Application note

Super resolution live cell imaging of the nervous system during development

Bram A.A.J.W. Willems[1] and Dušan Popov-Čeleketić[2]

[1] Department of Imaging Physics, Delft University of Technology, 2628 CJ Delft, The Netherlands
[2] Confocal NL, Science Park 406, 1098XH Amsterdam, The Netherlands, sales@confocalnl.com

The larval zebrafish serves as a powerful model for studying neural circuit development due to its transparency and suitability for fluorescent microscopy. Using GAIA Point *RE*scan confocal microscope, we performed super resolution imaging to investigate the structural and functional development of the zebrafish nervous system between three and five days post-fertilization. GAIA Point *RE*scan facilitated extended imaging sessions with minimal phototoxicity, offering new insights into vertebrate neural development. Imaging revealed key features, such as synaptic boutons and axonal projections, critical for circuit formation. Combining super resolution structural imaging with functional assays enabled the dynamic tracking of neural rewiring.

Keywords: Super resolution, confocal microscopy, live cell imaging, nervous system, zebrafish

INTRODUCTION

The vertebrate nervous system exhibits remarkable complexity, with its function inherently tied to the precise formation of neural circuits during development. While structural connectivity forms the basis of circuit activity, the interplay between synaptic function and structural reorganization remains poorly understood. Zebrafish *D. rerio* provide a versatile platform for addressing these questions, offering unique advantages such as transparency during early development and robust genetic tools. These features enable real-time imaging of developing circuits in vivo using advanced fluorescence microscopy.

We utilized GAIA Point *RE*scan confocal microscope to achieve super resolution imaging of the nervous system in zebrafish larvae between 3 and 5 days post-fertilization (dpf), a critical window for neurodevelopment. Using functionally active fluorescent markers, we could track the structural development of the zebrafish nervous system in conjunction with functional imaging assays.

Key features, such as synaptic boutons and axonal projections, were resolved with exceptional clarity, allowing us to capture structural changes associated with circuit refinement. This study underscores the power of super resolution live cell imaging to elucidate the intricate processes that govern neural circuit formation and highlights zebrafish as a compelling model for developmental biology and neurobiology.

SAMPLE PREPARATION

In order to image the structural detail of the nervous system during development, we used GAIA Point *RE*scan microscope. This setup uniquely enables super resolution imaging while limiting phototoxicity. Compared to fast functional imaging, GAIA requires longer acquisition times per frame due to the point scanning

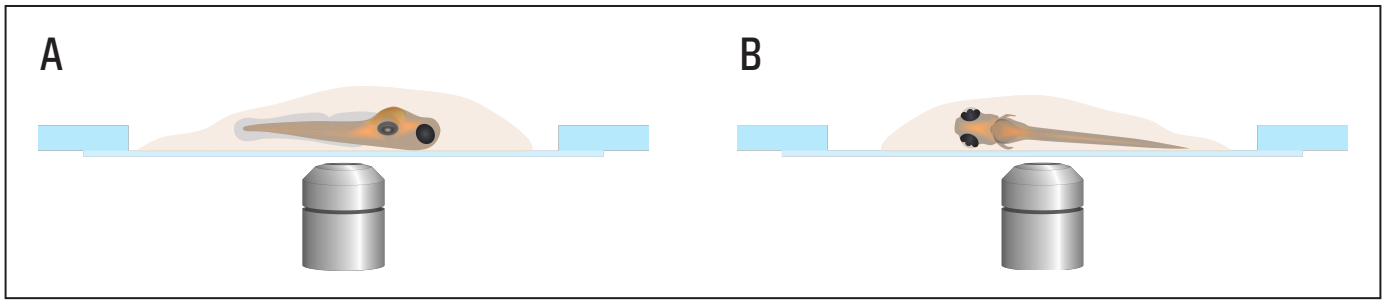

Figure 1. Larvae mounting positions in imaging dish. Mounting has been performed in 2% agarose with unparalyzed fish, not to interfere with CNS function. More dilute agarose mixtures can be used with the benefit of higher optical clarity, but require paralyzing agents to block movement. When using lenses with a short WD, it is important to mount the sample as close to the objective as possible for the highest image quality. A) Dorsal mounting enables high resolution imaging of the zebrafish brain without shadowing important structures through light absorbance and scatter in the eyes. B) Lateral mounting is ideal for imaging the central nervous system, organs and other structures in the trunk of the fish.

technology. Proper sample mounting is therefore particularly important for this type of live cell imaging, as inadequate sample preparation can significantly impact image quality. First of all, the typically reduced working distance (WD) of high NA objectives means that these thick samples have to be mounted as close to the cover glass as possible to match the WD. Furthermore, the longer acquisition times and higher resolution result in a higher sensitivity to movement artefacts. As such, the larvae have to be immobilized while keeping them alive during imaging. Here, larvae were mounted in 2% low melting agarose (Fig. 1) and kept in a humid environment within the imaging dish. Since the 3 to 5 dpf old larval fish are able to use diffusion of oxygen through their skin as means of respiration (Rombough, 2002), we were able to have them survive for at least 10 hours while being mounted. Alternatively, fish can be mounted in a lower concentration of agarose when paralyzed using neurotoxins, such as alpha-bungarotoxin, which target the neuromuscular junction, blocking muscle movement without interfering with other neural signalling circuits (Berg, 1972).

SUPER RESOLUTION IMAGING

Using super resolution imaging to study structural changes in neural circuits allowed us to clearly separate densely packed projections of axons and dendrites present within the neuropil (Fig. 2) or spinal cord (Fig. 3). Doing so in combination with functional imaging, we could track how synaptic activity impacts structural rewiring. In larval zebrafish synaptic terminals typically range from 100 to 600 nm in size within the neuropil (Svara et al., 2022), while motor neuron axons vary in diameter from less than 500 nm for secondary to about one micrometre for primary motor neurons. (Myers et al., 1986). As such, the increased resolution permitted us to observe key structures at the edge of the resolution limit, such as synaptic boutons and changes in axonal diameter. The large field of view (FN18) and deep imaging capabilities of GAIA are of particular benefit for imaging in vivo. Using objectives with longer WD allowed imaging beyond the diffraction limit of the large sections of a zebrafish larva up to 400 µm deep into the sample.

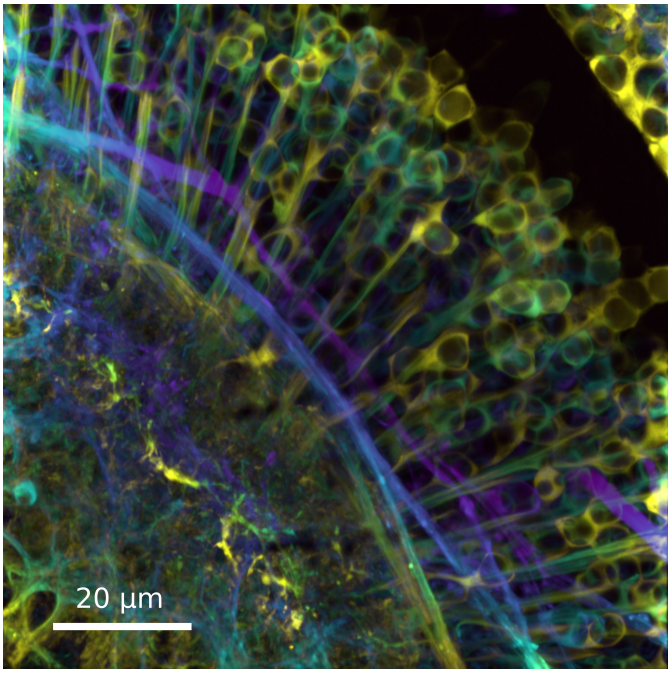

Figure 2. Interface between the neuropil and the surrounding stratum periventriculare (SPV). Maximum projection of 201 images spanning a 20 µm deep Z-stack from the most dorsal part of the optic tectum, imaged using a 60xSil/1.30NA objective with a WD of 300 µm. Within the neuropil (bottom left) individual axonic retinal ganglion projections from the eye can be distinguished in addition to dendrites innervating the neuropil from the surrounding SPV. The pan-neuronal cytosolic GCaMP6 expression shows voids in cell bodies where the nucleus is located. In some of the cells far smaller additional subcellular structures are visible devoid of GCaMP6 signal. The depth color coding of the single channel cytosolic GCaMP expression spanning the 20 µm from purple (shallow) to yellow (deep).

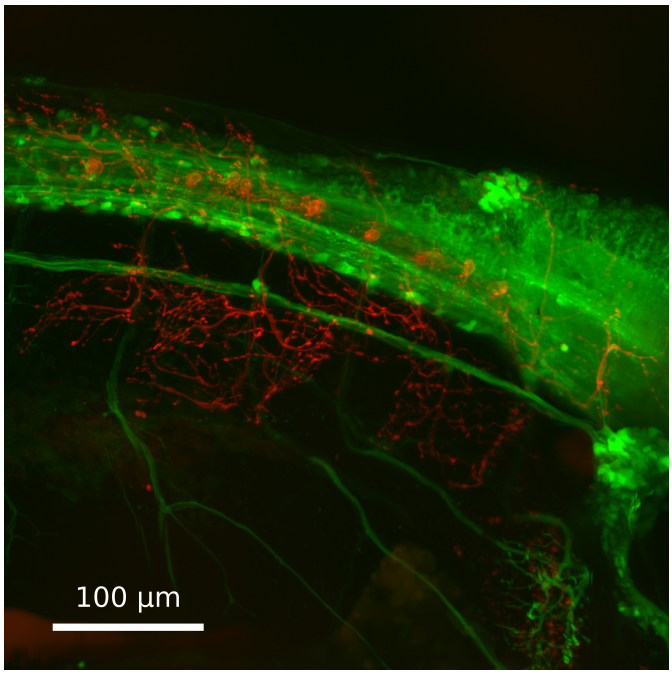

Figure 3. Primary motor neurons projecting from the central nervous system at the back of the hindbrain. Maximum projection of a 300 µm deep Z-stack containing 150 images per colour channel, imaged using a 30xSil/1.05NA objective with a WD of 800 µm. The central nervous system – 488 nm; panneuronal cytosolic GCaMP6 expression (green) – is imaged laterally, focusing on a region around the start of the spinal cord and the end of the brainstem. The lateral line nerve can be seen, clearly separated below the spinal cord. Primary motor neurons – 561 nm; tdTomato expression (red) – with cell bodies located within the spinal cord and brainstem project to the musculature of the fish.

CONCLUSION

Using GAIA Point *RE*scan confocal microscope, we performed super resolution imaging of the developing nervous system of 3, 4 and 5 dpf old zebrafish larvae in vivo. Utilizing the increased resolution, we were able to image key structures involved in the formation of neural circuits, beyond the diffraction limit. With the ability to image deep into the sample at super resolution, GAIA Point *RE*scan unit was capable of imaging the entire depth of a zebrafish larva up to 5 dpf. Laterally mounting the fish allowed imaging of the entirety of the body, while dorsal mounting put the majority of the central nervous system in reach, up to the yolk. Furthermore, photogentle imaging of the confocal unit allowed us to perform extended image sessions with a single sample, capturing multiple large image stacks in multiple channels without harming the fish.

ACKNOWLEDGMENTS

The use of zebrafish samples was made possible by the TU Delft Zebrafish Facility, and the authors would like to thank Daan Brinks and everyone from the support team for their aid in animal husbandry and care. Images were taken using GAIA Point *RE*scan confocal microscope in combination with a Hamamatsu camera. The authors are grateful to Janine Cravo and Melvin Le Bihan for their help during the imaging sessions in Confocal NL.

REFERENCES

Berg, D.K., Kelly, R.B., Sargent, P.B., Williamson, P., and Hall, Z.W. (1972). Binding of alpha-bungarotoxin to acetylcholine receptors in mammalian muscle. Proc. Natl. Acad. Sci. USA 69, 147-151. https://doi.org/10.1073/pnas.69.1.147

Myers, P.Z., Eisen, J.S., and Westerfield, M. (1986). Development and axonal outgrowth of identified motoneurons in the zebrafish. J. Neurosci. 6, 2278-2289. https://doi.org/10.1523/JNEUROSCI.06-08-02278.1986

Peter Rombough; Gills are needed for ionoregulation before they are needed for O2 uptake in developing zebrafish, *Danio rerio*. J Exp Biol 15 June 2002; 205 (12): 1787–1794. doi: https://doi.org/10.1242/jeb.205.12.1787

Svara F, Förster D, Kubo F, Januszewski M, dal Maschio M, Schubert PJ, Kornfeld J, Wanner AA, Laurell E, Denk W, Baier H. Automated synapse-level reconstruction of neural circuits in the larval zebrafish brain. Nature Methods. 2022 Oct; 19(11):1357– 1366. doi: 10.1038/s41592-022-01621-0