 10–20 mM in distilled water.

2.7. Observation of Membranes, Mitochondria, and Peroxisomes

1. Plasma membrane: 25–30 μM FM 4-64 (Molecular Probes).
2. Mitochondria: 2-(4-dimthylaminostyryl)-1methylpyridinium iodide (DASPMI, Molecular Probes or Sigma) at 10–25 μM in water.
3. Peroxisomes: antibody against the trifunctional peroxisomal FOX2 enzyme of *Neurospora (22)* or the sequence encoding the SKL tripeptide (peroxisomal targeting signal 1).

2.8. Electron Microscopy

1. Phosphate buffer stock solutions. Solution 1: 15.6 g $NaH_2PO_4 \cdot 2H_2O$ in 250 mL distilled water. Solution 2: 35.8 g $Na_2HPO_4 \cdot 12H_2O$ in 250 mL distilled water (keep both at 4°C). Just before use, mix 16 mL of solution 1 with 84 mL of solution 2 (0.4 *M*) and add 100 mL distilled water (final 0.2 *M*).
2. Glutaraldehyde: 2–6% in 0.2 M phosphate-buffer or in 0.1 M cacodylate buffer, both at pH 7.2.
3. OsO_4: 2–4% in 0.2 M phosphate-buffer (pH 7.2).
4. Uranyl acetate: 5% in distilled water. Store at room temperature, in the dark and in thick glass container.

5. Lead citrate: 0.2 g in 50 mL distilled water plus 0.5 mL 10 N NaOH. Store at room temperature in the dark.
6. Resin: 81 mL Epikote 812 ($C_{12}H_{20}O_6$; Merck), 50 mL DDSA (Dodecenyl succinic anhydride), 44.5 mL MNA (methyl nadic anhydride). Final use: add 0.4 mL DMP30 (dimethylaminomethyl phenol). Araldite (Merck).
7. Siliconized coverslips: Dimethyldichlorosilane 2% (Merk). Keep out of dust.
8. Formvar-coated slides. 1% formvar w/v in chloroform. Dip slide into solution and lift it as firmly as possible to avoid differences in thickness, and air dry. Discard if wrinkled or irregular.
9. Plastic-coated rings: In a tube with a diameter slightly larger than the diameter of an EM grid, cut several rings (1–2 mm high).

2.9. Silver Staining

1. Slides. Clean with 95% ethanol, dry and polish with lens paper. Coat slides with 0.9% (w/v) polystyrene from Falcon Petri dish dissolved in chloroform.
2. Silicon-treated coverslips: prosyl-28 (SCM).
3. Paraformaldehyde: 4% in distilled water (pH 8.2) plus 0.03% of sodium dodecyl sulfate (SDS).
4. Photoflo (Kodak): 0.4% in water.
5. Silver nitrate: 50% aqueous silver nitrate (Calbiochem) with a drop of gel developer (2% aqueous gelatin dissolved at 40°C and cooled).

3. Methods

3.1. Preparation of Strains for Meiosis and Microscopy

1. Store *Sordaria* wild-type and mutant strains either in tubes (4–5 years) or in small Petri dishes (1 year) at 4°C or at −80°C (*see* **Sections 2.1** and **2.2** for media). For fresh cultures, take a 2–5 mm strip of agar plus mycelium with either a sterile needle or a drawing pen (through flame of Bunsen burner) and place it on fresh medium (small Petri dish) at 23–25°C in light. Avoid ethanol sterilization because it prevents *Sordaria* growth. Fruiting bodies form after four days and develop synchronously over the entire Petri dish. The first asci in each fruiting body are synchronous and are all in meiotic prophase the fifth day. Prophase takes roughly 10 h and ascospores are formed two days later. Cultures fruit at room temperature, but timing will be dependant on temperature.

2. Make *Neurospora* crosses for cytology at 25°C in Petri dishes on synthetic crossing medium (*see* **Section 2.1**, Step 3 and **Note 2**). For each cross, grow the "female" parent (*matA* or *mata*) for five days at 25°C and then fertilize by adding conidia (in distilled water) from the parent of the different mating type *(23, 24)*. When used in a laboratory that cultivates other organisms, it is recommended to use the *fluffy* strain (from FGSC), which is perfectly fertile (by confrontation of two strains with opposite mating types), but which no longer makes asexual spores that easily contaminate other culture plates. The fruiting bodies develop synchronously and the first asci in meiosis are found 3 days after fertilization. Ascus development is not synchronous and by 6–8 days all meiotic stages are present in each fruiting body and the early-developed asci contain ascospores *(24, 25)*.

3.2. The Iron-Hematoxylin Procedure

The propionic-iron-hematoxylin method *(8, 10, 13, 18, 24–27)* gives very reliable and accurate staining of all fungal nuclei (*see* **Note 3**). As illustrated in **Fig. 7.2**, pachytene chromosomes (**Fig. 7.2A**), mitotic metaphase chromosomes of ascospores (**Fig. 7.2B**) and chromosomes of anaphase II as well as the corresponding spindle-pole bodies (arrows in **Fig. 7.2C**) are brightly stained when observed in a light microscope.

1. For general observation of divisions in asci, fix agar strips of 5–10 mm containing 10–20 fruiting bodies.
2. For detailed observations of meiotic stages fix only rosettes of asci, this allows faster and better spreading. To do so, open each fruiting body with forceps (press the bottom to keep asci together) and lift the rosette of asci out on the agar plate. Accumulate four to five rosettes and transfer them into fixative (*see* **Section 2.3**, step 3). Cavity slides are well suited to accumulate the rosettes before storage in small tubes half filled with fixative. Once fixed, the material can be kept at room temperature for 10–20 d or for 1–2 months when stored in a freezer.
3. Hydrolysis of rosettes of asci (*see* **Section 2.3**, Step 4 and **Note 4**): Transfer rosettes with fixative into a small centrifuge tube (glass). Remove fixative and add 5 mL of 1 N HCl. Heat 10 min at 60–70°C. Time is critical: to reach quickly the right temperature, heat the tube on a Bunsen burner (add thermometer into tube to control temperature) and stop hydrolysis rapidly by plunging the tube into ice. Rosettes can stay 1 d in 1 N HCl.
4. Hydrolysis of agar strips: Incubate at 70°C in a heater for 1 min, take out, let cool 2 min and wash the agar strips for 10 min in 3 vol of ethanol, 1 vol of acetic acid, and 1 vol of chloroform. They can stay 6–8 h in this solution before staining.

5. Staining. Take 5–10 rosettes of asci from HCl (use a Pasteur pipette; take care because they stick easily to glass), place on a clean slide and remove excess of acid with a filter paper (traces of HCl prevent staining). Add quickly a small drop of diluted ferric acetate solution (*see* **Section 2.3**, Step 1) and stir with a glass rod. Add two drops of hematoxylin (*see* **Section 2.3**, Step 2) and mix both solutions thoroughly with the glass rod (do not use metal, which can add iron). When the mixture turns brown, cover quickly with a coverslip (*see* **Note 5**). Gently tapping above rosettes spreads and breaks asci (best prophase figures are obtained when the ascus wall is broken). Press out excess stain with filter paper. The quality of the stain can be quickly assessed in a microscope and if necessary (i.e., if the nuclei are too pale), slight heating of the slide over flame improves staining (avoid cooking which precipitates hematoxylin). Seal with dental wax or glycerin.
6. Staining of agar strips: In a drop of diluted ferric acid solution, open perithecia one by one, squeeze out rosettes with needles and eliminate all wall debris. Add two drops of hematoxylin and proceed as above.
7. Observation. Chromosomes and spindle-pole bodies are normally highly contrasted in the light microscope (e.g., **Fig. 7.2**) but contrast can be improved when observed in phase contrast. Squashes remain good for several days. However, while only chromosomes (**Fig. 7.2A and B**) and spindle-pole bodies (**Fig. 7.2C**) are stained in fresh preparations, cytoplasm staining increases after two days.

3.3. The Acriflavine Procedure

Developed for *Neurospora* by Raju *(28)* this technique works for all fungi.

1. Hydrolyze fruiting bodies or rosettes of asci in 4 N HCl for 25 min at 30°C.
2. Wash samples in water.
3. Stain with acriflavine (*see* **Section 2.4**, step 2) 20–30 min at 30°C.
4. Wash three times in HCl-ethanol solution at 30°C, followed by three times in distilled water.
5. Squash rosettes in a drop of 10% glycerol.
6. When fruiting bodies are fixed, they must first be dissected in 10% glycerol and the wall removed before squashing asci in 10% glycerol.
7. Observe with an epifluorescence microscope at excitation 450 nm and emission 540 nm.

3.4. Immunocytology

The following protocols are identical for *Sordaria (12, 18, 29, 30)*, *Neurospora (10, 11, 31)*, and *Podospora (32, 33)* (*see* **Notes 6** and **7** plus **Section 2.5** for details). Examples of different antibodies and meiotic stages are shown in **Fig. 7.3**.

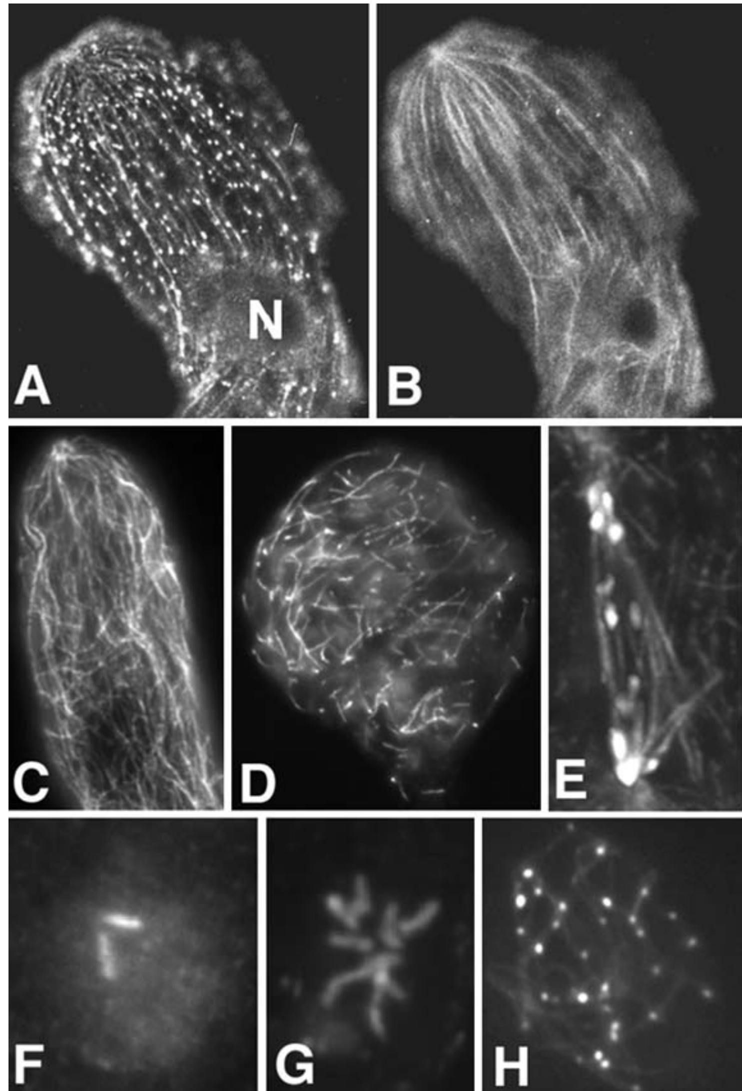

Fig. 7.3. Examples of antibody staining. (**A** and **B**) *Sordaria* prophase ascus, double stained with anti-actin (**A**) and anti-tubulin (**B**) and detected by confocal microscopy. (**C** and **D**) Untreated prophase ascus (**C**) shows dense layers of microtubules while an ascus after anti-actin drug treatment (**D**) shows aberrant shape (round instead of cylindrical) and aberrant microtubule organization. (**E**) Anaphase I spindle of *Neurospora* stained with anti-tubulin antibody merged with DAPI staining of chromosomes. (**F** and **G**) Prometaphase of post-meiotic mitosis: duplicated spindle-pole bodies are stained by anti-MPM2 (**F**) and chromosomes are stained by DAPI (**G**). (**H**) Leptotene nucleus double stained by Spo76-GFP (axes) and anti-Rad51 antibody shows numerous Rad51 foci located on unpaired chromosomes.

1. Fix rosettes of asci (*see* **Section 3.2**, Step 2) in fresh paraformaldehyde (3.7% for *Sordaria* and 7.4% for *Neurospora* and *Podospora*) 20–30 min at room temperature. Cavity slides are convenient because they allow easy removal of liquid with a glass Pasteur pipette that has been elongated in a flame to reduce diameter.
2. Wash with PBS: remove fixative with a pipette and add PBS. Keep rosettes in PBS while slides are made.
3. Place five to ten rosettes (with 7 μL PBS; use a P20 pipette) on a siliconized slide, add the polylysine-coated coverslip (polylysine side down) and, under a dissecting microscope, crush asci with a blunted hypodermic needle by tapping at the center of the rosette to disperse asci and finally break their bases.
4. With tweezers, remove the coverslip (to which asci remain attached because of the polylysine) from slide (watch polylysine side) and place it, asci up, in a large Petri dish (14 cm diameter, which allows easy handling) covered with parafilm.
5. Add 250 μL of PBS + Triton on the coverslip.
6. Prepare the next coverslip and again add 250 μL of PBS + Triton. Repeat until all needed coverslips are ready.
7. If different strains are used, mark location of each coverslip.
8. Add on each coverslip a large drop of lysolecithin and incubate at 37°C for 1 h.
9. Discard and rinse with PBS + Triton (250 μL per coverslip).
10. Discard and add primary antibody (*see* **Note 7**): first add 50 μL of PBS, make sure that the entire coverslip is covered, and then add 50 μL of antibody at correct concentration.
11. Incubate 12 h at room temperature and in dark (under aluminum foil or in box). To avoid drying, place wet filter paper around the coverslips.
12. Remove antibody and wash twice (15 min each) with PBS + Triton.
13. Incubate in secondary antibody + PBS (100 μL per coverslip) at 37°C for 45 min in the dark.
14. Remove and discard antibody, and wash with PBS + Triton.
15. Add 250 μL DAPI on each coverslip and stain for 10–15 min. Both DAPI solution and coverslips with DAPI must be kept in dark. Dim the room lights during handling.
16. Rinse each coverslip in distilled water and air-dry. Dab the last drop of liquid with filter paper.
17. Place each coverslip (polylysine side down) on a clean slide in a drop of antifade (*see* **Section 2.5**, Step 10), which reduces both fading and drying out of the preparations.

18. Seal the coverslip to the slide with nail polish and let dry in the dark for 1–2 h.
19. Store slides in a box either at 4°C (15–30 d) or at −20°C (up to two years).
20. View slides under fluorescent or confocal microscopy with excitation corresponding to the fluorophore on the secondary antibody. Use excitation at 364 nm for DAPI fluorescence. Software can be used to overlay the images (*see* **Note 8**). Examples are shown **Fig. 7.3**.

3.5. Observation of GFP- or RFP-Tagged Nuclei

The GFP coding sequence (p-EGFP-1, Clontech) is usually fused just after the last C-terminal amino acid predicted from the ORF (try amino-terminal fusions if no GFP is seen or if there is doubt concerning localization) (*see* **Note 9**).

1. Live-cell imaging of GFP-tagged vegetative cells: grow mycelia or protoplasts on sterile slides coated with a thin layer of medium, or use the inverted block method of Read's laboratory *(34, 35)*.
2. Live-cell imaging of GFP-tagged asci: lightly squash in a drop of 10% glycerol (*see* **Note 10**).
3. Fixed samples: Fix rosettes of asci in paraformaldehyde (3.7% for *Sordaria* and 7.4% for *Neurospora* and *Podospora*) at room temperature for 20–30 min. Use cavity slides to allow easy removal of liquid from rosettes.
4. Wash with PBS: slowly remove fixative with a pipette and add PBS. Keep rosettes in PBS while next slides are made.
5. Place five to ten rosettes with 7 μL PBS (use a P20 pipette) on a siliconized slide, cover with a polylysine-coated coverslip (polylysine side down) and, under dissecting microscope, crush asci with a blunted hypodermic needle (as in **Section 3.4**, Step 3).
6. Remove the coverslip from the slide with tweezers and place it, asci up, in a large Petri dish covered with parafilm (as in **Section 3.4**, Step 4). The asci remain attached to the coverslip through the polylysine.
7. Place 250 μL of PBS + Triton on the coverslip and prepare each of the next coverslips in turn, until all coverslips are ready.
8. Discard and add 250 μL of DAPI for 10–15 min. Keep in dark (*see* **Section 2.5**, Step 9).
9. Rinse each coverslip in distilled water in dim light and dab the last drop of liquid with filter paper.
10. Place each coverslip (polylysine side down) on a clean slide in a drop of antifade (*see* **Section 2.5**, Step 10).
11. Seal the coverslip to the slide with nail polish and let it dry in the dark for 1–2 h.

12. Store slides in a box either at 4°C (two weeks) or at −20°C. Staining remains visible for one year, but the brightness of GFP, RFP, and DAPI decreases after 6 months.
13. View slides in a fluorescent microscope with a GFP filter for GFP samples and red emission for RFP. After control for GFP specificity, pictures are taken with an FITC filter, in which asci are visible. This allows easier staging of nuclei. Deconvolution gives details not always seen with fluorescent microscopy (compare **Fig. 7.4B** with **Fig. 7.4A**). A confocal laser scanning microscope is necessary if two different structures are labeled (**Fig. 7.3A and B**). Prophase axes are less clear than with epifluorescence microscopy (compare **Fig. 7.4C** with **Fig. 7.4A and B**). Chromosomes seen by DAPI are less bright (**Fig. 7.4D**) than with GFP staining of axis proteins (e.g. cohesin Spo76/Pds5 in **Fig. 7.4A–C**). Also, GFP or RFP staining remains visible after antibody staining (e.g., anti-Rad51 in **Fig. 7.3H**).

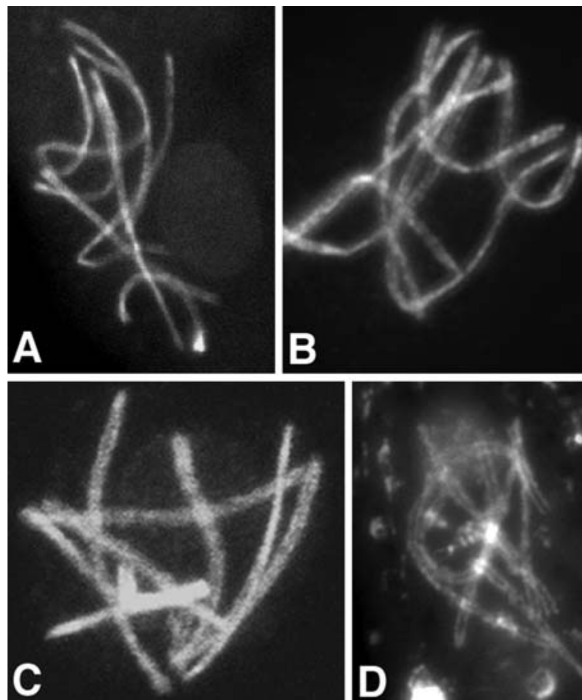

Fig. 7.4. Comparison of four methods to analyze pachytene synapsed chromosomes stained by Spo76-GFP. (**A**) Epifluorescent microscopy shows bright staining of the seven synapsed bivalents. (**B**) Deconvolution shows that Spo76 is not continuous but localizes as close, dense foci. (**C**) Confocal microscopy shows that staining is likely broader than axis. (**D**) DAPI staining is always less bright.

3.6. Drug Treatments

1. As fruiting-body development is synchronous in each Petri dish, a third of the agar containing fruiting bodies is dipped (fruiting bodies down) in drug solution, while the second third is treated with distilled water containing the appropriate concentration of solvent. The third part remains in the dish as a control *(29–31)*.
2. Apply nocodazole to fruiting cultures for 2–12 h. Cortical microtubules (examples in **Fig. 7.3B and C**) begin depolymerization after 15 min and spindle microtubules (**Fig. 7.3E**) after 12 h.
3. Treat with cytochalasin D overnight. Cortical actin filaments (**Fig. 7.3A**) are disrupted after 10 h. An example is given in **Fig. 7.3C and D**: in untreated asci, dense arrays of microtubules lie parallel to one another and converge at the ascus tip (**Fig. 7.3C**). After 10 h drug treatment, instead of being cylindrical, asci are round-shaped and microtubules are randomly arranged (**Fig. 7.3D**).
4. Latrunculin B arrests homolog movement after 2 h treatment.
5. Overnight treatment with BDM arrests spindle-pole body movement after spindle-pole body duplication (e.g., **Fig. 7.3F and G**).
6. After washing off the drug with water, recovery of the cytoskeleton network at different times, followed by fixation, can be monitored because asci develop normally in the treated fruiting bodies (*see* **Note 11**).

3.7. Organelles and Membrane Staining

1. Isolate asci as described in **Section 3.2**, Steps 1 and 2.
2. Plasma membrane: Place rosettes of asci in one drop of FM 4-64 for 1–2 h and squash.
3. Mitochondria: Add a drop of DASPMI on unfixed rosettes of asci, and squash.
4. Double staining with DAPI allows overlap of the blue DNA stain (blue emission) with the orange DASPMI stain (FITC filter).
5. Peroxisomes: prepare asci as in **Section 3.4**. Use antibody against the trifunctional peroxisomal FOX2 enzyme *(22)* at 1: 200 dilution.
6. Add the sequence encoding the SKL tripeptide (peroxisomal targeting signal 1) at the end of the GFP open reading frame (*see* **Note 9**). Transform wild-type or mutant strain. Prepare slides as in **Section 3.5**. SKL1-GFP brightly stains all peroxisomes of both vegetative and sexual cycles. Observe with GFP and FITC filter *(32, 33)*.

3.8. Electron Microscopy

1. Fix rosettes of asci as described in **Section 3.2**, Step 2 except using 2–5% glutaraldehyde in phosphate-buffer (pH 7.2) or in cacodylate buffer (pH 7.2) for 3 h at room temperature. Use a cavity slide because it allows easy removal of liquid from rosettes.

2. Wash three times with 0.2 M phosphate buffer or cacodylate buffer.
3. Transfer to a tube, and replace buffer with 2–4% OsO_4in the same buffer. Postfix at room temperature for 1 h under a fume hood and in closed tubes.
4. Wash rosettes once in buffer and three times in water.
5. Stain in 5% aqueous uranyl acetate overnight. This step reinforces the contrast of synaptonemal complex components. Omit this step for cytoplasmic or cytoskeleton studies.
6. Dehydrate rosettes through an ascending ethanol series from 5 min at low concentration to 20 min at higher concentrations.
7. Dehydrate further by incubating in a fume hood twice for 10 min in 1, 2-propylene oxide (C_3H_6O).
8. Infiltrate sample through a graded series of 1, 2-propylene oxide and low-viscosity epoxy resin (or Epon or araldite): 1 h in a 1:1 mixture of 1,2-propylene oxide and resin, then overnight in resin alone.
9. Discard resin, replace with fresh resin (plus dimethylaminomethyl phenol if using Epon), and incubate for 1 h.
10. Embed rosettes or parts of rosettes in capsules filled with resin and polymerize 12–15 h at 60°C.

3.9. Single Ascus Embedding

1. Prepare siliconized coverslips the day before and keep out of dust.
2. Rosettes of asci are fixed until Step 8 of **Section 3.8** (*see* **Note 12**).
3. Prepare slides: on each slide, glue two siliconized coverslips with small ribbons of magic tape (which is easy to remove) along two sides and put four small drops of resin on each coverslip (towards corner for easy handling of capsules, see below).
4. Prepare rosettes of asci: Put a large drop of resin on a clean slide and, with a needle or pen, add one to three rosettes of asci taken from Step 8 of **Section 3.8.**
5. Separate single asci from the rosettes under a dissecting microscope. The presence of resin allows easy dissection of rosettes with insect needles. Keep asci in resin while next slides are made.
6. Transfer each single ascus into one of the drops already prepared on coverslips and push it toward the surface of the coverslip, which saves time during sectioning because the ascus will be present in the first sections.
7. When all asci are transferred, polymerize slides at 60°C for 12–15 h.
8. After polymerization, turn the coverslips over and reattach with magic tape. The drop of resin will now be sandwiched between the slide and coverslip, which allows an easy screen of meiotic stages.

9. Select meiotic stage in a phase-contrast microscope. After osmium fixation, chromosomes and spindle-pole bodies are visible. Mark the coverslip to note the meiotic stage.
10. Turn coverslips over again and reattach with magic tape.
11. Fill capsules with resin. Add a strip of paper indicating the meiotic stage plus strain in each. (Use a lead pencil) Turn capsule onto the selected drop.
12. Polymerize slides with capsules at 60°C for 12–15 h.
13. Remove capsules from coverslips (they slide on silicon) and store at room temperature. Capsules we have prepared are still good after 30 years.

3.10. Serial Sectioning

1. Dip a clean slide into formvar solution, lift it as firmly as possible and air dry.
2. Cut the upper edge of the formvar film with a razor and float the formvar film off onto a clean water surface in a large glass bowl. The film will be clearly visible in oblique light.
3. Place single-slot EM grids onto the formvar film.
4. Pick up the film plus 10–12 grids on a parafilm sheet: use the parafilm to push the film plus grids underneath the water surface, and then pick up. Air-dry the grids sandwiched between the formvar and the parafilm.
5. Cut each grid out from the formvar with a thin needle: make several holes in the formvar around the grid and lift with tweezers without tearing the formvar film.
6. Trim the capsule face to the ascus wall. With the single ascus embedding method, asci will be located at the flat surface of the capsule.
7. Cut sections (30–80 nm thick) with a diamond knife. Cut individual ribbons of sections that correspond to the length of the single-hole grid (when too small, ribbons have a tendency to turn in water). Detach the ribbon by running an eyelash (mounted with tape or nail polish on a wooden stick) along the outside of the diamond edge. Turn off microtome momentarily.
8. During sectioning, note the number of sections in each ribbon and note also the place of single, missing, or folded sections. This helps later for nuclear reconstruction.
9. Pick up ribbons of serial sections with an uncoated single-hole grid: Hold the grid edge with tweezers, lower the grid (dull surface down) onto the water over the ribbons and pick up quickly with drop of water.
10. Lower the grid with ribbons either onto a formvar-coated single-hole grid (prepared in advance and hold with tweezers under a dissecting microscope) or onto a formvar-coated ring (*see* **Note 13**) and let dry.

11. As quickly as possible, turn the microtome back on: thermal drift during the time the microtome is turned off will cause variable thickness for the first section.
12. Stain grids with uranyl acetate for 30 min at 60°C followed by lead citrate for 20 min at room temperature (use plastic kit with slots).

3.11. Three-Dimensional Reconstitution of Meiotic Nuclei

1. In transmission EM (TEM), observe grids at low magnification to survey possible holes in the formvar. Minimize exposure near holes and in thick sections, because prolonged exposure in these regions will lead to rupture of the formvar and loss of the grid.
2. Take pictures from all sections in which the nucleus is visible (from 50 to 80 for prophase nuclei, depending on the nuclear shape and volume). Bookkeeping of the order of sections and the place where sections were single or missing helps greatly. Print all pictures of the nucleus.
3. Reconstruct chromosomes: starting with section **1**, trace each synaptonemal complex (SC) component visible on the picture on a thin plastic sheet with an ink pen. Addition of the section number allows easy checking of SCs or lateral/axial elements (LE) that cross the nucleus several times and that therefore are found in several places on each section. Trace the nuclear membrane as an outside marker.
4. Always trace the next section in overlap with the previous one. This corrects the possible distortion due to formvar and/or differences in section thickness. Ideally no section should be missing, but when SCs or LEs are cut perpendicularly, a lost section will not interfere with reconstruction or even with length measurement if it was noticed previously where the section was missing.
5. Once all SC components are traced (e.g., **Fig. 7.5A**), the nucleus can be analyzed for initiation of homolog recognition (e.g., **Fig. 7.5B** which shows homologous pair 3 aligned, and homologous pair 1 far apart), synapsis, recombination nodules (**Fig. 7.5C and D**), telomere position on the nuclear envelope, etc.
6. 3D rendering can be achieved by, for example, Vector Works and Zoom software (**Fig. 7.5A and B** and Refs. *8, 12, 36)*. With Zoom software, the nucleus can be turned, the path of each single SC or chromosome axis can be followed and the synapsis behavior of the different homolog pairs can be compared *(8, 36)*. *Movies showing rotations of 3D reconstructions of individual chromosome pairs and of an entire nucleus are provided on the companion CD for this volume.*

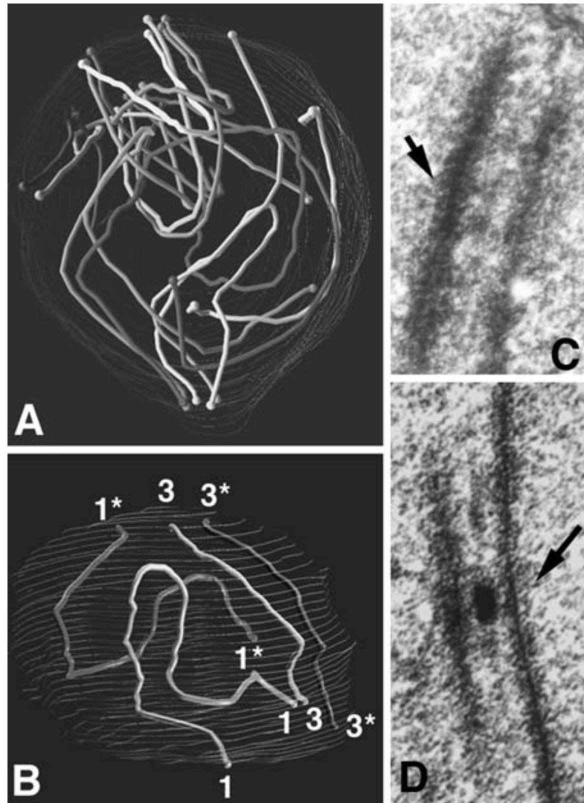

Fig. 7.5. Electron-microscope analyses of synaptonemal complex. (**A**) Early bouquet nucleus reconstructed from serial sections and treated by Zoom software to render a 3D image. *Movies showing rotations of 3D reconstructions of individual chromosome pairs and of an entire nucleus are provided on the companion CD for this volume.*(**B**) Early leptotene nucleus reconstructed from serial sections. The two pairs of homologous chromosomes show clearly that shorter pair 3 is already aligned when long pair 1 is still far apart. (**C**) *Neurospora* SC with small recombination nodule (*arrow*). (**D**) *Sordaria* SC with a large late recombination nodule (*arrow*).

3.12. Spreading and Silver Staining

1. Place five to six rosettes of asci on a silicon-treated coverslip in a drop of ice-cold paraformaldehyde for 4–5 min (*see* **Section 2.9**). Fixation time is critical.
2. Pick up the coverslip with a plastic-coated slide and knock the nuclei out of asci by tapping over the rosette with pencil or rubber hammer (*see* **Note 14**).
3. Freeze the slide on a cold plate or dry ice and quickly flip off the coverslip with a razor blade: some cells and nuclei will stick to the slide (freezing timing is critical).
4. Add a few drops of paraformaldehyde on the area previously covered by the coverslip and fix for 20–40 min. Mark the edges of the slide with an ink pen to indicate the area.

5. Remove the fixative, dip the slide into 0.4% Photoflo and let air-dry.
6. Mix one drop of silver nitrate with one drop of gel developer on a large coverslip.
7. Pick up the coverslip plus stain using the slide to be stained. Use gloves and use quick movement to avoid letting the stain run off. Incubate on a hot plate at 55°C for 2–3 min. (Higher temperature induces cracks in the plastic membrane.)
8. Wash off the coverslip with distilled water, taking care not to damage the plastic membrane, and wash the slide two to three times more in fresh water.
9. Dip the slide into 0.4% Photoflo and let air-dry.
10. Examine slides in light microcopy (phase contrast) to locate good SCs and mark their places with permanent-ink pen to guide where grids must be placed.
11. Cut the edge of the plastic membrane with a razor blade to lift one end, and float the plastic membrane off the slide carefully on a clean water surface in a large glass bowl. Lay single-slot grids on the marked areas. A tiny drop of superglue on the grid edge helps to keep the grid on the plastic membrane.
12. Pick up the membrane with a sheet of parafilm (*see* **Section 3.10**, Step 4) and let dry, covering to avoid exposure to dust.
13. Cut each grid out of membrane with a thin needle (*see* **Section 3.10**, Step 5) and store in a grid box before examination.

4. Notes

1. *S. macrospora* and *N. crassa* can also be collected in nature. *Sordaria* grows on dung and is easily isolated by placing small pieces of dry horse or cow dung on agar plates (see **Section 2.1**, Steps 2 and 3 for medium). After a few days, several fungi grow and fruit, among which *S. macrospora* and *S. fimicola* are the most common. As can be deduced from its name, *S. macrospora* makes larger ascospores than *S. fimicola*. *N. crassa* grows mostly on burned wood and sugar canes, but can also be found on bread when humidity is high. It produces large amounts of two types of powdery asexual spores (bright orange when made in light), which can be easily collected. *S. macrospora* does not produce asexual spores and can thus be cultivated with other organisms.
2. *Neurospora* fruiting is more strictly under nutritional control (nitrogen starvation) than for *Sordaria*, which can fruit in water (for more details see Ref. *21)*. Addition of filter paper

strips as a carbon source helps fruiting. Also, the concentration of amino acid supplements must be minimized because amino acid nitrogen inhibits crossing. Ascospores must be activated by heat before germination *(21)*.

3. The propionic-iron-hematoxylin stain was first developed by Lu to describe the course of meiosis in the ascomycete *Gelasinospora (26)* and further adapted by Lu and Raju *(27)* to stain meiotic nuclei of *Coprinus*. It is also perfect for *Podospora*, *Neurospora, Aspergillus, Ascobolus*, and *Sordaria* nuclei (e.g., *12, 13, 18, 20, 24, 25, 32*).
4. Because hematoxylin stains any acidic component of the cell, hydrolysis is necessary to lessen the cytoplasmic staining of the numerous ribosomes present in asci.
5. Hematoxylin alone does not stain, but becomes a stain by lake formation with ferric ion (the mordant). The quality of staining therefore depends on the proportion of the two components. Insufficient ferric ion makes faint stains and too much causes hematoxylin to precipitate. The usual proportion is a small drop of iron and two drops of hematoxylin, but this depends on the concentration of the ferric acetate solution. Try out the proportion on a slide before use: the mix must be dark brown.
6. The cell wall of intact asci is impermeable and thus interferes with antibody penetration and visualization. Asci can be exposed to wall-digesting enzymes (e.g., Novozyme) to facilitate antibody penetration, but this often results in the loss of some components (e.g., actin microfilaments or even microtubules) and is difficult to reproduce routinely. Therefore, asci are placed between the surface of a siliconized glass slide and a polylysine-coated coverslip and crushed with a blunted hypodermic needle. Asci remain mostly complete but their bases are open, which is enough to allow antibodies to enter the cell without, apparently, damaging the complex cytoskeleton network (**Fig. 7.3A and B** and Refs. *29, 30* for *Sordaria*; Refs. *10, 11, 31* for *Neurospora*).
7. For antibody staining, controls include the use of primary or secondary antibodies alone, or use of an inappropriate secondary for a given primary antibody. Double staining: always use the red secondary antibody to label the less abundant nuclear component (e.g., actin in **Fig. 7.3A**) and fluorescein for the other (e.g., tubulin in **Fig. 7.3B**). For capturing images, start with the red filter labeling, then fluorescein and end with DAPI (exposure to UV wavelengths fades sample quickly). When possible, use antibodies from mouse or rat. Rabbits routinely make antibodies against fungal cell walls and therefore antibodies made in rabbits also stain ascus walls.

8. Fluorescence from FITC or rhodamine-tagged secondary antibodies as well as from GFP- or RFP-tagged proteins is scanned with an epifluorescence photomicroscope equipped with appropriate filters. Images are captured by CCD camera. Pictures are accurate enough for most analyses (e.g., **Fig. 7.1B –D**, **Fig. 7.4A**). Z-sections help follow each chromosome/bivalent and allow 3D reconstructions (e.g., by ImageJ software). Series of optical pictures can be done "by hand" in the absence of a Z-sectioning mechanism: they are less regular but good enough to locate bivalents and see which is lying in back of the others.
9. For *Sordaria*, all GFP-tagged genes are inserted ectopically because homologous recombination is a rare event *(8, 12, 18)*. To be fully reliable, each tagged gene must complement the meiotic and sporulation defects with exactly the same efficiency as the complementing sub-clone when introduced in the corresponding mutant or deleted strain *(8, 12, 18)*. Also, ascus sizes, synapsis and ascospore formation of each strain containing a GFP-tagged protein in a wild type or a mutant background are compared to an isogenic strain lacking the GFP tag. Tagged constructs are first introduced by transformation in a wild-type strain and further crossed to the strain of interest (using spore-color markers to recognize hybrid fruiting bodies). Analysis of the 8-spored asci allows easy recovery of double mutants (in four wild-type : four mutant asci) with GFP or RFP-tags. Protocols for GFP or RFP tagging are identical for *Neurospora*, except that transformation must be done in strains lacking both the *rid* and *sad-1*(or *sad-2)*genes in order to avoid respectively RIP and MSUD silencing (*see* **Section 1**) of the duplicated sequences (see *10, 11, 34* for details and plasmids).
10. Contrary to mycelia or protoplasts, it is difficult to keep isolated rosettes of asci alive for more than 2 h. After 1 h, asci start developing vacuoles and slowly die away. This is enough to screen GFP localization or to follow post-meiotic mitoses but for detailed analyses of meiotic prophase, asci must be fixed (examples in **Fig. 7.1B –D**). GFP and RFP staining is brighter when strains are maintained in the cold for a couple of hours before cytology. Also, because fruiting bodies are thick-walled and black, strains can be grown in the light. In contrast, for mycelial or protoplast observations, grow strains in dark or reduced light.
11. GFP and RFP staining (e.g., cohesins Rec8 and Spo76/Pds5, or Rad51, Msh4) remain visible after overnight drug treatment.
12. First developed for *Neotiella (37)*, single-ascus embedding has since been used for both *Neurospora* and *Sordaria (6, 7,*

8, 12–16, 18, 19, 36). Ascus growth during prophase (*see* **Section 1**) is advantageously used for selection and storage of specific meiotic or mutant stages in large quantities and for several years. Another advantage comes from the fact that asci are close to the surface before sectioning, which saves a lot of time at the microtome.

13. Lowering a single-hole grid with ribbons over a formvar-coated ring with a hole larger than the grid is easier than overlap of coated and uncoated single-hole grids because the single-hole grid with ribbons often turns, provoking the loss of part of the sections. When dry, the grid is pushed out of the ring hole with a nail head that is the same size as the grid. First make several holes around the grid, then push with the nail head and take off with tweezers without tearing the formvar membrane.
14. A protocol for spreading SC of *Neurospora* was developed by Lu *(17)*. It also works for *Sordaria*, but in both organisms the breakage of meiotic cell walls remains a difficult step, which limits the routine use of the technique.

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