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Recording neuronal activity in 3D using calcium and voltage genetically encoded indicators

Walther Akemann[1] **Joséphine Morizet**[1] **Laurent Bourdieu**[1*]
[1] IBENS, CNRS UMR 8197, INSERM U1024, Ecole Normale Supérieure, Paris.

ABSTRACT

We have developed a non-linear optical microscope for recording 3D neuronal activity in a cortical column, from awake head fixed behaving mice, with unrivalled temporal resolution. This new technique, called Custom-Address Serial Holography (CASH), uses acousto-optic deflectors (AODs) synchronized with a regenerative amplifier operating at 40 kHz. In this configuration, we have shown that the AODs function as ultrafast phase and amplitude modulators, enabling each laser pulse to be spatially shaped at 40kHz. Spatial modulation of laser pulses enables digital holography of extended volumes in the microscope's focal plane for fluorescence excitation. We will demonstrate the possibility of recording the activity of neurons labeled with Genetically Encoded Calcium Indicators (GECIs) such as GCaMP6f with holographic volumes covering their cell bodies. Using holographic volumes covering only cell membranes, we will demonstrate the possibility of using Genetically Encoded Voltage Indicators (GEVIs) to record the activity of 3D neuronal networks. By updating aberration and diffusion correction synchronously with the scanning, we have also performed trans-cranial imaging over fields of view that go beyond the scattering memory effect. Finally, we will discuss the implementation of temporal focusing to improve the optical sectioning of the 3D-CASH holographic microscope.

Keywords: *Two-photon microscopy; acousto-optic deflectors; random access scanning; Genetically Encoded Calcium and Voltage Indicators; holography; 3D scanning.*

[*] ***Corresponding author***: *laurent.bourdieu@ens.fr.*

1. INTRODUCTION

The brain is a complex 3D meshwork of circuits, neurons and micron-scale neuronal processes, which processes information in a distributed way within milliseconds. Understanding brain function therefore requires, ideally, to be able to record brain activity in 3D, at high spatial and temporal resolution. Optical methods, which allow to target neurons in a flexible and minimally-invasive way, offer bright promises and have considerably developed in the last decades. However, recording large numbers of neurons at millisecond temporal resolution, with micron-scale spatial resolution in depth and 3D access remains an unmet challenge.

Non-linear microscopy permits submicron resolution over a depth of a millimeter in the brain. However, because of the need to concentrate light spatially to achieve non-linear effects, it is fundamentally a sequential method, in which the signal arising from a single focal excitation volume (voxel) is collected at each time-point. This imparts a trade-off between the dwell-time (D_t) at each voxel, the number of voxels recorded (N_v) and the frame rate (R) of the recording ($R=1/N_vD_t$). The dwell-time itself determines the total number of photons collected by voxel (N_p) and thus the signal to noise ratio of the recording, at best $\sqrt{N_p}$ when reaching shot noise limit. The total number of photons per time unit is really what matters to discriminate a change in signal in a given object.

A solution to the signal/frame rate trade-off is to keep the dwell-time high but to reduce the number of apparent pixels. While a typical image contains from 250 to 1000 kilo-pixels, the number of identifiable neurons or neuronal parts in the field of view is generally 3 orders of magnitude lower, less than a thousand. Pointing each laser pulse only at one of these objects of interest and recording the signal produced would therefore constitute the optimal solution to record at both high temporal resolution and high signal levels. This "Random-Access" strategy cannot be implemented efficiently with mechanical scanning devices

such as mirrors, because their inertia imparts millisecond-scale switch times between random voxels in the field of view. A technique allowing fast addressing of cells of interest in 3D, with millisecond sampling rate is thus needed. This Random-Access technique should also be compatible with in vivo recordings in head-fixed animal, where brain motion due to breathing, heart beat and muscle contraction will prevent stable recording if not severely mitigated.

2. METHODS

Random-Access scanning has been pioneered in the late 90s by the team of Peter Saggau using Acousto-Optic Deflectors (AODs) [1]. In their standard application as angular deflectors, an AOD used in the Bragg mode efficiently deflects light into the first diffraction order at an angle θ given by :

$$\theta = \lambda.F/v \qquad \text{Eq. 1}$$

where λ is the wavelength of the light, F (~100MHz) the acoustic frequency and v (~650m/s) the velocity of the steady-state acoustic wave inside the crystal. An AOD is working at a central frequency F_0 over a bandwidth $+/-\Delta F/2$ (15 to 20 MHz). The maximum scan angle $\Delta\theta$ is given by $\Delta\theta = \lambda.\Delta F/v$ (~40mrad). The use of an AOD with femtosecond lasers having a broad spectrum $\delta\lambda$ (~10-15nm) raises however issues of temporal and spatial dispersion, that can be compensated at the center of the FOV using diffractive elements [2,3,4]. A crossed AOD pair allows then Random-Access sampling of the FOV at an unmatched speed. The time to establish an acoustic wave in an AOD is given by t_s=L/v, where L is the AOD active aperture. Using large AOD with 13mm aperture to achieve larger FOV give t_s= 20µs. As the dwell time $D_t \sim t_s$, sampling of up 50 000 objects per second (or 50 per ms) can be implemented.

AODs have initially been used as pure deflectors, where the same acoustic frequency occupies the whole profile of the laser beam and where all light is thus deflected to a given angle with a flat wavefront (Eq. 1). However, an AOD can also be used in a non-steady state mode, where it acts as single axis wavefront shapers. We have shown that an AOD that is filled with an ultrasonic pattern of frequency $f_{AOD}(x)$ and amplitude $a_{AOD}(x)$ at a position x in the AOD provides a complex spatial modulation consisting of an amplitude modulation $T(a_{AOD}(x))$, where T is the characteristic amplitude transfer function of the AOD, and a phase modulation $\varphi_{AOD}(x)$, which is related to the frequency modulation $f_{AOD}(x)$ [5]. This shows that an AOD is more than just a deflector, but is in fact a real beam shaping device, acting both as a simultaneous amplitude and phase modulator. Therefore tilt (2D scan) and defocus (3D scan) stands as straightforward applications, while more complex 2D beam spatial modulations can also be achieved and used e.g. in microscopy for digital holography applications.

However, this hides a major difficulty and subtlety. Indeed, a spatially-varying frequency pattern in the AOD cannot be established in a steady-state mode, as an ultrasonic wave is a propagating wave. As the repetition rate of standard lasers in non-linear microscopy (~100MHz) is 3 orders of magnitude larger than the refresh rate of an AOD (~100kHz), consecutive laser pulses interact thus each with different wave patterns in the AOD. To solve this issue, we proposed to use AODs in a "synchronous mode", where the laser pulses are synchronized with the AODs using a low-repetition rate laser, such that the propagating ultrasound pattern is somehow frozen [5]. In this mode, complex phase and amplitude patterns can be generated by the AODs. It includes defocus modulation to achieve fast 3D scans. It allows also to generate complex intensity patterns in the FOV using digital holography [6,7].

3. RESULTS

We have developed a non-linear optical microscope for recording 3D neuronal activity in a cortical column, from awake head fixed behaving mice, with unrivalled temporal resolution. It can indeed address typically 20 cells at 1 kHz or 200 cells at 100Hz. This new optical technique, called Custom-Address Serial Holography (CASH), uses acousto-optic deflectors (AODs) with a regenerative amplifier operating at 40 kHz in the synchronous mode described above. In this configuration, we took advantage of the fact that the AODs function as ultrafast phase and amplitude modulators, to spatially shaped at 40kHz each laser pulse. Spatial modulation of laser pulses enables 3D addressing of neurons but also synchronous digital holography of extended volumes in the microscope's image plane for fluorescence excitation. Whereas defocus modulation allows thus fast 3D scanning at kHz sampling rate, complex shaping of the microscope PSF by wavefront modulation provide 3 key features : 1) it allows to maximize the fluorescence signal by collecting it from an extended cell volume and preventing fluorescence saturation in a diffraction limited spot; 2) it minimizes accordingly photobleaching and photodamage; 3) it allows to minimize motion artifacts. We have generated a library of 2D patterns consisting of 2D arrays of diffraction limited spots or of short segments. To this aim, we have designed different algorithms to determine optimal phase only or complex

patterns in the AODs to achieve the desired intensity patterns in the microscope FOV. These patterns can be adapted to the recording type as e.g. the cell dimension or the amplitude of the motion artifacts that is e.g. larger at large imaging depth. They are also adapted to the cell labeling. In the case of Genetically Encoded Calcium Indicators (GECIs), 2D square arrays of spots were optimal, while 2 line of spots positioned next to the cells can be used to estimate the neuropil contamination. In the case of Genetically Encoded Voltage Indicators (GEVIs), patterns consisting of several small parallel segments positioned near the plasma membrane on each side of the cell were found optimal in terms of SNR and motion artifact mitigation.

3D-CASH recordings of GCaMP6f expressing neurons in layer 2/3 and 5 of mouse primary visual cortex in response to moving contrast gratings reveal the layered organization of the cortex, in the structure of the neuron pairwise correlation matrix. 3D-CASH provides also the possibility of using Genetically Encoded Voltage Indicators (GEVIs) to record the activity of 3D neuronal networks at millisecond timescale, taking advantage of the most recent GEVIs optimized for spike detection. 3D-CASH also allows performing trans-cranial imaging over fields of view that go beyond the scattering memory effect by updating aberration and diffusion correction synchronously with the scanning, Finally, 3D-CASH is limited currently to samples sparsely labeled due to the reduced optical sectioning associated to the holographic illumination, an effect that we aim at reducing by implementing temporal focusing.

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