RAPTOGEN - 3D Random Access 2-PhoTon OptoGENetics

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ABSTRACT

Understand neuronal circuits in different brain regions is what neuroscience constantly aims, whereby functional imaging is now become an essential tool for neuronal detection and cerebral activity monitoring. In this framework, development of an optical system able to rapidly image the entire brain or to span different areas is still demanding. RAPTOGEN enables experiments which combine fast functional imaging and optogenetic, and includes custom stimulation as classical 2D raster scan, tailored beam shaping and 3D random access. We tested the imaging performances by acquiring signals from emitting fluorophores and we measured the electrophysiological behavior while detecting zebrafish larvae sample.

Keywords: Optogenetics; Random Access; 3D Stimulation.

1. INTRODUCTION

One of the most ambitious goal for the neuroscience is represented by the possibility to correlate the neuronal activity of different neurons distribution and to match them with repetitive behaviors. Large distributions of cells have to be imaged at once to observe the outcome general trend, while requiring single neuron resolution. In this contest, RAPTOGEN wants to:

- bring innovation and cost reduction, representing a viable alternative to the state-of-the-art bulky solutions for brain investigation.
- represent a good trade-off between visual field dimensions, acquisition time and image quality in a fast, simple and economic way
- flexible real-time decision-making strategy to optimize the parameters with respect to the experiment target.

The idea of RAPTOGEN consists on a fast and precise light-targeting strategy based on the combination of Acousto-Optic Devices (AOD) and galvanometric mirrors integrated in series, with a dedicated optical design. The first provides quickly control of the intensity and of 3D spatial distribution of light into the sample, while the second defines large FOV. Then, each selected region can be visualized and stimulated in fast mode, in order to study correlations of neurons in larger areas and with high resolution of results. Furthermore, moving the objective along the optical axis provides a wide axial range, while a fast-axial shift is obtained by driving the AODs with an acoustic wave with linearly varying frequency.

This self-standing system is designed as an add-on to be coupled to standard microscopes as confocal or light sheet, to upgrade them and to guarantee fast imaging and optostimulation requirements.
Indeed, although 3D random access scanning systems based on AODs have been used to perform fast 3D calcium imaging, no scan AOD-based applications in optogenetic stimulation have been reported yet.
In this regard, by means of a two-photon microscope we used the RAPTOGEN technology to observe 3D high-speed fluorescence imaging, calibrating the system in terms of FOV, optical resolution and dynamical performaces. We developed a software interface to control an electronic system based on Arduino 2 and a Digital Direct Synthesizer, realized in a compact and inexpensive way following the project design criteria. The two-photon excitation (TPE) allows to select with accurate precision the excitation volume within the entire animal brain. We have used the Zebrafish larvae to control and simultaneously image brain activity.

2. STATE OF THE ART

Two classes of light-targeting strategies have been mostly used for neuronal photostimulation, commonly identified as serial-scanned and parallel-patterned light-targeting methods [1-6]. The first strategy is mainly dominated by slow Galvo system which are limited by the inertia of the mirrors (peak sweeping rate of 200 Hz for closed-loop mirrors and 122 kHz for resonant ones). The second exploits Spatial Light Modulation (SLM), that is dramatically limited by the intrinsic slow refresh rate of the light modulator (200 Hz). These technical constraints affect

the capability to fast image and stimulate different neurons located in distant regions of the brain. The scanning dynamic can be sped up introducing AODs which do not have any inertial restrictions and allow to generate simultaneously multiple beams at different angles or to rapidly sweep a single beam, reaching up to MHz rates. In such a device, a radiofrequency (RF) is applied to a piezotransducer to generate a pressure wave that propagates through the device's internal crystal, acting globally as a diffraction grating for the beam with fine control over its deflection angle and intensity. Then, if the device is driven by multiple frequencies, a linear combination of gratings is produced, allowing to generate simultaneously different beams from a single one, with independent regulation in terms of spatial direction and intensity.

RAPTOGEN allows to interfere with neuron activity by light stimulation within different brain regions. The goal is controlling and simultaneously imaging brain activity of the sample to associate functional data to animal behaviour and to extract the relevant features of the induced activity across the brain.

We used Zebrafishes (Danio rerio) that is a popular vertebrate model organism used for neuronal research as neuropsychiatric disorders and neurodegenerative diseases. It can be imaged in their entirety with non-invasive optical methods, thanks to their transparency in larval state.

3. BREAKTHROUGH CHARACTER OF THE PROJECT

Brain activity is organized in highly complex spatio-temporal pattern involving many different neuronal cells. Current optogenetics methods act in a quite coarse way, by photostimulating large fields of view or, conversely, are restricted to the precise excitation of few neurons placed in spatially close positions. Thanks to the use of inertia-free acousto-optic deflectors, RAPTOGEN allows scanning of arbitrary 3D patterns, enabling photostimulation of cell patterns which might emulate natural neuronal activity more closely. 3D light patterning has been demonstrated previously using spatial light modulators based on liquid crystals [7]. However, with this technology stimulation patterns can be updated quite slowly, based on the refresh rate of the liquid crystal chip. RAPTOGEN instead allows modification of the stimulation pattern on the ms time scale. This will enable the realization of new optogenetics paradigms, where the cell pattern to be activated is changed dynamically, maybe in response to behaviour or to recorded neuronal activity.

4. PROJECT RESULTS

The system development was realized in synergy with a Leica Microscope (DM LFSA) to validate the system potentialities and to carry out two-photon imaging thanks to the optical system coupled to it. Although this system is sufficient for development work, Leica Microscope limits the FOV and introduces some aberrations. The measures presented in this work will therefore be limited by these working conditions but will be resolved when the development part will be completed and the system will be introduced as an add-on to a much more performing system, as foreseen for the next steps of the project. The setup is schematically represented in Fig. 1.

We optimized the chromatic dispersion and the beam deformation, caused by the interaction between a femtosecond laser with the AOD internal crystal. We evaluated the beam distortion by acquiring the profiles of the beam before and after the first AODs couple and evaluating the ratio between the standard deviation of the two fit $\sigma_{after\ AOD}/\sigma_{before\ AOD}$ = 2,23. It has been solved placing the second AODs couple symmetrically opposite to the first one, in order to compensate the effect with an inverted deflection of the broadband laser.

The transmitted power of the diffracted beam is a function of the deviation that the beam undergoes and then a function of the radiofrequency used as input to drive the AODs. The AOD couples have a colinear design for the central frequency with respect of the whole frequency band accepted. Therefore, the polarization as well as the input angle becomes critical, with a low tolerance for the input angle. In order to improve the system performances, we introduced a half wave waveplate before the first AODs couple to regulate the polarization of the input light. We mounted the AODs couple on roto-translational stages to increase the number of degrees of freedom of the AODs couple, improving power transmission efficiency.

The first calibration we did regards the galvanometric mirror in terms of voltage applied vs the field of view (FOV) effective length. Then we measured the maximum FOV achievable in the sample plane, which is affected by the physical constrain introduced by the tube lens aperture, placed inside the Leica microscope. It results to be (388±5) μm. We also measured the FOV achievable in our setup with the AODs scanning configuration, that is (54.8±0.6) μm.

Then, to quantify the optical features of the system, the Point Spread Function (PSF) has been measured scanning a specimen containing fluorescent beads (TetraSpeck microspheres, Invitrogen T7279, with radius r = 50 nm) with galvanometric mirrors. We measured intensity profiles, calculating the full width at half maximum (FWHM) of Gaussian fit results, along the radial and axial directions of the beads with the open-source software ImageJ. Fig. 2 shows the raw data points and the Gaussian model computed with the mean FWHM, obtained respectively along the lateral (left) and axial (right) directions. The lateral and axial FWHM are respectively (0,73±0,19) μm and (6,3±1,0) μm.

Once the software implementation was realized, we tested the system in terms of resolution and timing. The data have been measured, and reported in the Table 1, for a 2D configuration and a 3D one, called respectively single tone (ST) mode and linear sweep (LS) mode.

Tab. 1. Timing and resolution digital performances.

Config	Dwell time	Resolution	Max Scan Freq. (1 pt.)	Min Scan Freq. (2500 pts.)
ST	7 µs	2500 pts.	142.9 kHz	57.2 Hz
LS	15 µs	2500 pts.	66. kHz	26.7 Hz

In order to test the system devised, both in 2P imaging and optogenetic stimulation, we generated the Tg (elavl3:ReaChR-TagRFP) transgenic zebrafish line [8, 9]. This strain, owing to the pan-neuronal promoter *elavl3*, expresses in the plasma membrane of all differentiated neurons the fusion protein ReaChR-TagRFP, a combination of a light-gated cation channel [10-12] and a red fluorescent protein. Thus, exploiting TagRFP fluorescence, we were able to perform 2p raster scanning structural imaging of the entire larval brain, and taking advantage of ReaChR channel features, we tested the system for optogenetic stimulation in the intact zebrafish brain. Fig. 3 shows four frames separated from each other 10 µm, resulting from an image stack taken, moving the objective axially to change the focused plane.

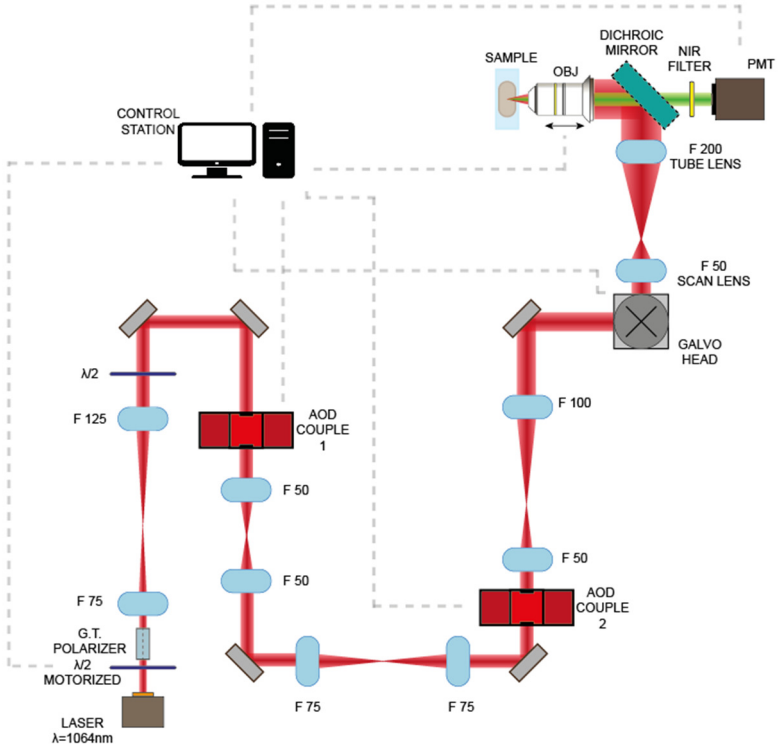

Fig. 1. Schematic view of the optical system.

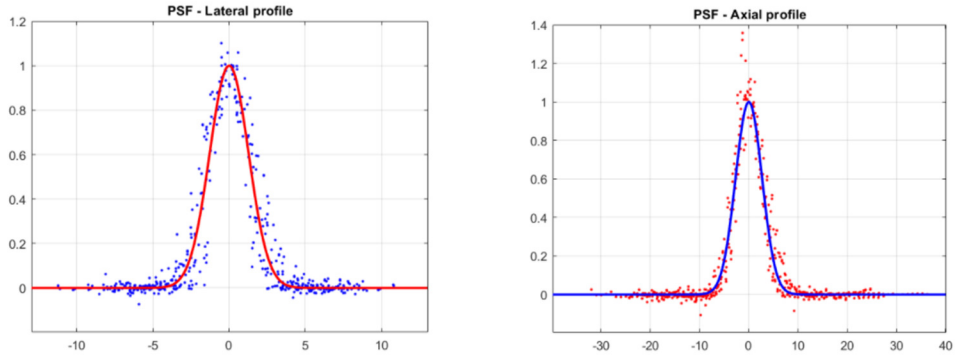

Fig. 2. Raw data points and the Gaussian model computed with the mean FWHM, obtained respectively along the lateral (left) and axial (right) directions.

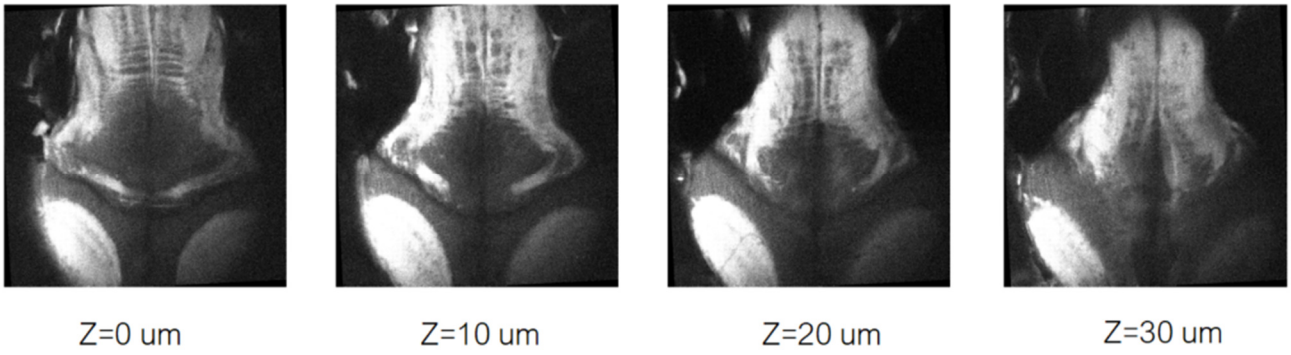

Fig. 3. Four frames of zebrafish brain imaged, separated from each other 10 µm.

5. FUTURE PROJECT VISION

5.1. Technology Scaling

At end of the Phase 1, we reach the Technology Readiness Level 5 (TRL 5) of the RAPTOGEN system, because we have integrated, build and validate the system in-vivo in a relevant environment. In fact, we used the system to image the whole brain of the zebrafish larvae in an electrophysiology laboratory. For the commercialization of the system, like add-on for optical microscope, we need to perform the following tasks:

- Engineer the whole system in order to improve the assembling aspect for the production considering the Add-On approach of the device;
- Validate the system on the operational environment to verify the usability aspect, the compliance of performance with respect to specifications and the compliance to the regulatory aspect required;
- Production of the system in a large scale and introduction on the Neuroscience and Microscopy markets.

5.2. Project Synergies and Outreach

In order to reach the markets involved to maximize the benefits, we need to include in our consortium a company or profile who knows the Neuroscience or/and Microscopy markets in depth, to drive and boost the introduction strategy and marketing campaign for the commercialization of the system. In the Phase 1, our consortium is composed of two partners. The industrial partner with knowledge of design and develop of devices in the field of photonics, microscopy and physics field and a research centre specialized in the Neuroscience research on organs and live animals. At the end of Phase 1, the design and the assembly of the RAPTOGEN system, with its in-vivo tests in a research laboratory, have been completed. For the marketing of the system, we need financial resource and partners to engineer, to validate on a normal environment and to qualify the system respect to the regulatory aspect.
To improve the dissemination of RAPTOGEN project results after the Phase 1 we need to perform this type of dissemination events:

- project-oriented dissemination focused on awareness rising about the project itself, its objectives and intended results, emphasizing the European collaboration needed to achieve results not possible at national level;
- dissemination actions devoted to show their relevance, not only within the scientific community but also for the industrial compartment;
- market-oriented dissemination, focused on the best use in order to reach the suitable stakeholders, such decision-or policy-makers.

5.3. Technology application and demonstration cases

The RAPTOGEN system impact the neuromicroscopy market and sector, currently governed by big players providing bulky and expensive microscopes. RAPTOGEN shift the concept of technological upgrading in microscopy to new paradigms, by launching in the market a unique device that can be simply added on to commercial optical microscopes and turn them into 3D two-photon microscopes for imaging and optostimulation, by reusing available laser sources and editing existing software. The system is a substantial technological upgrade to microscopes performances, while dramatically reducing experimental time and costs (90%) and increase the signal-to-noise ratio respect to traditional microscopes. RAPTOGEN represent a new simple microscope Add–On that can allow the designing of new experimental strategies in neuroscience research

and will ease the access for researchers to this experimental field.

5.4. Technology commercialization

Considering the TRL of the system at end of the Phase 1, we need to perform an engineer industrial design of the Add-On in order to develop a saleable product and make a synergic collaboration with microscopy-orientated company to sell the RAPTOGEN system. We have already contacted European companies interested to insert a system like this in their products portfolio and we are discussing with them in order to define the time to perform all the technical activities and the amount of required resource to define and minimize the time to market.

5.5. Envisioned risks

The consortium have all the technical knowledge to design, assemble and product the RAPTOGEN system, and in the case we have all the resource required to engineer and produce the RAPTOGEN Add-On we need to find microscopy market-orientated company in order to sell and distribute the device. We will mitigate this aspect by constantly monitoring the dynamics of the microscopy market and scientific conferences on this topic, both to reveal business opportunities and to look for microscopy market-orientated companies interested in distribution of the system.

5.6. Liaison with Student Teams and Socio-Economic Study

The coordinator of the RAPTOGEN consortium has a joint research laboratory with the Physics Department of the University of Florence where we develop instruments and devices in the field of Physics and Photonics. The joint laboratory is accessible to all students who want to deal with the world of industrial research to deepen their knowledge during their studies, and if they have innovative ideas, they have a stimulating environment in which to present and share their ideas with research and industrial actors. In particular, during the timeframe of the Phase 1, some students had the opportunity to collaborate with us during their study path and PhD to include the work in their thesis.

6. ACKNOWLEDGEMENT

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