Microscopy approaches in zebrafish

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In many scientific fields, research is reliant on microscopy to acquire, describe, and model fundamental processes. With this need for microscopy and multitudes of microscopy techniques existing, it can be challenging to identify the most suitable technique for a specific purpose. We here examine microscopy techniques to study cell fate control, vision development, and neuronal circuits covering various temporal, spatial, and computational scales in zebrafish.

Zebrafish has become a pivotal model organism across fields, providing new insights into our understanding from the cytoskeleton, over cells, to tissues, and organs. Characteristics such as high genomic similarity to humans and experimental accessibility to genetic manipulation allow in-depth studies and novel insights. However, the true strengths of zebrafish as model organism are that they develop *ex utero,* allowing them to be studied from the one-cell stage onwards, and that they are transparent, enabling non-invasive imaging from heartbeat to brain development [1]. Combining these features with a range of transgenic reporter lines has opened previously inconceivable opportunities to answer fundamental biological questions and push knowledge boundaries.

Due to these traits, the main type of microscopy used in zebrafish studies is light microscopy, mostly fluorescence-based and in more than two dimensions (2D). The produced microscopy data are highly complex with up to 6D, including space (x,y,z), wavelengths, time-points, and multi-points. Hence, a critical consideration is data handling and computational analysis to complement studies and reinform image acquisition, allowing truly data-driven microscopy.

We here examine the role of microscopy to study cell fate control, vision development, and neuronal circuits in zebrafish covering various temporal, spatial, and computational scales.

Light-sheet fluorescence microscopy to study early development

by Christiann Mosimann and Agnese Kocere (CU Anschutz, USA)

Even though zebrafish are a prime subject to study early development, capturing the complex coordination that forms the entire embryo in development has remained challenging due to the embryo's spherical geometry. The introduction of light-sheet fluorescence microscopy (LSFM) has enabled three-dimensional image acquisition of complex samples over time, making 4D-timelapse in multiple colours to capture developmental processes accessible [2]–[6].

LSFM applies a sheet of laser light that scans individual planes of a translucent sample, generating a sequence of optical sections that assemble to a 3D representation [3]. Illumination and acquisition are uncoupled and parallelised, enabling multi-lens setups to capture a sample suspended between lenses that can be rotated for 360-degree acquisition. In addition to capturing sample volume, the rapid scanning and distributed laser power result in significantly lower phototoxicity than confocal scanning microscopy, rendering LSFM well-suited to live-image developing zebrafish embryos with fluorescent reporters *in toto*. Consequently, LSFM of zebrafish embryos has revealed unprecedented details of vertebrate development, including details of endoderm migration, neuronal precursor paths, and heart tube formation [5]–[9].

Through an active, open-source-embracing LSFM community, the steps and components to build a growing variety of LSFMs have come into reach of interested labs worldwide. Initiatives like OpenSPIM (openspim.org) provide comprehensive parts lists, assembly instructions, and a growing user base to seek support [10], [11], [12]. Yet, pragmatically speaking, the complex optical assembly and maintenance of a custom LSFM setup are out of reach for most biology-focused researchers who wish to utilise rather than develop LSFM [10]: acquisition of the required parts is not trivial, several components require a precision workshop, and the entire build can take months from beginning to completion. Nonetheless, several commercial plug-and-play setups are available, with variable degrees of specialisations for select applications of imaging fixed or live cells, tissues, organoids, or embryos. To bring LSFM to the masses, the Flamingo LSFM (www.involv3d.org) can be shipped and loaned to interested labs for individual projects (currently USA only), reducing the limited access to LSFM hardware to shipping costs and desk space.

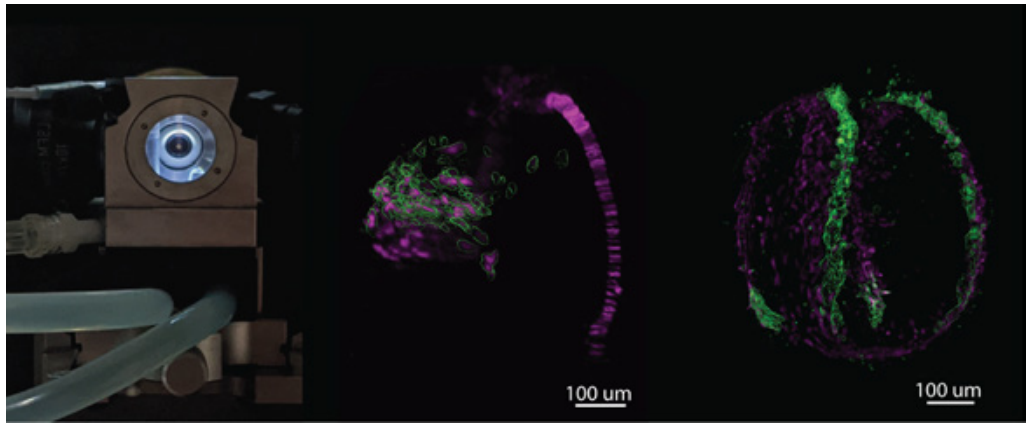

Figure 1.1.Through the looking glass: four-dimensional imaging of transgenic zebrafish embryos using the Zeiss Z.1 light sheet setup (left, with illuminated sample chamber). Engineered transgenic zebrafish expressing fluorescent reporters marking the notochord (middle panel, magenta) and hindbrain regions (magenta and green) at 24 hours of development, and the lateral plate mesoderm (right panel, magenta) and the progenitor cells forming the cardiovascular lineages (green) at 12 hours of development.

Our lab focuses on decoding the mechanisms of cardiovascular cell fate emergence from uncommitted lateral plate mesoderm in development and congenital disease [13]. We generate and apply novel transgenic zebrafish expressing fluorescent proteins under the control of gene-regulatory elements active in early progenitor cells. Once established through transgenesis, fluorescent reporters let us observe previously inaccessible developmental processes and cell types live in blue, green, or red fluorescence.

Mere LSFM users, we have been an early adopter of the commercial Zeiss Lightsheet Z.1 microscope that combines three lenses (two for illumination, one detection) with a climate-controlled sample chamber in a user-friendly box. With interchangeable detection lenses ranging from 10x to 63x, the Z.1 offers a versatile imaging platform for a variety of samples, but especially for developing zebrafish: suspended in agarose or in optically clear Fluorinated ethylene-propylene (FEP) tubing, we routinely image gastrulation-stage embryos (5.3 hours) as well as one-to-two day old zebrafish *in toto*, still or as time-lapse. Imaging transgenic reporter embryos, we captured the continuous formation of the zebrafish heart's ventricle, adding to previous observations based on imaging of fixed embryos [7]. 4D imaging and subsequent projections of spherical embryo data [5] also allow us to chart the emerging lateral plate mesoderm before it partitions into the heart, blood vessels, blood, and additional lineages [14].

Even though a significant tool in the arsenal of developmental biologists and zebrafish researchers, LSFM remains a complex application. Beyond challenging sample mounting and accessibility to fluorescent reporters that highlight the desired biological process, LSFM routinely generates exceedingly large datasets: multi-colour 4D imaging of one embryo (full capture for each channel at several angles every few minutes over hours) results in high triple-digit Gigabytes of imaging data – an ordinary desktop PC cannot handle the subsequent multiview alignment and post-processing [10]. Consequently, routine LSFM data assembly and output demand a significant investment into computational processing power, including a suite of software solutions to tackle individual steps ranging from open-source, such as ImageJ/Fiji-based plugins [15]–[17] or dedicated multiview packages to commercial programs such as Zeiss' ZEN, or Imaris. Training of new lab members in LSFM operation and analysis is a considerable endeavour that, due to its complexity, often takes longer than introductions to a conventional confocal microscope. While we all dream of big datasets, pragmatically speaking, individual lab members usually work with one-to-

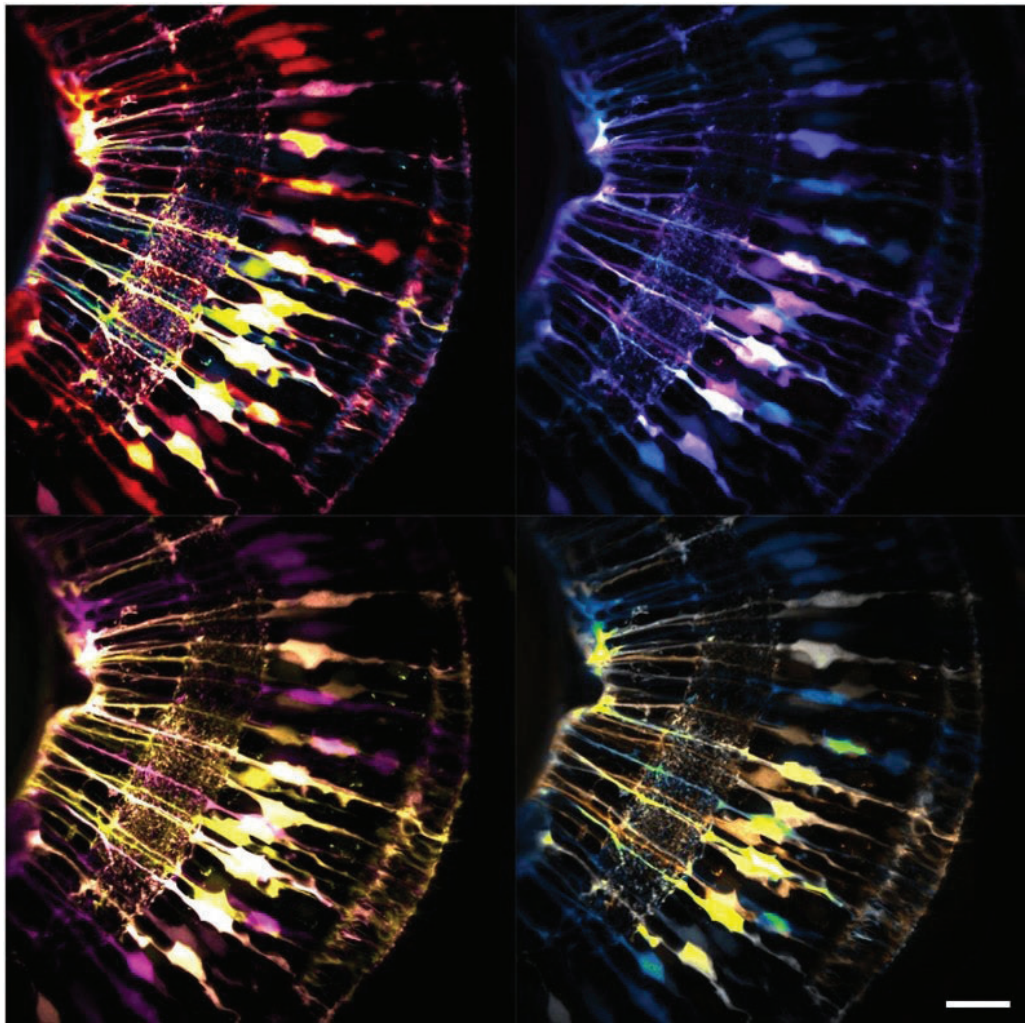

Figure 2.1. Visualisation of small-scale protrusions in MG in a 5-day old fish (scale bar 10µm).

three 4D datasets at a time and in series. While seemingly few, the processing time for each dataset, even without downstream analyses such as reporter co-expression, cell tracking, or morphometric analysis, occupies an imaging workstation for the duration of each job. Cloud or cluster computing services enable outsourcing of analyses, yet are not commonly in place for imaging-based analyses. The increasing growth in transcriptomics and genomics data, however, promise expansion of cloud computing, making it accessible for all applications in the future.

Despite these hurdles, LSFM adds a valuable dimension to live imaging using zebrafish. With the expansion of established fluorescent reporter transgenics, new staining methods, and increasingly accessible LSFM setups and expertise, zebrafish researchers have more opportunities than ever to illuminate early development.

AiryScan microscopy to study vision development

by Ryan B. MacDonald and Elisabeth Kugler (University College London, UK)

The retina, the light-sensitive tissue lining the back of our eyes, is constituted by various cell types that are highly interconnected to form visual circuits. Müller glia (MG) are specialised glial cells that are

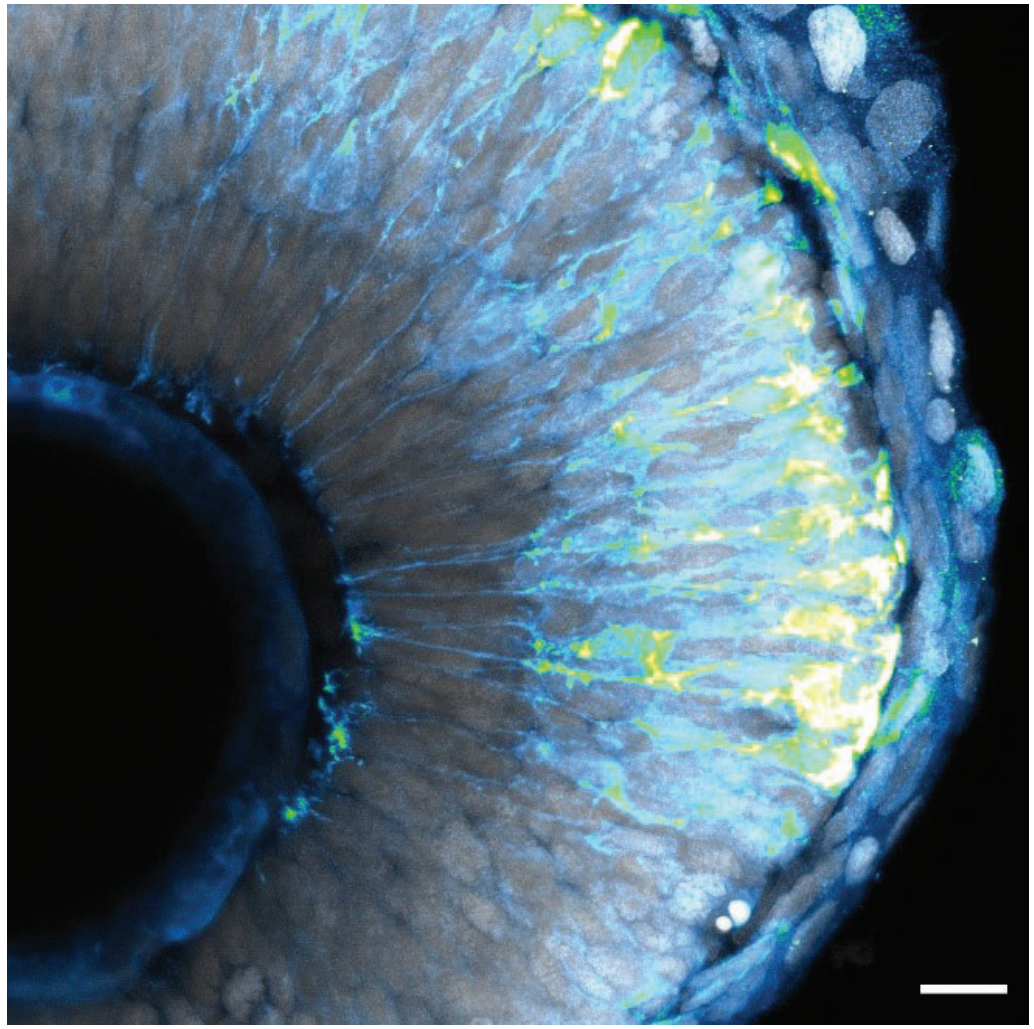

Figure 2.2. Multi-colour image acquisition, showing MG (green-blue) and retina nuclei (gray) in a 3-day old fish (scale bar 10µm).

responsible for supporting healthy retinal function throughout life. To perform this job effectively, MG have a complex cell shape with apicobasal polarity and defined subcellular regions that facilitate close associations with neighbouring cells and allows them to fulfil their specific supportive functions [18]. For example, these MG subregions enable close contact with photoreceptor neurons, containment of the cell nucleus, interaction with other neurons, and ensheathment of blood vessels [19].

Nevertheless, while MG shape is critical for their function, it remains challenging to fully visualise and comprehend their complex morphology without high-quality 3D visualisation (Fig. 2.1). Thus, our lab uses Zeiss AiryScan fluorescence microscopy, which enables image acquisition with 120nm lateral and 350nm axial resolution, to visualise glial cells in the developing and ageing zebrafish retina.

However, this state-of-the-art approach not only allows the visualisation of MGs, but also other retinal components such as cell nuclei (Fig. 2.2; average diameter 10 micrometers) or neurons in parallel. With this, we can start to unravel how glia cells relate to visual function and the importance of their interactions with other retinal components (Fig. 2.3).

Taking this further from observational to functional imaging, our lab also uses approaches to visualise

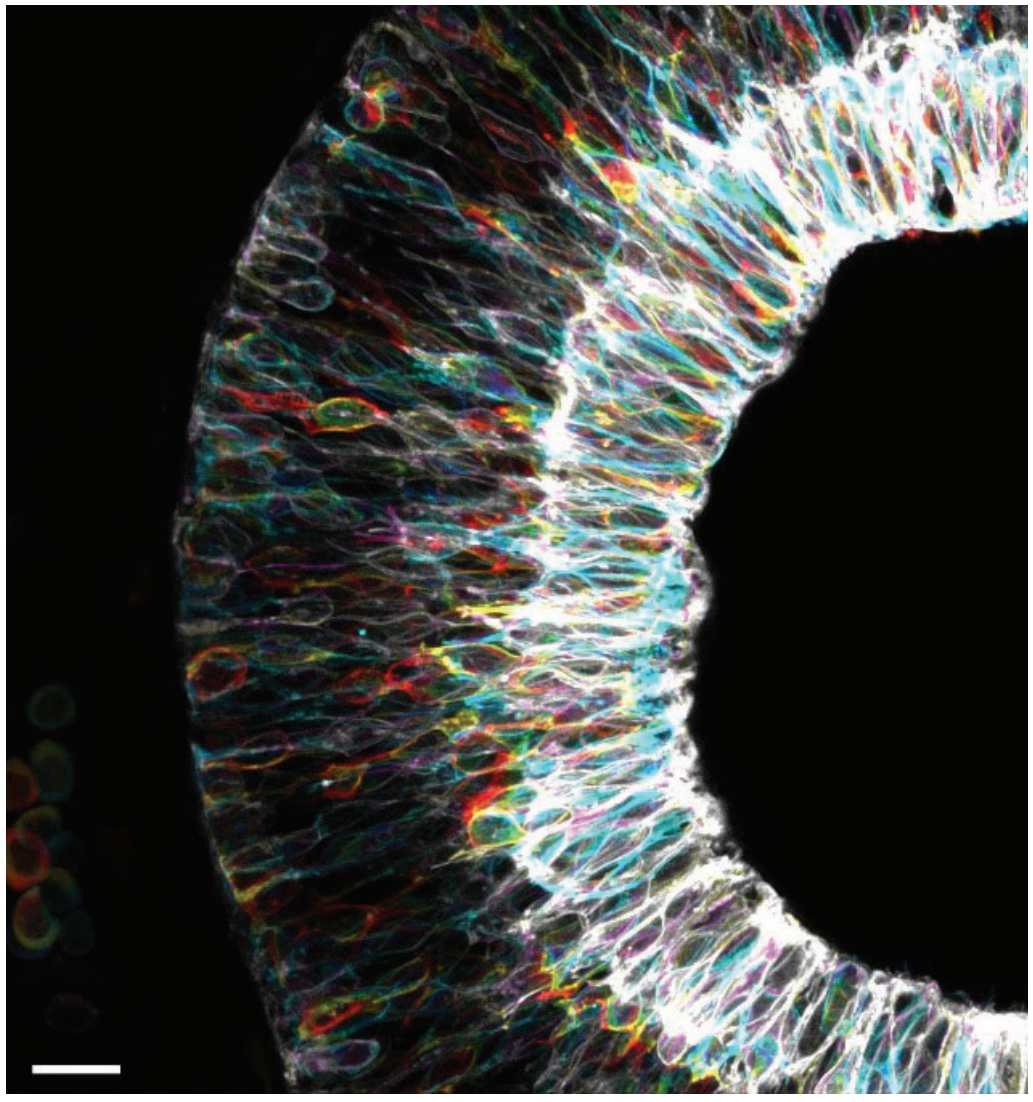

Figure 2.3. 3D depth-coded image of neurons in the eye, called retinal ganglion cells, in a 2-day old fish (scale bar 10µm).

neuronal calcium dynamics to unravel the temporal sequences of events in the developing retina around neuronal function and glial morphogenesis. Similarly, using cell- or tissue-specific manipulations enables us to understand the role of different retinal cells in the developing visual circuit.

Importantly though, like every microscopy technique, also Airyscan microscopy comes with its challenges. From an image acquisition perspective, we found particularly two aspects needing consideration across fields and experimentation: (a) Confocal and Airyscan microscopy suffer from substantial z-axis signal decay, which can be partially corrected during acquisition or post-processing. (b) When comparing Airyscan microscopy to other techniques, such as LSFM, the acquisition rate is slower, which can make it tempting to change acquisition settings to increase speed. However, this comes at the cost of sensitivity and resolution, making longer acquisition durations inevitable for the highest quality data. Together, Airyscan microscopy can acquire data with semi super-resolution, allowing the visualisation of small-scale cellular features that might be otherwise overlooked and the understanding of the role of glia in retinal development and disease.

Imaging and data analysis of brain function

by Nathalie Jurisch-Yaksi and Emre Yaksi (Norwegian University of Science and Technology, NO)

Understanding the function of brain circuits in health and disease is a major challenge. This is mainly due to the immense complexity of the brain that is emerging from connections between millions of individual neurons and glial cells. Furthermore, this complex network further interacts with the rest of the body (e.g. cerebrospinal fluid, metabolites, hormones) and environmental signals, making a holistic study of the brain very challenging. With its small and transparent brain, zebrafish provide a complementary alternative to mammals for studying the entire nervous system in living animals.

Our labs, individually and collaboratively, study the physiology of neurons, glia and the ventricular systems in brain development and function. Specifically, we study the role of internal brain states in sensory computations and regulating animal behaviour [20], [21], as well as the function of non-neuronal cells (glia [22], ependyma, Olstad [24] and D Gama [28]). To do this, we employ a combination of two-photon, confocal, LSFM, and epifluorescence microscopy in larval and adult zebrafish. Here, we discuss the advantages and disadvantages of these methods, and propose strategies on how to combine them to study brain development and function.

Mostly we utilise two-photon microscopy for recording neuron and glia functionglia function (Fig 3.1) [20]-[22]. Two-photon microscopy allows excellent optical sectioning and depth penetration up to several hundred micrometres in awake behaving (free and head-restrained) animals, non-invasively in both larval and juvenile zebrafish. Yet, most two-photon microscopes rely on scanning lasers, which limits the temporal resolution and signal-to-noise ratio. To deal with such limitations, many labs are now using LSFM (explained above) to visualise brain function at several hundred frames per second (FPS). In fact, LSFMs were used

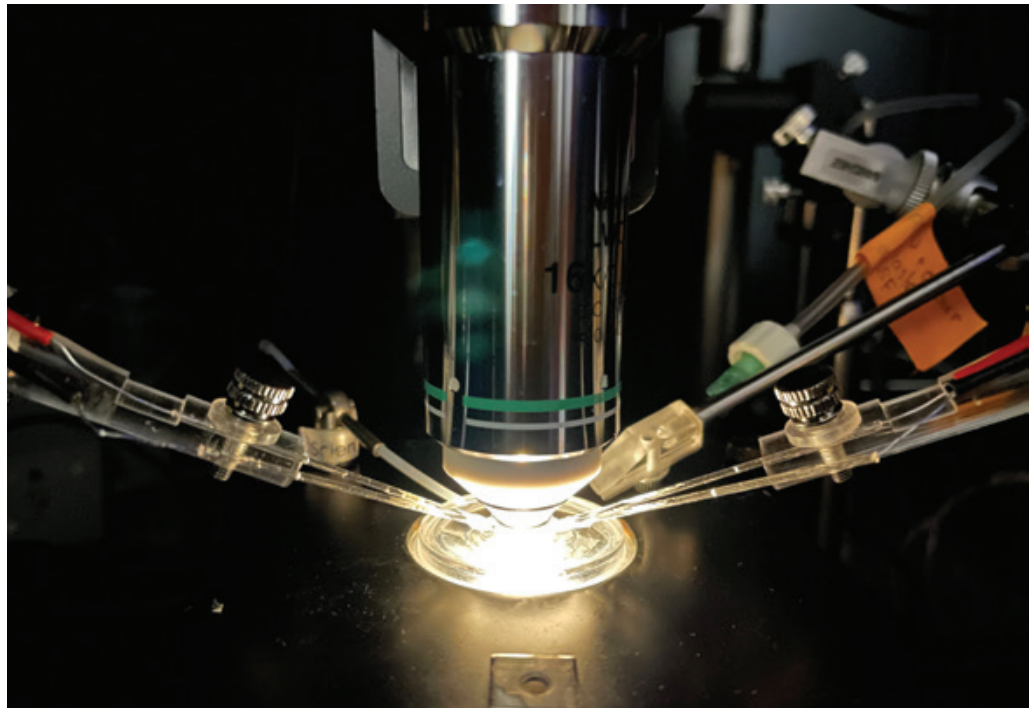

Figure 3.1. Two-photon microscopy is commonly used in combination with electrophysiological recordings. Here is a demonstration of glass electrodes that are used for stimulating and recording neural membrane potentials, while performing calcium imaging of neural activity (Photo credit: Anna Maria Ostenrath).

Figure 3.2.Tracking of the solitary motile cilia of five ependymal cells based on light sheet recordings in two-day-old animals.Time is colour-coded. (Olstad [24]).

to image the activity of all neurons and glia, across the entire vertebrate brain of larval zebrafish [23]. This was a major achievement for neuroscience! In our labs, we use LSFM up to 950 FPS to image very fast biophysical processes such as the beating of cilia [24], [25] (Fig. 3.2), lining the brain ventricles or the nose. It is important to note that LSFM requires intricate sample preparation, which is easier to employ in smaller transparent larval fish. Moreover, most LSFMs rely on visible light, which has inadequate tissue penetration and can be problematic while imaging thick samples. Finally, visible light exposure is an additional concern when studying how it impacts on animals' behavioural states, when studying how brain computations lead to behaviour as the light itself impacts the behaviour. Several labs now employ two-photon light-sheet microscopes, which give better optical sectioning and deeper tissue penetration than visible light and are not detectable by animals, therefore not affecting their behaviour.

A major advantage of confocal microscopes is the range of lasers and filters coming with them, and their broad availability in core facilities (Fig. 3.3), which often means better excitation and spectral separation of different fluorophores. Hence confocal microscopy is our method of choice when collecting anatomical or histological images from brain samples with multiple colours. Moreover, excellent spectral separation is an advantage when used for simultaneous functional imaging of multiple cell types in the brain (e.g. neurons and glia) that express indicators of activity in different colours.

However, advanced microscopes might not be available for most scientists in the world. Here, epifluorescence microscopes, some of which can be built for a few hundred dollars, can be very effective to study brain function. Epifluorescence microscopes cannot do optical sectioning of the tissue and present substantial light scattering - some of these can be dealt with, using a few tricks. For example, we employ data analysis tools such as principal component- or independent component analysis to separate the dynamic neural signals coming from different locations in the sample. In brain regions with clear functional topography (e.g. mice/fish/fly olfactory bulb, rodent barrel cortex) this approach has been successfully used to collect excellent quality neural activity data [26], [27]. We also employ fluorophores with limited expression

Figure 3.3. Confocal stack of cilia and axons in a one month old zebrafish brain, with depth encoding. Staining done with an antibody against glutamylated tubulin [28].

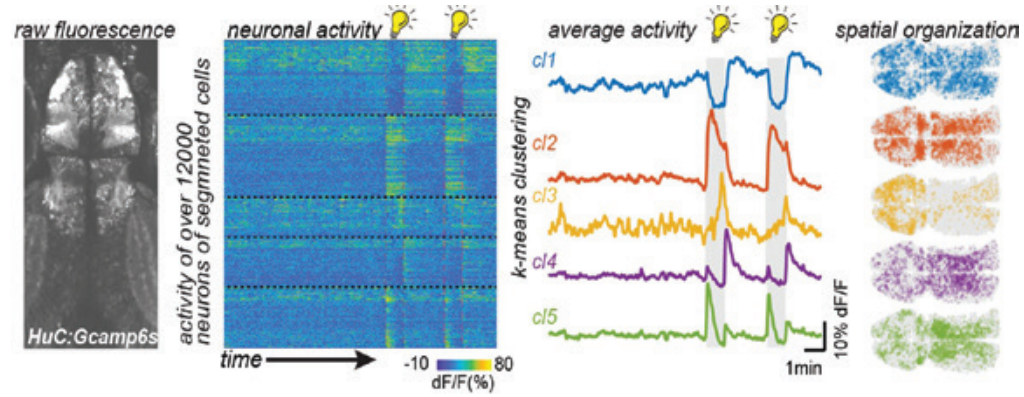

Figure 3.4. Output of a 2p volumetric calcium imaging experiment done in four-day-old zebrafish with two light stimulations. It shows the complexity of the dataset and possible analyses allowing to recover the calcium dynamics in individual cells, similarities in responses across cell populations and their location within the brain.

to neuronal nuclei, or genetically defined cell types, which helps greatly for identifying individual neurons or cell types despite the scattered light. Hence, we recommend scientists to consider available and relatively cheap epifluorescence microscopes, which often can be sufficiently good to answer many questions.

It is important to note that functional brain imaging relies on fluorescent calcium indicators (Fig. 3.4), which are well established, yet they are indirect and slow reporters of neural activity. Moreover, calcium is an ion that serves multiple functions in neurons and can be differentially released by different organelles independent of neural activity (*i.e.* ER, mitochondria). In fact, the source and function of glial calcium signals are still not fully understood, which complicates glial calcium imaging further. Hence, it is important to keep this in mind, when interpreting neuronal and glial calcium signals. Certainly, novel voltage indicators will contribute to a better interpretation of neural signals. Glial calcium imaging still needs further studies that will help us better interpret the functional relevance of these signals.

Future Directions

The examples discussed demonstrate not only the breadth of fundamental questions studied in zebrafish but also the range of techniques required to do so.

Similar to the ever more scientific questions and techniques, optics and microscopy are ever-changing fields. This is highlighted by the debut of concepts such as tissue clearing and its combination with LSFM to push image quality boundaries even further [29].

Current and future work also aim to advance the frontiers of *in vivo* microscopy in larvae and adults as well as expand the library of functional imaging tools.

However, with evermore dimensions and data, one monumental challenge in research is how to effectively handle, share, and analyse microscopy data.

The size and complexity of data sets require better computational frameworks, standardised software, and dedicated training of scientists. Many labs write their own software packages to collect, store, analyse, and interpret results. It is not that uncommon for an hour-long experiment to generate hundreds of Gb of data, which then requires multiple steps of processing before scientists can assess results. Luckily several teams generate open-access tools (e.g. FIJI, OASIS, CalmAN) that facilitate analysis of large data sets. Yet, long-term visualisation and interpretation of large datasets remains a considerable challenge. One approach to reducing data duplication and supporting reproducibility is to openly share

microscopy data and analysis approaches, based on the FAIR principle, namely Findability, Accessibility, Interoperability, and Reusability [30].

An increased focus on data sharing and analysis can provide new data insights and the discovery of important features that we might not know yet. What is clear however is that the increased digitisation and standardisation of microscopy data creates new opportunities towards the integration of different imaging modalities and the creation of multidimensional in silico datasets. Building on these, image-based computational "avatars" could be built to simulate and further understand biological phenomena.

Conclusion

We here showcased a wide range of techniques used in zebrafish to study - *and answer* - fundamental biological processes. What becomes clear is that one approach, technique, or model organism cannot provide all answers, but that combinations and multidisciplinary approaches are what spearhead scientific breakthroughs.

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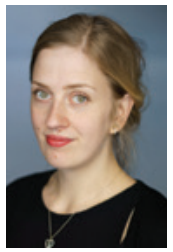

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