Aus dem International Graduate Program Medical Neurosciences der Medizinischen Fakultät
Charité
UNIVERSITÄTSMEDIZIN BERLIN

DISSERTATION

ESTABLISHING A NEW, GENETICALLY TRACTABLE VERTEBRATE BRAIN MODEL WITH RICH BEHAVIOUR - DANIONELLA TRANSLUCIDA

DANIONELLA TRANSLUCIDA, EIN TRANSPARENTER UND GENETISCH MANIPULIERBARER MODELORGANISMUS MIT KOMPLEXEM VERHALTEN ZUM STUDIUM DES VERTEBRATEN-GEHIRNS

Zur Erlangung des akademischen Grades Doctor of Philosophy (PhD)
VORGELEGT DER MEDIZINISCHEN FAKULTÄT CHARITÉ - UNIVERSITÄTSMEDIZIN BERLIN

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Dissertation date
04.06.2021

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Abstract

Understanding how the brain orchestrates behaviours is a major objective in systems neuroscience. This quest involves accomplishing the following tasks: First, to characterise the behaviour of interest. Second, to identify the neurons and their networks responsible for the behaviour. Third, to study the computations performed by these neurons and fourth, to reveal the underlying mechanisms. As of yet, tackling all of these steps in adult vertebrates has been very challenging due to the size and opacity of their brains. Molecular component of cells, especially lipids and proteins, have light scattering properties and prevent excitation and retrieval of fluorescent signals in deeper brain areas. As a result, only limited optical access can be achieved using microscopy techniques in adult vertebrate brains. In this thesis I introduce a freshwater teleost fish, *Danionella translucida* (DT), as a new laboratory species. Unlike other vertebrates, it remains small and transparent throughout adulthood, with a majority of its cells accessible to optical recording techniques. Furthermore, DT shows rich social behaviours e.g. sexual behaviour, shoaling, schooling, fighting and, remarkably, vocalisation. This thesis focuses on foundational experiments to establish DT as a new model organism for systems neuroscience. First, I characterise essentials of DT behaviour, in particular its ability to vocalise. Second, I demonstrate genetic tractability to tailor DT for anatomical and functional circuit studies using Tol2-mediated gene insertion of a calcium-sensor and Crispr/Cas9-targeted gene editing for depigmentation. Third, I implement a proof-of-principle experiment to show that circuit functionality during sensory stimulation can be tested in the immobilised transgenic animal using two-photon calcium imaging. DT's optical features combined with rich behaviour and genetic amenability open the way to investigate the underlying mechanisms for neural computations performed by single cells. Hence, establishing DT as a new model organism throughout this thesis enables targeting the fourth and ultimate goal of systems neuroscience in the adult vertebrate.

Deutschsprachige Zusammenfassung

Im Forschungsbereich systemische Neurowissenschaften ist eine der grossen Herausforderung zu verstehen, wie das Gehirn Verhalten dirigiert. Dieses Ziel erfordert vier Schritte beginnend mit dem Charakterisieren eines interessanten Verhaltens. Zweitens, müssen dazu entsprechend relevante Neurone und neuronale Netzwerke identifizieren werden. Drittens gilt es die Verrechnungen innerhalb beteiligter Neurone zu studieren und letztlich die dahinter steckenden Mechanismen zu erkennen. Durch die Grösse und Undurchsichtigkeit von Gehirnen adulter Wirbeltiere war es bis dato unmöglich all diese Ziele gemeinsam in einem Modell zu studieren. Zellulaere Bestandteile, insbesondere Lipide und Proteine, haben lichtstreuende Eigenschaften und verhindern das Anregen und Messen von Fluoreszenzsignalen in tieferen Bereichen des Gehirns. Als Folge koennen nur sehr begrenzte Ausschnitte des erwachsenen Wirbeltiergehirns optisch untersucht werden. In dieser Doktorarbeit etabliere ich einen neuen Modelorganismus - den teleosten Süsswasserfisch *Danionella translucida* (DT). Anders als die meisten Vertebraten bleibt die DT auch während erwachsener Entwicklungsstadien klein und transparent, welches die Mehrheit ihrer Neurone optischen Methoden zugänglich macht. Desweiteren zeigt die DT komplexe Verhalten wie Fortpflanzungsverhalten, koordiniertes Schwimmen in der Gruppe, Kampfverhalten und beachtlicherweise Vokalisierungsverhalten. Ziel dieser Arbeit war es grundlegende Experimente durchzufuehren, um die DT als neuen Modelorganismus für systemische Neurowissenschaften zu etablieren. Dazu charakterisiere ich als Erstes die Grundzüge verschiedener Verhalten mit Schwerpunkt auf dem Vokalisierungsverhalten. Zweitens zeige ich, dass die DT genetisch manipulierbar ist, um sie für optische Studien neuronaler Netzwerkanatomie und -funktionalität anzupassen. Dazu inseriere ich mit Tol2-Transgenese einen Kalziumsensor in das Genom der DT und unterbinde mit Crispr/Cas9 ihre Pigmentierung durch Genveränderung des Enzyms Tyrosinase. Drittens, demonstriere ich in einem proof-of-principle Experiment am transgenen Tier, dass die DT sich während sensorischer Stimulierung unter dem Zwei-photonen Mikroskop auf Aktivitätseigenschaften in einzelnen Zellen innerhalb neuronaler Netzwerke untersuchen lässt. Die genetisch veränderbare DT eignet sich durch ihre optischen Eigenschaften und ihr komplex ausgebildetes Verhalten sehr gut zur Untersuchung neuronaler Netzwerke und der mechanistischen Verarbeitung innerhalb individueller Zellen. Damit trägt das Etablieren der DT als neuen Modelorganismus innerhalb dieser Arbeit direkt zur Adressierung der vierten Fragestellung systemischer Neurowissenschaften bei.

CHAPTER 1

INTRODUCTION

1.1 Central goals of systems neuroscience

All living creatures are constantly confronted with a vast abundance of environmental stimuli they have to react to, in order to survive. Stimulation is taken up by different organs and sensory modalities involving a multiplicity of neuron types to deliver and process incoming information. The same applies to responsive behaviours that have to be planned in different parts of the brain depending on their type of action e.g. changing a gaze, communicating with conspecifics or showing responsive motor behaviours such as escape. With often millions of neurons, each having hundreds or thousands of synapses, we are far away from understanding how the brain orchestrates behaviours in humans or animals. To explain how the brain processes sensory information we need to learn how neurons are interconnected across large parts of the brain. Drawing on this anatomical knowledge it is crucial to study the underlying mechanisms for neuronal computations in the entirety of single cells within circuits, required for generating behaviours. Altogether, systems neuroscientists aim to investigate how distributed neuronal groups are governed by feed-forward, recurrent and feed-back connectivities and computations. As of yet, monitoring each single neuron across the entire brain is experimentally challenging. Brain size (see figure 1.1) and complexity of most animal brains make the individual neuron inaccessible for optical techniques and only locally accessible for electrophysiological methods. Hence, a model organism enabling optical access across the entire brain at single-cell resolution during behavioural and neuronal maturity is in demand.

1.2 Model organisms for circuit studies

According to Krogh's principle, "for such a large number of problems there will be some animal of choice... on which it can be most conveniently studied". Throughout the history of neuroscience choosing a model organism with attention to their anatomical properties, behavioural powers and the methodological access, was crucial for investigating the mechanisms of neural computations from molecular to system level. For studying neuronal networks the refinement and advancement of optical techniques and optical probes (Nakai et al., 2001) improved our experimental access and understanding. Two-photon microscopy (Denk et al., 1990) revolutionised optical techniques offering enhanced spatial resolution and penetration depths superior to classical confocal microscopy (see figure 1.2). Depending on the model organism neuronal network architecture and activity can be optically studied at different spatial scales.

1.2.1 Model organisms for circuit studies at small and large scale

Studying neural systems could supposedly be implemented best in human experimentees, showing behaviours that can be explicitly instructed which are challenging or even impossible to extend to other animals. However, linking quantitative behaviour with single neuron measurements at large scale *in vivo* is technically, physiologically and ethically infeasible in the human, which is why extensions through

suited model organisms are required. For examining the neuronal computations supporting cognitive functions with physiological and psychological methods non-human primates are invaluable. Nevertheless, understanding circuit mechanisms in their large, complex and typically genetically unmodified brains is challenging because the identities and wiring partners of recorded neurons are often unknown. The smaller marmoset promises to advance studies combining genetic manipulation with functional brain accessibility. Though the brain is lissencephalic (lacking gyri) it possesses the shared neural architecture of primates (de la Mothe et al., 2006, Chaplin et al., 2013). The species' compatibility with gene editing techniques has provided a path for transgenic marmosets (Sasaki et al., 2009). Despite its relatively small body size, one disadvantage is their brain size (ranging between rat and cat brains with ca. 30 x 35 x 25 mm) making optical whole-brain studies at cellular resolution also impossible (Mitchell and Leopold, 2015). Rodents are the currently dominant mammalian animal order in neuroscience (representing about 95 percent of all lab animals). With the technical miniaturisation and refinement they have replaced equally inexpensive but bigger, as for example feline animals. As a result chronic recordings and reduced numbers of experimental individuals allowed improved experimental conditions. Studies in rodents benefit from almost 40 years of experience in gene-editing since the first transgenic animal was generated using cloned DNA micro-injection into the murine pronuclei (Gordon and Ruddle, 1981). The laboratory rat (and its various strains), even more than the mouse, is easily trainable and attractive to study due to its complex behaviour and neural networks strongly resembling that of other mammals including us humans. One major disadvantage however, like in marmosets, is their brain size making non-invasive, optical sub-cortical recordings at cellular resolution extremely challenging. Certain deep-brain imaging strategies in rodents involve physical insertion of optical devices as a gradient-index lens (GRIN) or a micro-prism to optically access deep layers as the murine hippocampus, thalamus or hypothalamus at cellular resolution (Attardo et al., 2015). Hence, rodents remain subpar for optical population studies across the entire brain even with modern optical techniques as multi-photon imaging. A promising rodent-like (although closer to moles or hedgehogs) candidate for imaging is the Etruscan shrew. With its small body (about 40 mm) and brain size (5 mm x 3,5 mm x 4 mm) it presents a decent model for imaging the adult mammalian cortex. Like in rodents however, additional invasive devices remain indispensable for large-scale neuronal activity recordings in deeper brain areas.

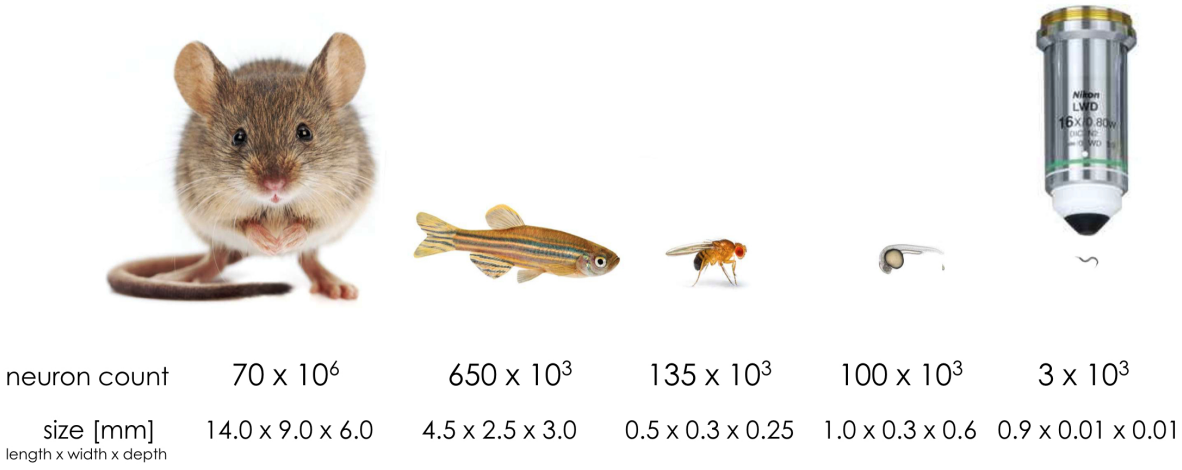

Figure 1.1: Common model organisms employed for neural network studies.
Organisms are sorted by total neuron count of their brains in decreasing order from left to right. The widely established mammalian lab mouse (*Mus musculus*) suited for sub-cortical studies employing invasive optical devices like a GRIN lens, the vertebrate adult zebrafish (*Danio rerio*) with optical access to superficial areas of the teleost brain due to skull ossification, the invertebrate fruit fly (*Drosophila melanogaster*) enabling whole-brain imaging in the invertebrate brain after removing the cuticle and fat, the larval zebrafish allowing for whole-brain imaging in the vertebrate during early development and the tiny nematode worm (*C. elegans*) submitting optical investigation of the entire nervous system.

All of these mentioned model systems show complex behaviours and are thus interesting to study. However, for understanding how neurons fundamentally function within networks (for sensory processing and the generation of behaviour) they all share to find their limitation in brain size and opacity. While optical imaging evolved as the method of choice for monitoring many spatially distributed neurons, the size and opacity of mammalian brains forced researchers to focus on smaller regions. Due to restricted access to a large fraction of neurons only small or superficial circuits, e.g. in the cortex, can be examined. More suitable model organisms for circuit studies would be optically accessible at large scale due to their small body and brain size as well as more advantageous optical properties.

Over the past few decades advanced optical recording techniques combined with electrophysiological methods and computational neuroscience enabled detailed description of endogenous and evoked network activity. At the same time, non-standard model organisms have become indispensable for improving our understanding in systems neuroscience. Enabling sufficient optical access to the majority of their neurons the full potential of advanced imaging techniques such as multi-photon microscopy as well as anatomical and functional probes could be exploited for network studies at large scale.

Two paramount model species for holistic neuronal network studies are the invertebrates *Caenorhabditis elegans* (*C. elegans*) and *Drosophila melanogaster* (*Drosophila m.*). For *C. elegans* the entire nervous system of the 1 mm small hermaphrodite nematode with only 302 neurons has been reconstructed from electron microscopy data sets in the late eighties already (White et al., 1986, Durbin, 1987). Their unique experimental tractability combined with relatively sophisticated behaviours and available genetic toolkits allow calcium imaging in the entire nervous system in unprecedented detail (Schrödel et al., 2013). This promises to uncover universal operational principles across nervous systems of different species. In the much more complex *Drosophila m.*, one of the biggest whole-brain image data sets at nano-scale resolution has been captured offering a view of all cells and connections (Zheng et al., 2018). Currently, the connectome of the adult brain counting 135'000 neurons in total has been reconstructed (Xu et al., 2020) as well as that of larvae (Schneider-Mizell et al., 2016, Gerhard et al., 2017). Although not transparent as *C. elegans*, the fruit fly is suited for investigating the neural circuit basis of behaviour due to its simplicity, small size and genetic tractability that resulted in a large set of transgenic lines with specific cell subset labeling. However, removing the proboscis, antennae, trachea and the surrounding cuticle and fat is mostly required to optically expose the central brain. In conclusion, exhaustive works in invertebrates have improved our understanding on how small and local circuits collectively influence bigger networks. However, explaining global network states in more complex brains as those of vertebrates has so far remained relatively challenging.

For studying neuronal networks in the vertebrate brain, the larval zebrafish proved a groundbreaking model organism enabling functional whole-brain imaging recordings (Ahrens et al., 2012, Ahrens et al., 2013, Naumann et al., 2016 see figure 1.3). As for *Drosophila*, the entire larval zebrafish brain was recorded at nano-scale resolution using serial electron microscopy (ssEM, Hildebrand et al., 2017). Homologies between the mammalian and teleost brain areas have been established based on topological and functional data (Wullimann and Mueller, 2004). Exploiting gene-editing techniques as Tol2-mediated transgenesis and subtype-specific promoters (Kawakami et al., 2004) allowed monitoring neural circuit activity and optogenetic manipulations to assess causal relationships between neural patterns and behaviour (Suster et al., 2009, Baier and Scott, 2009). Larval zebrafish were seen to perform short-term memory, associative learning and social learning in simple behavioural paradigms, promising surprisingly powerful potential of the teleost brain (Roberts et al., 2013). However, one major disadvantage in zebrafish is the developing skull after early developmental stages, which diminishes optical penetration and the detection of activity signals from single neurons in deeper brain areas. As a result we can only study the whole brain of larval zebrafish which limits our understanding to how immature networks function during relatively simple behaviours.

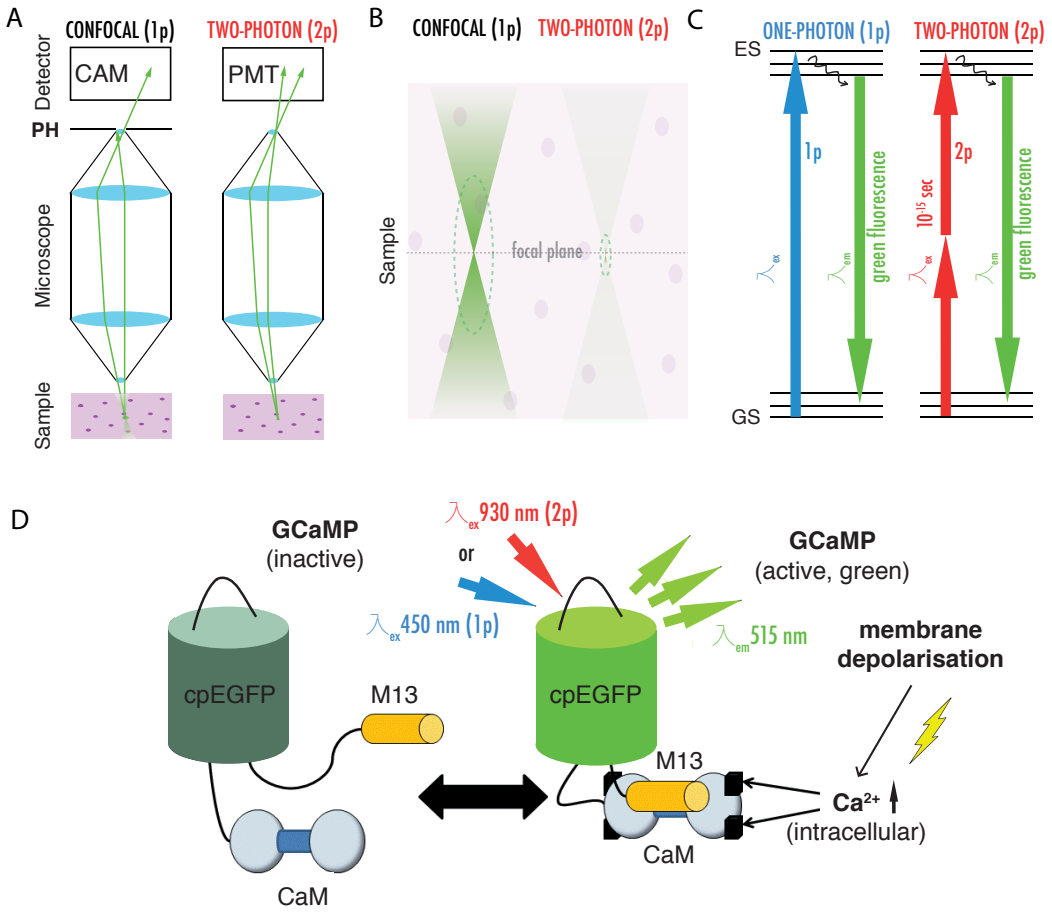

Figure 1.2: Mechanisms of one and two-photon microscopy and the calcium sensor GCaMP for neuronal activity labeling. **A** One-photon (1p) confocal microscopy uses a pinhole (PH) to increase optical resolution by detaining out-of-focus light before it can enter the detector. Two-photon microscopy (2p) in contrast takes advantage of the non-linear effects of photon absorption. 2p-microscopy excites small volumes in the focal plane, from which emitted light is being consecutively detected in single pixels on photomultiplier tubes (PMTs). **B** One-photon microscopy excites relatively large volumes of tissue above and below the focal plane, promoting photobleaching of out-of focus fluorophores and background noise. Two-photon excitation is mostly limited to the focal plane, presenting an advantage over confocal microscopy. **C** Jablonski diagram of 1p (left) and 2p (right) excitation of a green fluorescent molecule. On the left, one single photon of visible blue light excites a fluorophore from its ground state (GS) to its electronic state (ES). After losing energy due to non-radiative transitions within the electronic states (wavy arrow indicates relaxation) green fluorescent light is being emitted. The energy of fluorescent photons falling back to ground state is lower than the excitation energy. On the right, following the same principle two-photons of lower energy (infrared range) are necessary to excite the molecule to its electronic states in order to create fluorescent emission. Both photons need to be absorbed within a few femtoseconds. Hence, highly energetic lasers are required for pumping high photon density pulses. **D** The calcium-sensor GCaMP consists of a circularly permuted EGFP bound to the calcium-binding protein calmodulin and an interface molecule called M13. Membrane-depolarisation causes neuronal calcium influx which induces fluorescence based on a conformational change of the calmodulin-M13 complex activating cpEGFP via a linker protein (see closer proximity between components on the right) increasing the fluorescent properties of the protein. Independent of 1p or 2p excitation in the visible blue (450 nm) or infrared (930 nm) regime GCaMP emits green fluorescent light with peak intensities at around 515 nm.

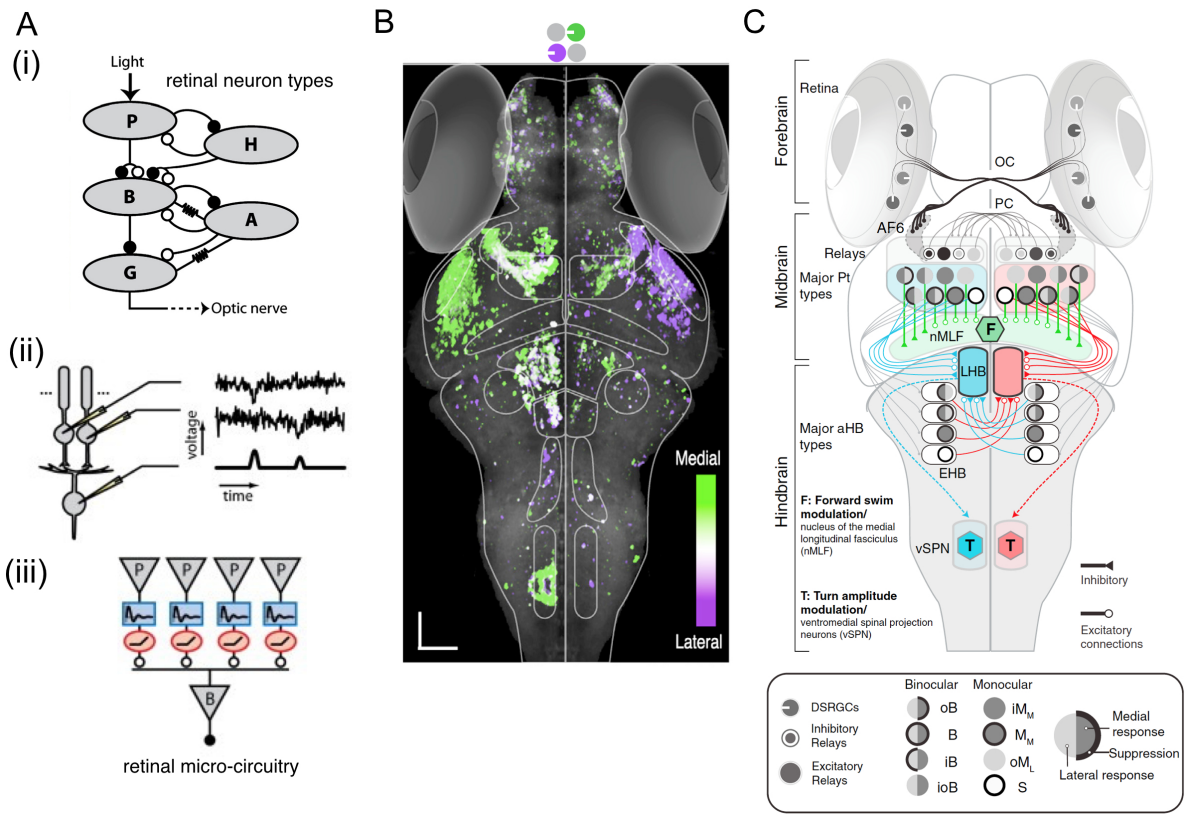

Figure 1.3: Mechanistic network functions investigated on sub-cellular scale in the murine retina (left) and on single-cell/ population-scale across the whole brain of larval zebrafish (middle, right).
A (i) to (iii) Computational mechanism within a local, sensory neural network in the retina (reprinted from Gollisch and Meister, 2010) **A(i)** Schematic drawing of the connections between the five classes of retinal neurons (photoreceptors (P), horizontal cells (H), bipolar cells (B), amacrine cells (A), and ganglion cells (G)). **A(ii)** Retinal micro-circuit performing computational task after dim light stimulation. Accumulated noise in rod photoreceptors causes distinct activation in upstream bipolar cells as a response to single photons embedded in noise. Voltage traces show distinct photon responses. **A(iii)** Circuit diagram shows temporal band-pass filtering and thresholding in photoreceptors before forwarding the summed photon signal to bipolar cells (circuit signs: neuron (triangle), temporal filter function (rectangle), instantaneous rectifier (oval), sign-preserving/inverting synapse (closed/open circle). Processing sequence in this micro-circuit has been seen to cause selective neuronal response only if sufficient evidence is encountered that a photon event has occurred. **B to C** Whole-brain calcium imaging combined with behavioural analysis for modeling a sensorimotor circuit for optomotor response (OMR) in the larval zebrafish (reprinted from Naumann et al., 2016). **B** Correlated activity map for monocular stimulation with leftward moving bar gratings recorded with two-photon imaging. **C** Quantitative whole-brain circuit model for OMR showing diverse neural response types for differential eye-specific and direction-specific motion processing (legend: arborization field 6 (AF6), pretectum (Pt), nucleus of the medial longitudinal fasciculus (nMLF), anterior hindbrain (aHB), region in rhombomere 1 (RoL), anterior rhombencephalic turning region (ARTR), ventral spinal projection neurons (vSPNs)).

1.2.2 Limits of current approaches and what we need

In summary, to understand neuronal networks in the adult vertebrate, scientists were so far limited to local circuits at small scale. One example are local microcircuits in the murine retina (see figure 1.3, A (i) to (iii)). In contrast, studies of large and distributed circuits could so far only be implemented in the fully developed invertebrate or larval vertebrate brain. Whereas the entire nervous system of *C. elegans* can be monitored during simple behaviours, it shows adverse differences in anatomical organisation

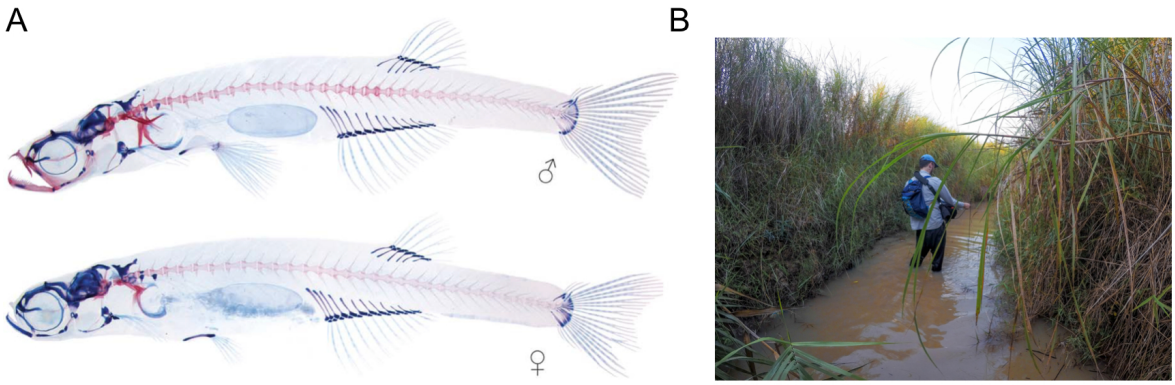

Figure 1.4: *Danionella dracula* bone structure and the DT habitat.
A) The lateral view of male and female *Danionella dracula* skeletons cleared and double stained for bone (alizarin red) and cartilage (alcian blue) showing paedomorphosis (reprinted from Britz and Conway, 2016). **B)** Side branches of the lower Bago river between Yangon and Bago in Myanmar, natural home to *Danionella translucida*.

and behavioural complexity as an invertebrate compared with vertebrate organisms. For larval zebrafish a major drawback is their time-limited translucency that enables whole-brain studies only during early developmental stages (see figure 1.3, B and C) withholding information on how the fully wired vertebrate brain functions during maturity. Yet, what is currently missing is a combination of imaging method and well-suited model organism that allows for studying the functioning of brain-spanning neuronal networks in the adult vertebrate.

1.3 *Danionella translucida* as a potential model organism for circuit studies

For a neuroscientist interested in how distributed neuronal populations interact to process information in vertebrates, the most convenient model organism would offer access to its neuronal activity at temporally and spatially fine resolution across its whole brain. The ideal model organism would enable whole-brain neuronal imaging in vertebrates even during adulthood. The solution to this challenge would be a species that combined optical access and small size in adulthood with a rich behavioural repertoire. Being able to introduce transgenes into model organisms was instrumental to many major advances in neurosciences in the last years (Luo et al., 2018). Therefore, the ideal model species, would also be amenable to genetic manipulation, such as the introduction of fluorescent activity reporters or the targeted knock-out of unwanted genes. Here, I'm introducing a new non-standard model for studying neuronal networks that possesses exactly these highly beneficial properties: *Danionella translucida* (DT).

1.3.1 What was known about DT before

Danionella translucida is a small freshwater fish from Myanmar and as a member of the danioin cyprinids a close relative of the established model organism, zebrafish. DT live in side branches of rivers such as the Bago, where secondary waters can be relatively shallow, turbid and weakly torrential, depending on seasonal rainfall. DT was found to be among the smallest living vertebrates with a size mostly below 12 mm (Roberts, 1986). Britz et al. suggested that Danionella's miniaturised size might be associated with their paedomorphic bone development, resulting in a mostly cartilaginous skeleton (Britz and Conway, 2009, Britz et al., 2009, Britz et al., 2014, Britz and Conway, 2016). Danionella's lifelong near-transparency is a remarkable optical property and the underlying reason for its species' sub-group name *translucida*.

1.3.2 Goals of the study

The primary goal of this thesis is to establish *Danionella translucida* as a new tool for studying how neural networks, widely distributed across the adult vertebrate brain, process sensory information and control behaviour. The properties of DT already mentioned make it a very promising tool for circuit neuroscience based on optical imaging. However, several challenges have to be overcome before DT could be applied for studying the functioning of the vertebrate brain. (i) As an initial step it is crucial to demonstrate that DT can be kept and bred in the laboratory, requiring the development of strategies for breeding and husbandry. (ii) We need to uncover DT to exhibit interesting, biologically relevant behaviours under the controlled conditions of the laboratory. (iii) Because genetic manipulations are instrumental to many modern approaches in systems neuroscience, we need to explore and establish DT's genetic tractability. (iv) Lastly, because we intend to perform imaging experiments over extended periods of time, we need to develop an immobilization strategy compatible with modern imaging techniques.

CHAPTER 2

RESULTS

2.1 Summary of the study results

In Schulze et al., 2018 I present a study in which, together with my colleagues, I tackled and solved these challenges, and established DT as a new model organism in the laboratory suited for circuit studies in the adult vertebrate brain. To establish DT in the laboratory, we first explored strategies to breed, rear and hold DT outside of their natural habitat. Therefore, refining established zebrafish protocols and observing DT for behaviours different from zebrafish for tailoring breeding conditions were crucial. Different from zebrafish that can be set up in reproductive tanks for pairing, we found the communal breeding DT to be a crevice spawner (Gale and Gale, 1977) laying a smaller quantity of eggs, organised as clutches. To promote DT egg laying, we installed spawning tubes that enabled DT to enter and hide in breeding tanks. As a result we gained an increased quantity of eggs. Larval upbringing was explored and optimised by developing a rearing strategy with rotifers for live feeding and minimum flow in between day 5 to 10 post fertilisation. Developing *Danionella* rearing and husbandry as an initial step enabled establishing a stable colony and the introduction of DT as a laboratory model. To characterise the small DT brain size in detail a high resolution MRI was used from which the exact brain volume was determined to be 0.6 µL. Further, we found the basic DT neuroanatomy to be conserved across teleosts analysing Nissl-stained slices, which also showed the absence of an ossified skull in the adult animal. Employing an automated cell detection scheme determined the number of neurons in the adult brain to be in the range of 650'000. We further tested DT for behaviours known to require maturity. Despite it's miniaturised dimension during adulthood we found DT show a rich behavioural repertoire. After observing DT to generate acoustic signals, we characterised their vocalisation behaviour regarding its time of occurrence, potential function and temporal features. We found only male DT to vocalise which happens predominantly during the first hours of the day coinciding with the reproductive peak time in our colony. Moreover, vocalisation seems to be motivated socially and could be linked to fighting behaviour in between males. Finally, we characterised vocalisation patterns to consist of short, steeply ongoing pulses (< 2 ms) that occur at two predominant frequencies (60 Hz and 120 Hz). Pulse repetitions often last only 30 ms (3-4 pulses, called bursts). In rare cases however these groups of pulses were seen to last up to a few minutes, which we termed burst trains. Remarkably, DT vocalisation complements the behavioural repertoire of adult zebrafish and suggests to play a role in acoustic communication. As a result further optical experimentation focused on laying the groundwork for studying the auditory circuit in response to conspecific vocalisation. Two other collective behaviours which occur only during later developmental stages are shoaling and schooling. Shoaling and schooling describe swimming at close proximity within a group but only schooling unfolds more coordinated with body axis alignment leading to seemingly choreographed swimming. We showed that DT accomplishes both behaviours beneficial for predator avoidance, foraging and mating. The ability to execute shoaling and schooling was disrupted in case of darkness and indicated them to be clearly visually mediated. For tackling genetic manipulation in the zygote the establishment of earlier mentioned reproduction settings was crucial and enabled microinjections at an efficient quantity. Therefore, genetic techniques could be implemented during a short

experimental time window (of three hours in the morning). We showed that two transgenic approaches in the DT work to generate transgenics by adapting Tol2-mediated gene insertion and Crispr/Cas9-evoked gene disruption (Kawakami et al., 2004, Ran et al., 2013). Tol2-mediated transgenesis was successfully implemented for introducing the fluorescent calcium indicator GCaMP6f into the DT genome. In the zebrafish GCaMP6f has been shown to be panneuronally expressed under the promoter NeuroD. Pursuing the same goal, we used the GCaMP6f:NeuroD plasmid (provided by the Wyart lab that studies the zebrafish) and achieved sparse and mosaic signal across the DT brain with relatively strong expression in the cerebellum and telencephalon. To further increase DT's optical transparency and reduce the chance of light-induced heating and tissue damage during imaging, we set out to eliminate sparse melanophore pigmentation. We successfully tackled this challenge by generating an Illumina short-read genome sequencing library and implementing Crispr/Cas9-mediated gene knockdown of the pigmentation enzyme tyrosinase. As a result we obtained fully depigmented DT. Taking direct advantage of DT's genetic tractability we used GCaMP6f expressing fish to demonstrate them to be a suited and potent model for optical investigations. As a proof-of principle experiment, I conducted single-cell calcium imaging using two-photon microscopy in transiently GCaMP6f-expressing DT. Besides spontaneous activity in the telencephalon I recorded sensory stimulus-evoked activity in the hindbrain. As acoustic vocalisation in DT indicates an inherent relevance for auditory processing of conspecific sounds I used an acoustic cue that mimicked conspecific vocalisation for sensory stimulation. This work has been published in Nature Methods - Schulze et al., 2018. Within this cumulative thesis the publication approach and results are being aggregated within the frame of the Charité's top-journal promotion format. More details can be found in the publication attached (see chapter 6).

CHAPTER 3

DISCUSSION

During this thesis project, I introduced *Danionella translucida* as a new model organism (see chapter 6). The aim of this thesis was to establish a new model organism in neuroscience, *Danionella translucida*, such that it could be used by laboratories interested in neuronal circuit studies. This miniaturised and transparent teleost displays rich collective behaviours and is genetically tractable, thereby creating a range of new opportunities for studying the functioning of an adult vertebrate brain at single cell resolution.

3.1 Discussion and outlook

My initial achievements during this thesis were establishing rearing and husbandry in the laboratory and characterising DT morphology. Afterwards, I analysed DT's behavioural repertoire to tackle the first goal in systems neuroscience, finding rich DT behaviours. Secondly, I tested and adapted genetic tools that enable the introduction and knock-out of genes for adjusting DT for imaging studies. And thirdly, I implemented one such imaging study in a proof-of-principle experiment showing spontaneous and stimulus-correlated neural activity. I here briefly discuss those steps with addition of potential improvements for future experiments.

I developed spawning and reproduction conditions based on communal husbandry tanks that make DT simple to rear and easy to adapt, in particular for laboratories that have zebrafish colonies already established. With the smallest known vertebrate brain, detected in a high resolution MRI, DT promises to facilitate the first adult vertebrate whole-brain connectome studies.

Despite having the smallest vertebrate brain, I found that DT are capable of complex and collective behaviours - such as vocalisation, shoaling, schooling and sexual behaviour - displaying a behavioural repertoire unmatched by larval zebrafish. Meeting this primary requirement in systems neuroscience, their remarkable ability to vocalise is particularly interesting, because it strongly suggests that these fish are able to hear and that their brains process acoustic communication signals. Therefore, in combination with optical imaging of neural activity it might be possible to study their auditory processing in individual cells across their entire brain. My work clearly demonstrated visually mediated behaviour when testing coherent swimming in groups during light and darkness. Performing visually mediated shoaling and schooling, despite their naturally turbid habitat, further favours future circuit studies to target visual processing and the underlying mechanisms. Its transparency across the entire body including the trunk fosters network studies throughout the central and peripheral nervous system.

The second main goal during this thesis was to achieve genetic modification in DT in respect of experimental applications, a key requirement for model organisms. I tailored DT's phenotype for optical experimentation using two-photon calcium imaging. The here achieved ability to genetically manipulate DT strongly encourages neurobiological studies e.g. optical studies of circuit anatomy and functioning in DT. The late developmental stage beneficial for studies of matured brains and behaviour posed a challenge for pervasive expression patterns due to increasingly differentiated cells across the brain.

Achieving dense panneuronal expression of fluorescent genes will be time-consuming and a task ideally shared by multiple laboratories to accelerate the expansion and manifestation of DT as a widely-used and easily accessible model system. Different panneuronal, ubiquitous and subpopulation-specific promoters will have to be tested. Aiming to promote the generation of many different transgenic lines, together with my colleague Mykola Kadobianskyi, I have generated a whole genome model for DT based on genome and transcriptome sequencing (published in Nature Scientific Data - Kadobianskyi et al., 2019).

Thirdly, I aimed to demonstrate DT's suitability for studying neuronal activity using two-photon calcium imaging. I performed a proof-of-principle experiment imaging single neuron activity in the DT brain and clearly demonstrated that DT is highly qualified for these kind of studies. The here proposed imaging immobilisation will have to be adjusted for testing other behaviours that require responsive movements e.g. motor behaviours in order to read out behavioural responses during neuronal activity recordings in the tethered animal. Two-photon activity traces showed extended decay times due to relatively slow GCamp6f kinetics. Similar kinetics can be observed in zebrafish using the same calcium sensor. To tackle relatively slow GCaMP6f kinetics, faster activity reporters can be used in the future. The new GCamp6f(u) has four times faster decay times than GC6f (Helassa et al., 2016) and the new jGCaMP7 allows better detection of individual spikes in bigger neuron populations (Hod Dana, 2019). With their continuous refinement GEVIs become more attractive for functional circuits studies. For example, Voltron shows improved brightness and photo-stability promising extended imaging durations (Abdelfattah et al., 2019) and ASAP-3 targets somata enabling sub-millisecond activation kinetics and repetitive sampling, which could be shown using 2p microscopy in behaving mice (Villette et al., 2019). With the smallest among vertebrate brains, we were able to optically access at least half of Danionella's brain volume at low excitation intensities (less than 15 mW). To retrieve fluorescent signals from the more ventral brain regions, at more than about 700 micron in depth, adaptive optics (Papadopoulos et al., 2017) or longer wavelengths as in tree-photon microscopy (Ouzounov et al., 2017) might be used in future experiments. For auditory stimulation we here used a simple DT vocalisation mimic to probe neural activity related to sensory processing. The ascending auditory pathway has been shown to be conserved across taxa (Mueller, 2012). Regarding future studies of the fully developed, ascending auditory pathway for processing conspecific vocalisation a range of acoustic DT mimics can be used. Therefore, the distinct nature of DT vocal patterns facilitate the generation of auditory cues and enable strategic stimulus presentations. Until transgenic lines in their many flavors are readily available, synthetic dyes e.g dextran- or AM-conjugated activity-independent or activity-indicating fluorophores can be locally injected for anatomical and functional studies during future experiments. Alternatively, virus-mediated tracing has been shown to work in larval zebrafish (Zou et al., 2014) and could be a promising tool for studying DT neuroconnectivity and neural activity, independent of successful transgene expression in targeted subpopulations.

To fully understand activity data from individual cells and populations, neuronal tracing studies will help to identify neuronal connectivities within networks. Once volumetric, anatomical whole-brain data have been acquired, these can be used to generate a DT brain atlas similar to the Z-brain (Randlett et al., 2015) and ZBB (Marquart et al., 2015) generated for zebrafish. Such an atlas enables registering an individual's brain anatomy to a general template and allows for comparing functional activity data across individuals.

Lastly, I would like to mention a non-neuroscientific research area as a potential field of study for DT enriching the scene of non standard model organism. DT could be an interesting addition to the set of gerontology models for comparative studies. Body size and life expectancy typically correlate across taxa (Kirkwood and Austad, 2000). The average mouse lives for about 3 years which corresponds to roughly 80 years lifespan in humans (Flurkey et al., 2007). Being more than a magnitude of order smaller than the *Mus musculus*, DT show extreme longevity with a lifetime of about 4 years. Thus, DT could be an interesting lab system for the study of aging, especially when combined with non-invasive in vivo imaging of cells across the whole body. In conclusion, DT's remarkably small size, rich and sophisticated behaviour and genetic access make it a promising model organism for neural circuit studies and possibly further biological fields of research.

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CHAPTER 4

EIGENSTÄNDIGKEITSERKLÄRUNG MIT EIDESSTATTLICHER VERSICHERUNG

Ich, Lisanne Schulze, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: "Establishing a new, genetically tractable vertebrate brain model with rich behaviour - *Danionella translucida*."/ "*Danionella translucida*, ein neuer, genetisch manipulierbarer Modelorganismus mit komplexem Verhalten zum Studieren des Vertebraten-Gehirns." selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe. Alle Stellen, die wörtlich oder dem Sinne nach auf Publikationen oder Vorträgen anderer Autoren/innen beruhen, sind als solche in korrekter Zitierung kenntlich gemacht. Die Abschnitte zu Methodik (insbesondere praktische Arbeiten, Laborbestimmungen, statistische Aufarbeitung) und Resultaten (insbesondere Abbildungen, Graphiken und Tabellen) werden von mir verantwortet. Ich versichere ferner, dass ich die in Zusammenarbeit mit anderen Personen generierten Daten, Datenauswertungen und Schlussfolgerungen korrekt gekennzeichnet und meinen eigenen Beitrag sowie die Beiträge anderer Personen korrekt kenntlich gemacht habe (siehe Anteilserklärung/ Contributions). Texte oder Textteile, die gemeinsam mit anderen erstellt oder verwendet wurden, habe ich korrekt kenntlich gemacht. Meine Anteile an etwaigen Publikationen zu dieser Dissertation entsprechen denen, die in der untenstehenden gemeinsamen Erklärung mit dem Erstbetreuer angegeben sind. Für sämtliche im Rahmen der Dissertation entstandenen Publikationen wurden die Richtlinien des ICMJE (International Committee of Medical Journal Editors; www.icmje.og) zur Autorenschaft eingehalten. Ich erkläre ferner, dass ich mich zur Einhaltung der Satzung der Charité – Universitätsmedizin Berlin zur Sicherung Guter Wissenschaftlicher Praxis verpflichte. Weiterhin versichere ich, dass ich diese Dissertation weder in gleicher noch in ähnlicher Form bereits an einer anderen Fakultät eingereicht habe. Die Bedeutung dieser eidesstattlichen Versicherung und die strafrechtlichen Folgen einer unwahren eidesstattlichen Versicherung (§§156, 161 des Strafgesetzbuches) sind mir bekannt und bewusst."

Signature (graduate student, Lisanne Schulze):

Berlin, 29. January 2021

Die hier eingereichte Dissertation mit dem Thema: **"Establishing a new, genetically tractable vertebrate brain model with rich behaviour - *Danionella translucida*."/ "*Danionella translucida*, ein transparenter und genetisch manipulierbarer Modelorganismus mit komplexem Verhalten zum Studium des Vertebraten-Gehirns."** basiert auf der Arbeit publiziert im Journal Nature Methods. **Die Erstautorschaft wird geteilt mit Joerg Henninger.** Wesentliche Teile der Arbeit zur Etablierung eines neuen Modelorganismus im Bereich Neuroscience wurden von Lisanne Schulze selbststaendig durchgefuehrt.

Signature (Benjamin Judkewitz, stamp)

Signature (Joerg Henninger)

Signature (Lisanne Schulze)

Lisanne Schulze war hauptverantwortliche Wissenschaftlerin fuer die Publikation Schulze et al., 2018:
Lisanne Schulze, Jörg Henninger, Mykola Kadobianskyi, Thomas Chaigne, Ana Isabel Faustino, Nahid Hakiy, Shahad Albadri, Markus Schuelke, Leonard Maler, Filippo Del Bene, Benjamin Judkewitz, Transparent Danionella translucida as a genetically tractable vertebrate brain model, 2018, Nature Methods

Beitrag im Einzelnen zur Etablierung des neuen Modelorganismus *Danionella translucida*:

Lisanne hat die Einführung von DT in das Labor vollzogen, wobei sie künstliche Haltungsbedingungen, Fortpflanzungsstrategien und die Möglichkeit zur Aufzucht identifiziert hat. Durch das Analysieren von DT Reproduktionverhalten ermöglichte sie den Aufbau einer Laborpopulation sowie die Grundlagenschaffung zur transgenen Veränderung in der Zygote. Die Analyse von Gehirnvolumen erfolgte durch die Nutzung des Kleintier MRTs an der Charite. Die Anzahl von Neuronen im DT Gehirn wurde von ihr in gefärbten slices unter der Anfertigung und Nutzung eines automatisierten Detektier- und Quantifizierungsschemas in MATLAB bewerkstelligt, wobei dieser Code mit Unterstuetzung von Thomas Chaigne entworfen wurde. Die Anaylse neuronanatomischer Areale und Konservierung wurde von ihr basierend auf der Untersuchung Nissl-gefärbter slices im Vergleich mit Daten anderer teleoster Fische (Topologischer Atlas, Wullimann 1996) und mit Unterstuetzung von Leonard Maler durchgefuehrt. Lisanne hat bei DT Vokalisierungsverhalten identifiziert. Das Analysieren und Quantifizieren von Vokalisierungseigenschaften in Python wurde in Kooperation mit Jörg Henninger durchgefuehrt (Analyse allgemeiner Vokalisierungsmuster, Quantifizierung von Vokalisierungsfrequenzen und -intervallen, Quantifizierung von Vokalisierungspulsen zur Identifikation von Vokalisierungszeiten, Identifikation eines Verhaltensdimorphismus auf dem basierend nur männliche DT vokalisieren, Untersuchung einer sozialen Funktion von Vokalisierungsverhalten in Gruppentanks, Analyse männlichen Aggressionsverhaltens im Zusammenhang mit Vokalisierung). In Kooperation mit Ana Faustino wurden Video- und Audioaufnahmen blind auf Kampfverhalten und Vokalisierungssignale getestet. In Rücksprache mit Thomas Chaigne wurde ein MATLAB Skript zur Quantifizierung und statistischen Auswertung des koordinierten Schwimmverhaltens erstellt. Das Erlernen der Tol2-Transgenese Technik erfolgte im Wyart Lab Paris (ICM). Daraufhin hat Lisanne diese Technick im Judkewitz Labor etabliert und die erfolgreiche Erstellung erster transgener DT mit Expression eines Aktivitätsmarkers (GCaMP fuer calcium imaging) erzielt. Das Erzeugen einer Illumia Sequenzierungs Bibliothek (finanziell gefördert durch Markus Schuelke) wurde zusammen

mit Mykola Kadobianskyi durchgeführt. Ebenso das Anfertigen von spezifischen single-guideRNAs zur gezielten Genmanipulation mit Crispr/Cas9 zum Erreichen transgener, depigmentierter DT (Crispr/Cas9 Materialien und Diskussionen zum Thema von und mit Filippo Del Bene und Shahad Albadri). Injektionen wurden zeitweise parallel mit Nahid Hakiy durchgeführt, um das Generieren, Screenen und Aufziehen transgener Tiere zu beschleunigen. Der Aufbau eines custom Zwei-photonen Mikroskops erfolgte zusammen mit Benjamin Judkewitz, Ioannis Papadopoulos und Maximilian Hoffmann. Das Entwerfen einer stabilen Immobilisierungsmethode zur optischen Untersuchung adulter DT über mehrere Stunden sowie optische Untersuchung neuronal Aktivitaet unter dem Zwei-Photonen Mikroskop wurden selbststaenidg etabliert, durchgefürht und analysiert. Lisanne war die Hauptverantwortliche bei der Einführung von DT in das Labor und der Etablierung des neuen Modelorganismus für circuit studies. Sie hat vorrangig und essentiell dazu beigetragen die Forschungsarbeit an der DT im Judkewitzlabor aufzubauen und für andere Labore vorzubereiten. Zur vollständigen Transparenz werden im obigen Text besonders Kooperationen aufgeführt.

Signature, date, stamp (PI, Benjamin Judkewitz)

Signature, date (graduate student, Lisanne Schulze)

CHAPTER 5

JOURNAL SUMMARY LIST

The here described project was published in **Nature Methods** (Schulze et al., 2018), number one out of seventy-nine among the following journals listed. Hence, this cumulative thesis is being handed in as a top-journal promotion according to Charité's standards.

Journal Data Filtered By: **Selected JCR Year: 2018** Selected Editions: SCIE,SSCI Selected Categories: **"BIOCHEMICAL RESEARCH METHODS"** Selected Category Scheme: WoS

Gesamtanzahl: 79 Journale

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
1	NATURE METHODS	64,324	28.467	0.205090
2	Nature Protocols	40,341	11.334	0.081330
3	BRIEFINGS IN BIOINFORMATICS	5,750	9.101	0.018020
4	CURRENT OPINION IN BIOTECHNOLOGY	15,326	8.083	0.023020
5	LAB ON A CHIP	31,678	6.914	0.046810
6	ACS Synthetic Biology	4,890	5.571	0.019270
7	MOLECULAR & CELLULAR PROTEOMICS	17,603	4.828	0.038550
8	BIOINFORMATICS	107,600	4.531	0.205400
9	PLoS Computational Biology	26,646	4.428	0.085350
10	BIOCONJUGATE CHEMISTRY	15,840	4.349	0.020240
11	BIOLOGICAL PROCEDURES ONLINE	762	4.094	0.000830
12	Biomedical Optics Express	9,547	3.910	0.021750
13	JOURNAL OF CHROMATOGRAPHY A	61,427	3.858	0.044730
14	METHODS	21,871	3.782	0.025700
15	JOURNAL OF PROTEOME RESEARCH	21,216	3.780	0.035820
16	Journal of Biophotonics	3,309	3.763	0.006210
17	New Biotechnology	2,938	3.739	0.004640
18	Biotechnology Journal	5,615	3.543	0.009790
19	Journal of Proteomics	9,688	3.537	0.020630
20	CYTOMETRY PART A	4,326	3.433	0.007370

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
21	ANALYTICAL AND BIOANALYTICAL CHEMISTRY	30,057	3.286	0.035910
22	Acta Crystallographica Section D-Structural Biology	21,054	3.227	0.018480
23	Plant Methods	2,605	3.170	0.004780
24	PROTEOMICS	14,097	3.106	0.019090
25	Journal of Breath Research	1,741	3.000	0.002700
26	Expert Review of Proteomics	1,749	2.963	0.003340
27	IEEE-ACM Transactions on Computational Biology and Bioinformatics	2,781	2.896	0.005850
28	Clinical Proteomics	720	2.892	0.001940
29	JOURNAL OF CHROMATOGRAPHY B-ANALYTICAL TECHNOLOGIES IN THE BIOMEDICAL AND LIFE SCIENCES	22,077	2.813	0.019690
30	Drug Testing and Analysis	2,705	2.799	0.005180
31	JOURNAL OF NEUROSCIENCE METHODS	17,224	2.785	0.016110
32	ELECTROPHORESIS	13,528	2.754	0.010850
33	JOURNAL OF MAGNETIC RESONANCE	11,633	2.689	0.011020
34	Journal of Biological Engineering	1,076	2.667	0.002110
35	JOURNAL OF BIOMEDICAL OPTICS	13,787	2.555	0.016940
36	Biomicrofluidics	3,914	2.531	0.007650
37	BMC BIOINFORMATICS	30,607	2.511	0.045720
37	MOLECULAR AND CELLULAR PROBES	1,950	2.511	0.002110
39	ANALYTICAL BIOCHEMISTRY	38,439	2.507	0.013800
40	Proteomics Clinical Applications	2,002	2.324	0.003700

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
41	Bioanalysis	3,331	2.321	0.005560
42	JOURNAL OF BIOMOLECULAR SCREENING	2,934	2.297	0.004290
43	JOURNAL OF MASS SPECTROMETRY	5,129	2.267	0.004890
44	JALA	972	2.241	0.002040
45	SLAS Discovery	347	2.192	0.001130
46	SLAS Technology	188	2.048	0.000330
47	RAPID COMMUNICATIONS IN MASS SPECTROMETRY	11,867	2.045	0.009700
48	PHYTOCHEMICAL ANALYSIS	2,641	1.963	0.001970
49	BIOLOGICALS	1,511	1.960	0.002330
50	BioChip Journal	420	1.950	0.000720
51	Molecular Imaging	1,078	1.942	0.001190
52	IEEE TRANSACTIONS ON NANOBIOSCIENCE	1,450	1.927	0.002640
53	IET Nanobiotechnology	890	1.925	0.001000
54	JOURNAL OF FLUORESCENCE	4,053	1.913	0.003680
54	JOURNAL OF IMMUNOLOGICAL METHODS	11,710	1.913	0.007870
56	JOURNAL OF MOLECULAR GRAPHICS & MODELLING	7,117	1.863	0.003930
57	Methods in Enzymology	24,871	1.862	0.019180
58	TRANSGENIC RESEARCH	2,684	1.817	0.002920
59	JOURNAL OF MICROBIOLOGICAL METHODS	9,939	1.803	0.008350
60	Proteome Science	978	1.795	0.001090
61	BIOMEDICAL CHROMATOGRAPHY	4,216	1.748	0.004620

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
62	JOURNAL OF VIROLOGICAL METHODS	7,508	1.746	0.008670
63	BIOTECHNIQUES	6,451	1.659	0.003630
64	PLANT MOLECULAR BIOLOGY REPORTER	2,986	1.604	0.003320
65	Algorithms for Molecular Biology	719	1.585	0.001740
66	CHROMATOGRAPHIA	4,597	1.552	0.002930
67	COMBINATORIAL CHEMISTRY & HIGH THROUGHPUT SCREENING	1,414	1.503	0.001120
68	ASSAY AND DRUG DEVELOPMENT TECHNOLOGIES	1,207	1.420	0.001900
69	Journal of Spectroscopy	626	1.376	0.001410
70	JOURNAL OF LABELLED COMPOUNDS & RADIOPHARMACEUTICALS	1,825	1.291	0.001950
70	PROTEIN EXPRESSION AND PURIFICATION	5,010	1.291	0.004280
72	JOURNAL OF CHROMATOGRAPHIC SCIENCE	3,112	1.216	0.002900
73	Acta Crystallographica Section F-Structural Biology Communications	1,948	1.199	0.004400
74	Current Bioinformatics	442	1.189	0.000550
75	PREPARATIVE BIOCHEMISTRY & BIOTECHNOLOGY	817	1.117	0.001100
76	JOURNAL OF LIQUID CHROMATOGRAPHY & RELATED TECHNOLOGIES	2,569	0.987	0.001650
77	JOURNAL OF COMPUTATIONAL BIOLOGY	4,056	0.879	0.003300
78	Current Proteomics	216	0.768	0.000220
79	Methods in Microbiology	842	0.714	0.000270

CHAPTER 6

PUBLICATION

Publication: Transparent *Danionella translucida* as a genetically tractable vertebrate brain model

https://doi.org/10.1038/s41592-018-0144-6

https://doi.org/10.1038/s41592-018-0144-6

https://doi.org/10.1038/s41592-018-0144-6

https://doi.org/10.1038/s41592-018-0144-6

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CHAPTER 7

CURRICULUM VITAE

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

CHAPTER 8

PUBLIKATIONSLISTE

Publication references Lisanne Schulze:

Maass PG, Rump A, Schulz H, Stricker S, **Schulze L**, Platzer K, Aydin A, Tinschert S, Goldring MB,Luft FC et al. (2012) A misplaced lncRNA causes brachydactyly in humans.The Journal of clinical investigation 122:3990–4002

Schulze L, Henninger J, Kadobianskyi M, Chaigne T, Faustino AI, Hakiy N, Albadri S, Schuelke M,Maler L, Del Bene F et al. (2018) Transparent danionella translucida as a genetically tractable vertebrate brain model.Nature methods15:977 **(top-journal promotion)**

Kadobianskyi M, **Schulze L**, Schuelke M, Judkewitz B (2019) Hybrid genome assembly and annotation of danionella translucida.Scientific data6:1–7 **(not included in dissertation due to top-journal promotion)**

Acknowledgements

During my thesis I was supported and influenced by my PI Benjamin Judkewitz, whose aim it was to find the best model organisms available for imaging the vertebrate brain and study its neuronal networks. He was a major support during writing the first *Danionella translucida* paper. Basically equally crucial during my project, was my colleague Jörg Henninger, postdoc in the JLAB, and the second biological scientist joining the lab to introduce *Danionella translucida* to neuroscience. Acting in concert with me throughout most of my thesis project he was incredibly helpful with his patience and experience in analysing data similar to audio signals having worked with electric fish before. Additionally, I would like to thank Mykola Kadobianskyi for his investment of time and devotion during the shared sequencing project. He did a great job and was a loyal fellow in the lab. Thomas Chaigne was of great support for generating the scripts for automated neuron counting and analysing *Danionella t.* shoaling behaviour. As a specialist Ana Faustino was a perfect partner for discussing social behaviour in teleosts. Nahid Hakiy was an indispensable support, especially while starting the lab together seeking successful breeding and rearing strategies for *Danionella t.* as an inevitable first step. Filippo Del Bene and Shahad Albadri (Sorbonne University, Paris) provided us with their previous knowledge on using Crispr/Cas9 in the zebrafish. Having worked with fish over decades, Leonard Maler (University of Ottawa) shared his precious experience and knowledge on the teleost brain. Additionally, I would like to mention Ioannis Papadopoulos who started together with me in Benjamin's lab at the time there were two office tables for three people and no lab yet for being a great companion and guidance through the early phase of my PhD. I enjoyed the adventurous journey of setting up a new lab from scratch together with Benjamin and entering the world of a new model organisms for neuroscience. Apart from my lab members and cooperators I would further like to thank Claire Wyart and Eva Naumann for discussing *Danionella t.* so vividly and in Claire's case for inviting me to her lab in Paris to learn the experimental basics for genetic modifications in the teleost zygote. Last but not least, my deep gratitude to my partner for her patience and stamina to endure the obligatory phases of frustration and her commitment to encourage my perspectives, enlighten my mood or simply distract my mind. I'm very thankful for and happy about your incredibly weird brain.