Two-photon light-sheet microscopy for high-speed whole-brain functional imaging of zebrafish neuronal physiology and pathology

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ABSTRACT

We present the development of a custom-made two-photon light-sheet microscope optimized for high-speed (5 Hz) volumetric imaging of zebrafish larval brain for the analysis of neuronal physiological and pathological activity. High-speed volumetric two-photon light-sheet microscopy is challenging to achieve, due to contrains on the signal-to-noise ratio. To maximize this parameter, we optimized our setup for high peak power of excitation light, while finely controlling its polarization, and we implemented remote scanning of the focal plane to record without disturbing the sample.

Two-photon illumination is advantageous for zebrafish larva studies since infra-red excitation does not induce a visual response, that otherwise would affect the neuronal activity. In particular, we were able to record whole-brain neuronal activity of the larva with high temporal- and spatial-resolution during the nocturnal period without affecting the circadian rhythm. Analyzing the spatially resolved power spectra of GCaMP signal, we found significant differences for several frequency bands between the day/night phases in various brain regions.

Moreover, we studied the fast dynamics that characterize the acutely induced pathological epileptic activity of the larvae, identifying the brain structures that are more susceptible to the action of the epileptogenic drug.

In conclusion, the high speed two-photon light-sheet microscope that we developed is proving to be an important tool to study both the physiological and the pathological activity of the zebrafish larval brain without undesired visual stimulation.
1 INTRODUCTION
Light sheet (LS) microscopy emerged in the last years as a powerful technique able to visualize large biological specimens with high acquisition rate, thanks to its parallelized detection. Furthermore, its associated good optical resolution and low phototoxicity allow to acquire images in rich detail and for prolonged times. Here we describe the development of a custom-made two-photon (2P) LS microscope optimized for high-speed (5 Hz) volumetric imaging and we present preliminary data regarding its application to the study of zebrafish larva (ZL, Danio rerio) neuronal physiology and pathology.

2P LS microscopy is a variant of the traditional (one-photon) LS technique that exploits the 2P absorption effect for fluorophore excitation. This technique uses near infrared (NIR) light for excitation: a wavelength range that is not visible for the larva. This aspect is important because, if specific countermeasures (usually involving complex excitation geometries) are not undertaken, the visible excitation light usually employed in LSM is collected by the larva eyes, thus eliciting visual response that can confound the neurophysiological data. This aspect is critical in specific situations, such as the study of circadian rhythm and of epileptic seizure activity. Regarding the former, the exposure of the larva to visible light during the nocturnal period can disrupt the circadian cycle. While, regarding the latter, the epileptic brain is particularly susceptible to the external stimuli, such as flashes of light. This stimulation can act such as an external trigger for the onset of the epileptic seizure, therefore altering the fine mapping of the brain spatial susceptibility pattern of neuropathological hyperexcitability. In these situations, 2P LS microscopy shows a clear edge as a technique able to record whole-brain activity without unwanted external stimulations and with high temporal resolution and cellular spatial resolution.

2 METHODS
The custom-made 2P LS microscope used for imaging and the methods for circadian rhythm acquisition were already described in: Giuseppe de Vito, Chiara Fornetto, Pietro Ricci, Caroline Müllenbroich, Giuseppe Sancataldo, Lapo Turrini, Giacomo Mazzamuto, Natascia Tiso, Leonardo Sacconi, Duccio Fanelli, Ludovico Silvestri, Francesco Vanzi, Francesco Saverio Pavone, “Two-photon high-speed light-sheet volumetric imaging of brain activity during sleep in zebrafish larvae,” Proc. SPIE 11226, Neural Imaging and Sensing 2020, 1122604 (February 21, 2020). DOI: https://doi.org/10.1117/12.2542285; here we will report the description for the sake of clarity.

NIR excitation 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 galvo, used to generate the LS, and a closed-loop galvo, used to scan the LS in the dorso-ventral direction. The beam is finally relayed to a pair of excitation dry objectives, placed at the two lateral sides of the larva, by a scan-lens, a tube-lens and two pairs of relay lenses. The objectives are alternatively illuminated depending on the instantaneous polarization state of the incoming light, that is deflected by a polarizing 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 of a 150 μm thick portion of the larval brain was performed at the volumetric frequency of 5 Hz, with a pixel size of $2 \times 2 \times 5 \mu m^3$. After acquisition, the data were manually inspected and movement artifacts were removed. For the circadian rhythm acquisitions, we observed 6 transgenic ZL expressing GCaMP6s-H2B, a nuclear-localized pan-neuronal calcium indicator, in homozygous albino background. The larvae were maintained on 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 paralyzed with a myorelaxant agent, included in agarose gel and mounted on a custom-made glass support immersed in thermostated fish water.

For the epileptic seizure acquisitions, we observed 12 additional transgenic ZL of the same strain, exposed to different convulsant concentrations. Larvae were maintained according to standard procedures. Stock solutions of pentylenetetrazole (PTZ), were prepared by dissolving in milliQ, while the final concentrations used in the experiments were obtained by diluting each stock in fish water. We recorded 5 minutes of physiological neuronal activity before adding to the imaging chamber the convulsant agent at the desired concentration. Upon drug administration, we recorded 5 minutes of neuronal activity every 10 minutes for one hour, thus acquiring a total of 30 minutes of aberrant neuronal activity during seizure onset and propagation.

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.
For the data analysis of the circadian rhythm acquisition, we computed the pixel-based 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-interests (ROIs) 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. 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.

For the data analysis of the epileptic seizure acquisitions, we drew ROIs around individual cells on different z-planes and manually inspected the ΔF/F traces of calcium activity. In addition, we computed the pixel-based temporal standard deviation of the ΔF/F traces and we semi-automatically aligned the resulting z-stacks. Then, we analyzed these results separately for each PTZ concentrations using a general linear mixed model implemented in R. For this model, we used the PTZ exposure time as fixed factors and the individual larva as random factor.

### 3 RESULTS AND CONCLUSIONS

High-speed volumetric 2P LS microscopy is challenging to achieve, due to constrains on the signal-to-noise ratio (SNR). In order to do so, we optimized our setup for high peak power of the excitation light. Aiming to confine the excitation power, both spatially and temporally, we excited the sample alternatively from the two sides, using a PBS and an EOM. Furthermore, the orientation of the polarization plane of the excitation light was optimized to exploit the polarization-dependent selection rules of the 2P absorption process to orient the fluorescence light emission preferentially toward the direction of the detection objective.

We employed a large-NA water-immersion detection objective to maximize fluorescent signal collection. To overcome motion artifact due to compression waves generated by the detection objective scanning during high-speed volumetric imaging, we used an ETL lens to operate remote focusing in detection. Finally, we increased the SNR demagnifying the image to increase the number of photons collected by each camera pixel.

Using this setup, we were able to record whole-brain neuronal activity of the ZL with high temporal- and spatial-resolution during the nocturnal period without affecting the circadian rhythm. As a preliminary result, we characterized the power spectra of neuronal calcium traces of 4 dpf ZL during the day/night phases and found significant differences in various brain regions.

Among these regions, we observed an increment in neuronal activity for frequencies slower than <0.1 Hz for a rostral subregion of the optical tectum during the nocturnal period. This effect is similar to the observed increment in the infra-slow band in 7-14 dpf ZL dorsal pallium observed during the sleep phase analogous to mammalian slow wave sleep.

Moreover, we studied the fast dynamics characterizing pathological pharmacologically-induced acute epileptic activity of the ZL. In this case, using invisible NIR excitation, we were able to record the epileptic activity untangled from the additional neuronal stimulation that would be otherwise induced in a pathological brain context by the light flashes perceived during the laser scanning. The ΔF/F traces of calcium activity recorded from single cells clearly show the establishment of an epileptic activity starting from mild concentration of PTZ.

To identify brain structures that are more susceptible to the development of abnormal neuronal activity, we have identified the pixels that display the strongest regression factor between the PTZ exposure time (as independent variable) and the ΔF/F variance (as dependent variable) for each PTZ concentration employed. By manually inspecting the resulting maps we noted that the center of the susceptibility patterns gradually shifts along the caudal-to-frontal direction as the PTZ concentration increases. This result indicates that the time-dependent recruitment of the brain volume by the PTZ epileptic activity exhibits spatial characteristics that are linked to the PTZ concentration.

In conclusion, the high speed 2P LS microscope that we developed is proving to be an important tool to study both the physiological and the pathological activity of the ZL brain in contexts where unwanted visual stimulation induced by the excitation light can be particularly detrimental.

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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. Image and legend text reproduced from: Giuseppe de Vito, Chiara Fornetto, Pietro Ricci, Caroline Müllenbroich, Giuseppe Sancataldo, Lapo Turrini, Giacomo Mazzamuto, Natascia Tiso, Leonardo Sacconi, Duccio Fanelli, Ludovico Silvestri, Francesco Vanzi, Francesco Saverio Pavone, “Two-photon high-speed light-sheet volumetric imaging of brain activity during sleep in zebrafish larvae,” Proc. SPIE 11226, Neural Imaging and Sensing 2020, 1122604 (February 21, 2020). DOI: https://doi.org/10.1117/12.2542285
Figure 2
Left: single planes from time-averaged volumetric acquisitions of zebrafish larval brain after PTZ exposure. The plane number is indicated on the images, where 0 indicates the most dorsal acquired plane. Adjacent planes are spaced 5 μm apart. The PTZ concentration is reported on the top of the images. Images on the same row were acquired from the same larva. Right: ΔF/F traces of calcium activity recorded from the cells indicated by color-matched circles on the brain images on the left.