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Two-photon high-speed light-sheet volumetric imaging of brain activity during sleep in zebrafish larvae

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ABSTRACT

Although it is well known that zebrafish display the behavioural signature of sleep, the neuronal correlates of this state are not yet completely understood, due to the complexity of the measurements required. For example, when performed with visible excitation light, functional imaging can disrupt the day/night cycle due to the induced visual stimulation. To address this issue, we developed a custom-made two-photon light-sheet microscope optimized for high-speed volumetric imaging. By employing infra-red light (not visible to the larva) for excitation, we are able to record whole-brain neuronal activity with high temporal- and spatial-resolution without affecting the sleep state.

In two-photon light-sheet microscopy the maximum achievable frame rate is limited by the signal-to-noise ratio. To maximize this parameter, we optimized our setup for high peak power of excitation light, while finely controlling its polarisation, and we implemented remote scanning of the focal plane to record without disturbing the sample.

Using this setup, as a preliminary result, we characterized the intensity spectra of neuronal calcium traces of 4 days post fertilisation larvae during the day/night phases. We aim to extend these results to multiple brain regions and frequency bands.

1 INTRODUCTION

Even though it is well known that teleost fish, including larval zebrafish, display the behavioural signature of sleep[1], the neuronal correlates of this state are not yet completely understood[2]. This is mainly a consequence of the complexity of the measurements needed: the absence of a neocortex makes it difficult to interpret the results of electroencephalogram recordings, while long-term intra-cranical recordings are invasive and can affect the biological process under investigation. Functional imaging has been successfully exploited to study neuronal states in zebrafish[3,4], however, when performed with visible excitation light, the day/night cycle can be disrupted due to induced visual stimulation. To address this issue, we employed two-photon (2P) light-sheet (LS) microscopy[5,6]. In particular, we developed a custom-made 2P LS microscope specifically optimized for high-speed volumetric imaging. By employing infra-red light (not visible to the larva) for excitation, we are able to record whole-brain neuronal activity with high temporal- and spatial- resolution without affecting the sleep phase. Here we report the preliminary results obtained with this instrument to observe neuronal correlates of the nocturnal phase in the whole-brain of zebrafish larvae.

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2 METHODS

We observed 6 transgenic larval zebrafish expressing GCaMP6s-H2B, a nuclear-localized pan-neuronal calcium indicator[7], in homozygous *albino* background[7–9]. The larvae were maintained on an artificial light/dark cycle (also during image acquisition) and observed at 4 days post fertilization (DPF). We recorded 5 minutes of neuronal calcium activity every 20 minutes for about 4 hours of artificial day and 4 hours of artificial night between the switch-over time. Immediately before the acquisition the larvae were paralysed with a myorelaxant agent, included in agarose gel and mounted on a custom-made glass support immersed in thermostated fish water. Fish maintenance and handling were carried out in accordance with European and Italian law on animal experimentation (D.L. 4 March 2014, no. 26), under authorization no. 407/2015-PR from the Italian Ministry of Health.

The imaging was performed with a custom-made 2P LS microscope. Excitation infrared light is generated by a pulsed Ti:Sa laser. The attenuated beam passes through an Electro-Optical Modulator (EOM) used to switch its linear polarization state. The beam is then deflected by a pair of galvanometric mirrors: a fast resonant mirror, used to generate the LS, and a closed-loop mirror, used to scan the LS in the dorso-ventral direction. The beam is finally relayed to a pair of excitation dry objectives by a scan-lens, a tube-lens and two pairs of relay lenses. The objectives are placed at the two lateral sides of the larva and alternatively illuminated depending on the instantaneous polarisation state of the incoming light, that is deflected by a polarising beam-splitter (PBS) placed between the tube-lens and the first relay lens. A half-wave plate is used on one of the two excitation arms to ensure that the polarization planes of the incident light from both sides are coincident. The emitted green fluorescence light is collected by a water-immersion objective and spectrally filtered. The image is then demagnified and relayed to a sCMOS camera, after passing through an electro-tunable lens (ETL).

Imaging was performed at the volumetric frequency of 5 Hz, with a pixel size of $2 \times 2 \times 5\ \mu m^3$, and a field of view of about $1 \times 1 \times 0.15\ mm^3$. After the acquisition, the data were manually inspected and movement artefacts were removed. We then computed the pixel-based relative (i.e. divided by the value at f = 0 Hz) Fourier power spectrum for each acquisition using a custom-made python script. Then we generated 3D-stacks by retro-projecting the per-pixel band values and we semi-automatically aligned them.

Finally, we manually traced regions-of-interest on the 3D-stacks corresponding to anatomical structures and computed their mean values. We compared these values using a general linear mixed model implemented in R[10]. For this model, we used the period of the artificial day/night cycle and the binarized acquisition time as fixed factors and the individual larva as random factor.

3 RESULTS AND CONCLUSIONS

In 2P LS microscopy the maximum achievable frame rate is limited by the signal-to-noise ratio. To maximize this parameter, we optimized our setup for high peak power of the excitation light. Therefore, we used a PBS and an EOM to alternatively excite the two sides of the sample. In this way, the excitation power is more tightly confined both in the spatial-dimension and in the temporal-dimension. Furthermore, we observed that the spatial distribution of the emitted fluorescence light is anisotropic, due to the polarisation-dependent selection rules of the 2P absorption process. We exploited this effect by rotating the polarisation plane of the excitation light to achieve maximal emission toward the direction of the detection objective.

To maximize the recorded signal, we employed a large-NA immersion objective for detection, since this kind of objectives can collect more photons with respect to dry ones. In addition, we employed an ETL lens to remotely change the objective focal plane to avoid compression waves impinging on the sample that would otherwise be generated by objective scanning during high frame-rate acquisition. Finally, we demagnified the generated intermediate image to maximize the number of collected photon by individual camera pixel, thus increasing the SNR of the electronic readout.

Using this setup, we could characterize the Fourier power spectra of the neuronal calcium traces in the larval brain during the day/night phases, observing differences in various regions [Fig. 1]. In particular, as a preliminary result, we report an amplitude decrement during the night phase in an oscillation band around 10 s in the Anterior Rhombencephalic Turning Region (ARTR). The nervous activity in this region displays periodic oscillations and the ARTR activation precedes turning movements of the larva[11,12]. Therefore the observed decrement is consistent with the fact that zebrafish larvae display reduced locomotor activity during night.

In conclusion, we developed a microscopy setup able to acquire whole-brain calcium imaging with high spatial and temporal resolution and without altering the sleep state. We employed this microscope to characterize the differences in the neural activity between day/night phases of 4-DPF zebrafish larvae. We aim to extend these results to more brain regions and to other frequencies bands.

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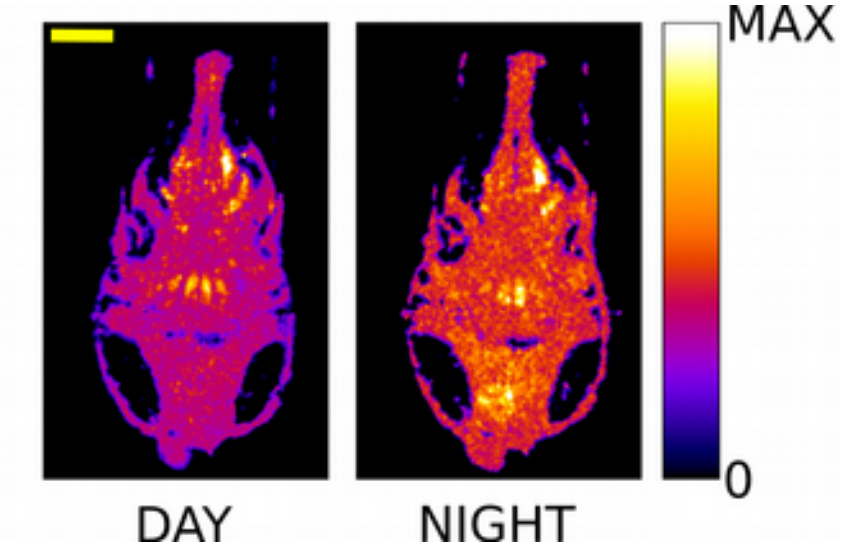

Figure 1
Single planes of volumetric stacks generated by retro-projecting the values of the relative power spectrum in the band of 7 s - 9 s. The images show the same larval brain during the diurnal (left) and the nocturnal (right) phases. The values (in arbitrary units) were colour-coded as indicated by the colour bar on the right. Scale bar: 200 μm.