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Set-ups to induce by light a memory in in-vitro neuronal cultures

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ABSTRACT

To study the brain and the related neuronal network activity, many attempts were made to design and develop platforms able to induce and record neuronal signals. However, many brain processes - like memory formation and storage - and diseases - like amnesia or epilepsy - need more basic studies. For these, a bottom-up approach is needed, starting from 2D in-vitro neuronal cultures. In this work, we will present two experimental systems able to optogenetically interact with 2D neuronal networks with patternized light. One system consists in a Digital Light Projector (DLP) integrated in a microscope setup, which can illuminate neurons from the top; the other, is a compact and transportable photonic chip, properly designed to illuminate neurons plated on its surface.

Keywords: hybrid, neural, interface, digital, light, projection, photonic, chip

1. INTRODUCTION

In order to study the brain and the related neuronal network activity, many attempts were made to design and develop platforms able to induce and record neuronal signals. Many were developed for in-vivo applications, in order to study processes and behaviours of the complex neuronal networks in the brain 1–14. However, many brain characteristics - like memory formation and storage - and many diseases - like amnesia or epilepsy - need more basic studies. For these, a bottom-up approach is needed, starting from 2D in-vitro neuronal cultures. In this frame, different kind of systems can be implemented, able to interact with single neurons (or even specific parts of them) and read their responses, in elecrophysiological or optical way 15–22. Between the optical techniques, optogenetics is one of the most powerful, that let the control of neuronal activity with high temporal and spatial resolution: it consists in the expression in cells of special light sensitive proteins, namely *Channelrhodopsins* (ChRs) that act like ionic channel on the cell surface. When these proteins are illuminated by a specific wavelength and intensity, they let the entrance or exit of ions, thus inducing excitation or depression of the neuronal activity 23–26. For this work, we used ChR2 channelrhodopsin, which induces neuronal excitation under illumination with about 460 nm of wavelength.

The work is carried out within the BACKUP project 27, whose aim is to create a hybrid platform formed by a neuromorphic photonic chip and a biological neuronal network. From a biological point of view this platform allows the study of some features and activities of simple 2D neuronal network. In particular, we focus our attention on the process of memory formation. Memory is formed after an event induces repeated stimuli on some neurons inside a neuronal network. These stimuli induce long term potentiation (LTP) on the involved cells which then form the so-called engram 28, 29: this is essentially a sub-network that is related to that specific memory. We thus want to induce the formation of an engram in vitro through patternized light and optogenetics. With this aim, we developed two experimental systems able to illuminate 2D neuronal networks with patternized light. One system consists in a Digital Light Projector (DLP) integrated in a microscope setup, which can illuminate neurons from the top; the other, is a compact and transportable photonic chip, properly designed to illuminate single neurons plated on its surface.

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2. MICROSCOPY SETUP

The microscopy setup consists in a microscope system (X-Light V2, Crest optics), which includes a confocal spinning-disk module with an integrated Super Resolution module, based on structured illumination microscopy, in upright configuration. The system is equipped with 7 solid state LED sources that cover the spectrum from violet (395 nm) up to red (640 nm) arranged in the SPECTRA Light engine. As shown in figure 1, the light coming out from the SPECTRA (green lines) is guided through an optical fiber to the super-resolution/confocal modules. Here, a dichroic filter selects the desired spectrum. Thanks to a mirror, the light is reflected down to the objective, which focalizes the light on the sample. If the sample has fluorescence indicators, the emitted light (red lines in the figure) can be collected by the objective, and arrives to a CMOS camera, passing through the dichroic mirror and other emission filters. The camera is a Prime BSI Back-Illuminated BSI Scientific sCMOS with 95% of quantum effciency, with 2048 x 2048 pixels with 6.5μm x 6.5μm pixel area. The system can be used in widefield (i.e. without confocal nor super resolution acquisition), confocal mode or super-resolution mode. For the confocal mode, it has a double pinhole pattern spinning disk, one with 40 μm holes, and one with 70 μm ones, which can be inserted in the path or kept away. With spinning disk, the confocal resolution with 60x NA 1.4 oil immersion objective is <800nm. For the super-resolution mode, there are many patterns of structured illumination masks provided, whose interference with high frequency spatial details of the image lets the reconstruction of the final image with a lateral resolution of ∼120 nm and an axial resolution of ∼300 nm. The system is equipped with two water immersion objectives with 60x (LUMPLFLN60XW, NA 1, WD 2mm) and 20x magnification (UMPLFLN20XW, NA 0.5, WD 3.5mm), a 100x immersion objective (UPLSAPO100XO, NA 1.4, WD 0.13 mm) and a dry long working distance 10x objective (LMPLFLN10XLWD, NA 0.25, WD 21mm). The control of the system and acquisition is performed through MetaMorph acquisition and analysis software. Taking advantage of a free entrance in the rear part of the system, a Digital Light Projector (DLP) was integrated with the microscpe set-up (figure 1d).

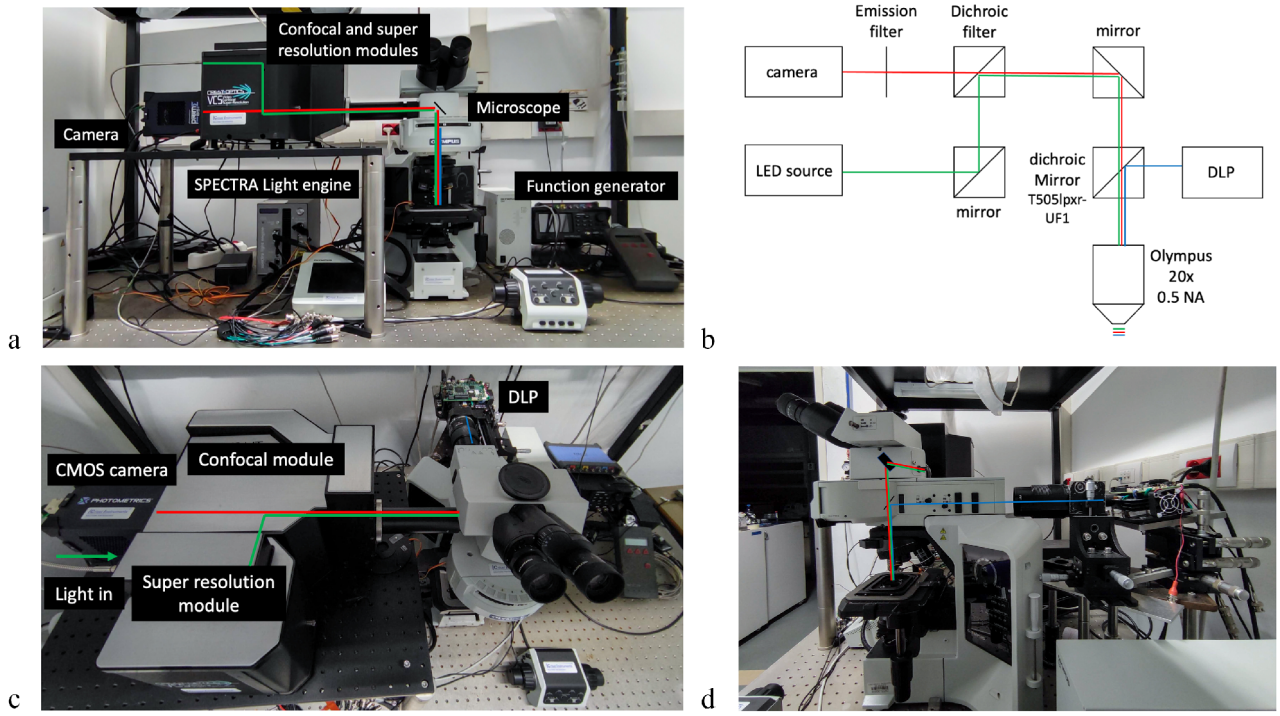

Figure 1. In figure are explained in detail the different parts of the system used in this work. A frontal (a), up (c) and lateral (d) view of the system are shown, together with a scheme of the optical path (b). In the figures, in green is represented the excitation light, in red the light emitted by the sample and in blue the light coming from the DLP.

3. CELL CULTURES

Cortical and hippocampal neuronal cultures were prepared from wild type mice (C57Bl6) embryos at E 17.5 days of age. In brief, cortices and hippocamp are isolated from embryos and incubated for 20 min at 37°C in Trypsin/EDTA 0.25%. Cells are dissociated with a plastic pipette, collected by centrifugation (800 RPM, 5 min) and resuspended in Neurobasal supplemented with B27-supplement and Penicillin/Streptomycin/Glutamine and plated on poli-L-Lysine coated coverslip. Neurons are plated on 12 mm diameters glass coverslips and, in order to let light excitation, are infected with pAAV-Syn-ChR2(H134R)-YFP at days in vitro (DIV) 2-4. We performed the experiments starting from 10-11 DIV, when the network is sufficiently developed and the virus is correctly expressed in all the neurons.

4. DLP SYSTEM

The system is a DLP E4500, which includes 3 LEDs, optics, a WXGA DMD (Wide Extended Graphics Array Digital Micromirror Device) and a driver board. The light engine can produce approximately 150 lumen at 15 W LED power consumption. The blue LED (488 nm) that will be used in this work has a power of 600 mW. The light from the LEDs impinges on the DMD which has 1039680 mirrors arranged in 912 columns by 1140 rows with a diamond array configuration. Each of these mirrors has two main possible inclinations that reflect the light in a different direction. This system allows to get patternized illumination with preloaded and custom patterns that can be chosen through the DLP E4500 software. Moreover, these patters can be pulsed in time, with both an internal or external trigger, with a nominal precision down to μs. The system supports 1-, 2-, 3-, 4-, 5-, 6-, 7-, and 8-bit images with a 912 columns $\times$ 1140 rows resolution. These images are pixel accurate, meaning that each pixel corresponds to a micromirror on the DMD. The light coming from the DLP system is collimated and aligned to the optical path of the microscope from the rear port of the system. As can be seen in figure 1d, the DLP is positioned on a 5-axis stage, while a macro TAMRON 90mm AF2.5 objective is mounted on a 3-axis stage in order to collect the DLP light and create the desired image on the sample plane. The light is directed down to the sample thanks to a dichroic mirror (Chroma T505lpxr-UF1) which acts like a high-pass filter, reflecting all the wavelengths smaller than 505nm. In this way, we can use the DLP and in the same time record signals in yellow, red and far-red. The alignment of the system was performed in order to obtain blue patterned illumination on the sample plane of the microscope, with high contrast and enough intensity to excite the neurons, using 10x and 20x objectives. Considering the aberrations of the optics in this system, the minimum resolution is 3.5 μm and the minimum distance between two points to get a contrast higher than 10 is 15 μm. In order to excite single neurons, spots can be created in the desired positions. For the measurements presented in this work, we used spots of 13-10 pixels of diameter in the original image loaded to the DLP. The area of influence of the light excitation has a diameter that change in the range of 19-44 μm with the 20x objective and 29-68 μm with 10x objective. The measurements that will be shown in this work were performed with the 10x objective, with an excitation intensity of about 3 mW/mm^2, enough to excite ChR2 23,30–35.

4.1 Calcium experiments

After the system setup and alignment, we performed some experiments to prove that the DLP system is able to optogenetically excite the neurons of our in-vitro neuronal cultures. There are many fluorescence indicators that can be used in order to optically detect the neuronal activity. We decided to use X-Rhod-1AM calcium indicator whose excitation and emission peak are around 580 nm and 603 nm respectively 36–39: its spectral characteristic allows using the DLP to excite the indicator and recording its emission through the dichroic filter, that reflects the blue light from the DLP but transmits wavelengths higher than 505 nm. We performed calcium recording on 10-13DIV neurons plated on glass coverslips. Neurons were loaded with 1μM X-Rhod-1AM for 30 minutes in darkness in HBSS supplemented with Ca2Cl (2mM) and MgCl (1mM). Coverslip were then washed with HBSS supplemented with Ca2Cl (2mM) and MgCl (1mM) for 30 min and placed onto the stage of the microscope, ready to be excited by the DLP. In order to perform the experiments, we used two optical lines of excitation (580 nm for X-Rhod-1AM and 488 nm from Channelrhodopsins) and one reading line (603 nm for X-Rhod-1AM emission spectrum). The different wavelengths were selected through dichroic mirrors and band pass filters as shown in figure 2. We will refer to the different filters configurations with the terms *RFP configuration* for the excitation and acquisition of X-Rhod-1AM signal and *YFP configuration* for the acquisition of the fluorescence

indicating the infection with ChR2. To not have artefacts in our data given by a weak blue signal that leaks and arrives to the camera during the excitation, we triggered the DLP on when the camera is off, as represented in figure 2. A special BNC breakout cable gives access to different status of the camera and trigger options, among which the "Read out" output of the camera indicates that the camera is currently digitizing. Thus, we decided to connect this output of the camera to the trigger in port of the DLP. In the DLP software we selected the external negative trigger option, with a pattern of 20 ms long with a frequency of 3Hz. First of all, we check the health of the neuronal culture in transmitted light, while in YFP and RFP configurations we check the correct infection with ChR2 and loading of X-Rhod-1AM respectively (figure 2). Through Metamorph software we set up a timelapse acquisition with 1.5 Hz frequency. Doing so, each time the camera finishes to acquire one frame, the DLP starts two pulses of the stimulation pattern. The DLP pattern can be switched on and off manually: for each acquisition we wait about 100 frames (66 seconds) of background acquisition, then we switch on the DLP pattern for 20 frames (13 seconds, 40 pulses in total) and eventually we continue recording for a total timelapse of 300 frames. Images are saved in Multi-Tiff mode, are analysed in Metamorph for a first check, and then loaded in MATLAB for data analysis.

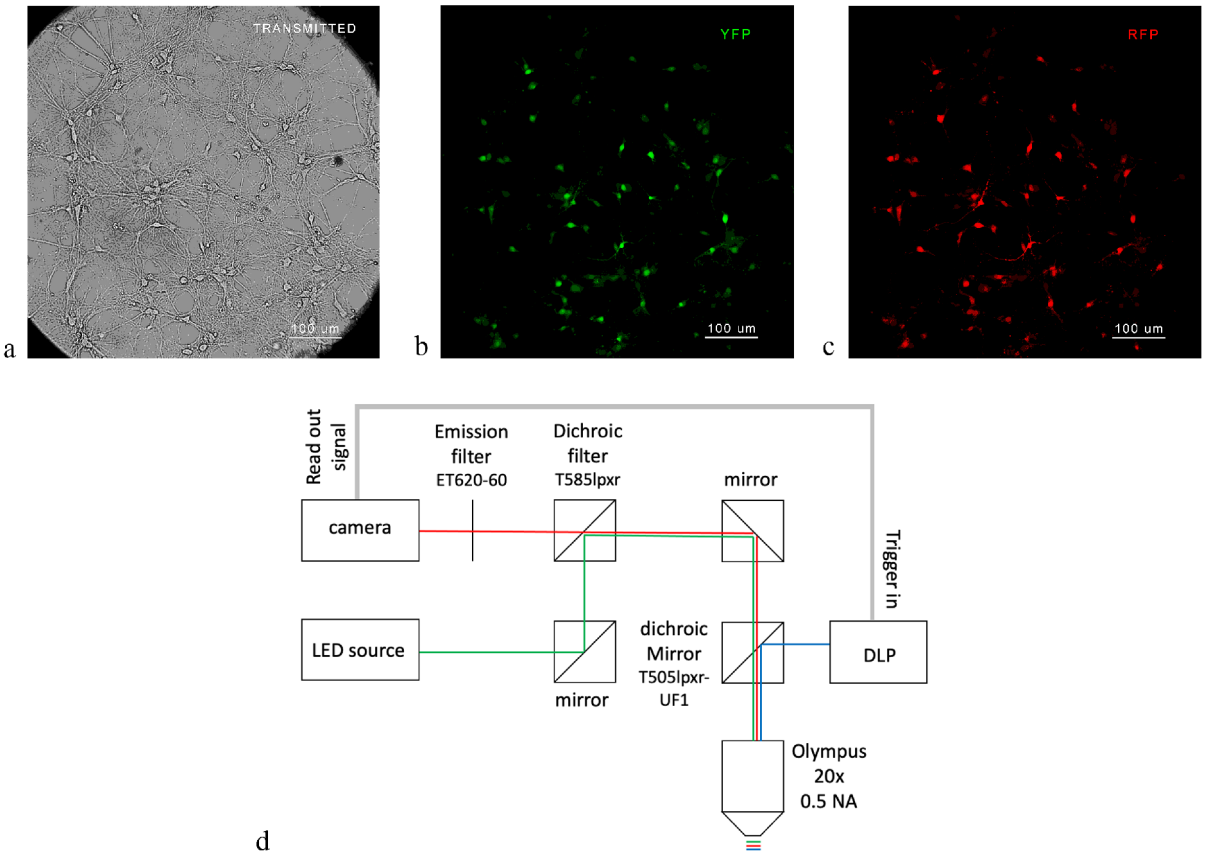

Figure 2. A culture under the microscope seen with 20x objective in transmitted light (a), YFP configuration (b) and RFP configuration (c). These images were taken without crops of the image got by the camera sensor: this is the reason for the black corners. (d) The setup used for calcium signal acquisition. To acquire the images of X-Rhod-1AM signal, we used RFP configuration: the green LED excites the sample and red light is collected, filtered with a bandpass filter and brought to the camera. The optogenetic excitation was performed with blue light coming from the DLP, reflected to the sample thanks to a dichroic mirror. The camera and the DLP were triggered in order to avoid artefacts in measurements.

4.2 Signal analysis

In literature there are a lot of works in which people aim to analyze calcium signals in order to understand the neuronal network behaviour 40–49. Since usually in these experiments there are many neurons involved, each

with its fluorescence trace to be analysed in time, we decided to implement our own analysis in MATLAB, in order to have a direct control over the analysis process of our data: we implemented automatized processes with always the opportunity by the user to check the correct ROI selection and trace analysis. The main steps are the following:

- automatically localize the neurons in the images;
- reconstruct time traces of the signals and analyse them;
- eventually reconstruct from traces the behaviour of the network.

Finding ROIs

After importing the Multi-Tiff images, we start localizing neurons in the field of view. To do so, first of all we divide the intensity range of the image in a finite number of levels (50 in our case). Then, we use each of these levels as a minimum threshold to create a binary image. This image will present a lot of individual areas, some correctly located above neuronal bodies, others on wrong features or processes. Thus, on this binary image we use the *regionprops* Matlab function to get informations about the regions found, like area, centroid, minimum and maximum axes, the proportion between the axes, the list of the pixels and the maximum of the intensity. Then, we impose conditions on these parameters in order to select just the correct regions that will become our ROIs. Since we are performing this analysis for each intensity levels, we then filter duplicates looking at the distance between the centroids of the ROIs. Then, the user is asked to check the found ROIs and to select those that are not correct: in order to not miss neurons, we decided to use less restricted thresholds in the previous steps and leave the user to clear the found ROIs. Another way is to restrict more the conditions with the possibility that some neurons will be missing. In case of a spot illumination, also the centroids of the spots are saved. In figure 3 are shown the centroids of the final ROIs. Then, for each frame of the Multi-Tiff images, the pixels' intensity average of each ROI is computed in order to get the trace of the calcium fluorescence in time. From the raw traces, the positions of the ROIs and the position of the spots, is then possible to perform the desired analysis on the calcium response of the neuronal network after a given stimulation.

Trace analysis

First of all, for each trace, we interpolated data up to the first frame of excitation. We then normalized the entire trace to this interpolation line in order to flatten the baseline. The expected calcium response is characterized by a fast increment of the fluorescence simultaneous to the start of the light excitation and a slow decay to the basal level at the end of the stimulus. We will refer to this kind of responce as a *spike* signal. On the normalized trace we can then compute different parameters:

- $\Delta F/F_{rest}$: where ΔF is given by the maximum of the fluorescence signal in the time window of the excitation minus its value just before the start of the stimulation, while F_{rest} is the average of 50 frames before the excitation. We will refer to this quantity as *response intensity*;
- Σ: is the maximum absolute variation of the trace before the stimulus;
- $corr_{std}$: the correlation of the trace with a spike-shape trace (figure 4)
- $corr_{sat}$: the correlation of the trace with a saturated-shape trace (figure 4)

Looking at these values, different thresholds can be imposed in order to select which traces are representative of the neuronal responses and which one are not. For example, we decided to consider as representative responses the traces with a $\Delta F/F_{rest}$ >0.003 and $\frac{\Delta F/F_{rest}}{\Sigma}$ >1. This last condition ensures that a calcium spike will be considered as response just if it is higher than possible oscillations of the fluorescence before the stimulation. As can be seen from figure 4, some of our traces have a spike like behaviour, while others do not come back to the baseline level but keep a high level of fluorescence; some have a middle way shape. We think that this behaviour is mainly due to the indicator that holds calcium and do not release it during our acquisition time. Since we

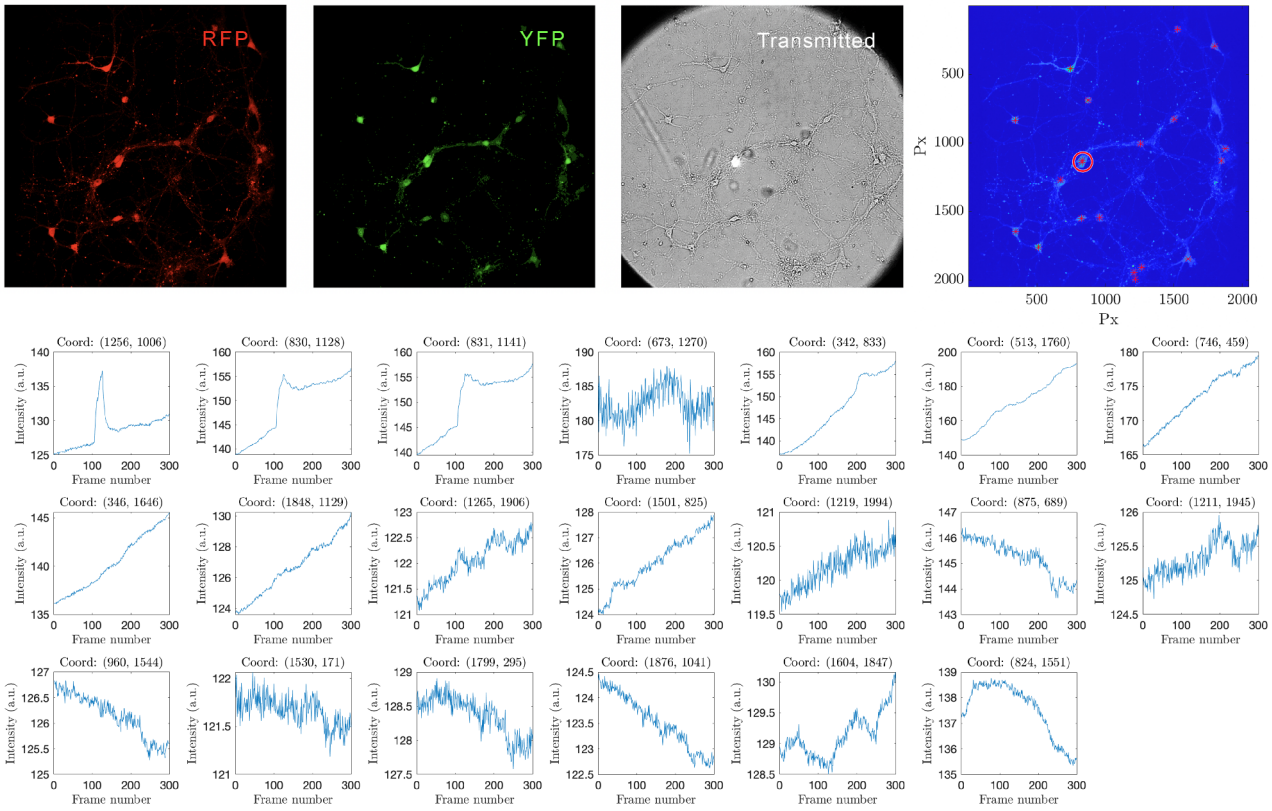

Figure 3. (Top panels) From left to right are shown the fluorescence signals in the field of view of the calcium indicator (in RFP configuration), the infection with ChRs (YFP configuration), the image in transmitted light with the spot from the DLP on the excited cells and the centroids of the found ROIs after the analysis. (Bottom panels) Raw traces got from the analysis of the ROIs. The coordinates refer to the centroids of the analysed ROIs.

were interested in neurons response and not in the kinetic of the system, we decided to consider as responses all traces that show, at the time the stimulation started, a big discontinuity, i.e. $\Delta F/F_{rest} >> 0$.

Another useful analysis, is to compute the calcium response of the image pixel by pixel: in this way it is possible to see the response of both the neuronal bodies and the processes. This analysis consists simply in getting the calcium trace of each pixel of the image along all the frames of the Multi-Tiff image, computing $\Delta F/F_{rest}$, and plot these values in a 2D image, as shown in figure 5 (right image). In the figure on the left, is shown the level of infection with ChR2 of the cells through, the fluorescence intensity of YFP.

As can be seen in the example reported in figures 3 and 5, 3 neurons responded to the illumination: 2 of them are located under the spot, while one neuron responded also if not illuminated. This can be explained by two main reasons: one or more processes were near the excited cells and were thus illuminated, or the recorded signal is the result of an existing link between the illuminated cells and the responding one.

This kind of analysis can be performed with 1 or more excitation spots or also illuminating big areas of the field of view (figure 6). Also in these case, some of the not illuminated neurons respond, and the reason is the same as before. In particular, in the case of the half field illumination, it can be seen that the neurons that are responding on the right are connected with neurons on the left by clearly visible processes. In both the cases, not all the illuminated neurons respond: this can be due to several factors, such as the level of infection with ChR2 or problems related to the calcium indicator, like a signal already saturated before excitation or a lack of sensibility for too low responses. These kinds of patterned illumination can be of big importance in fundamental biological studies in which one or more cells must be excited or inhibit at a desired time. If from the DLP is set a temporal pattern able to induce long term potentiation on neurons, the system is potentially able to create an

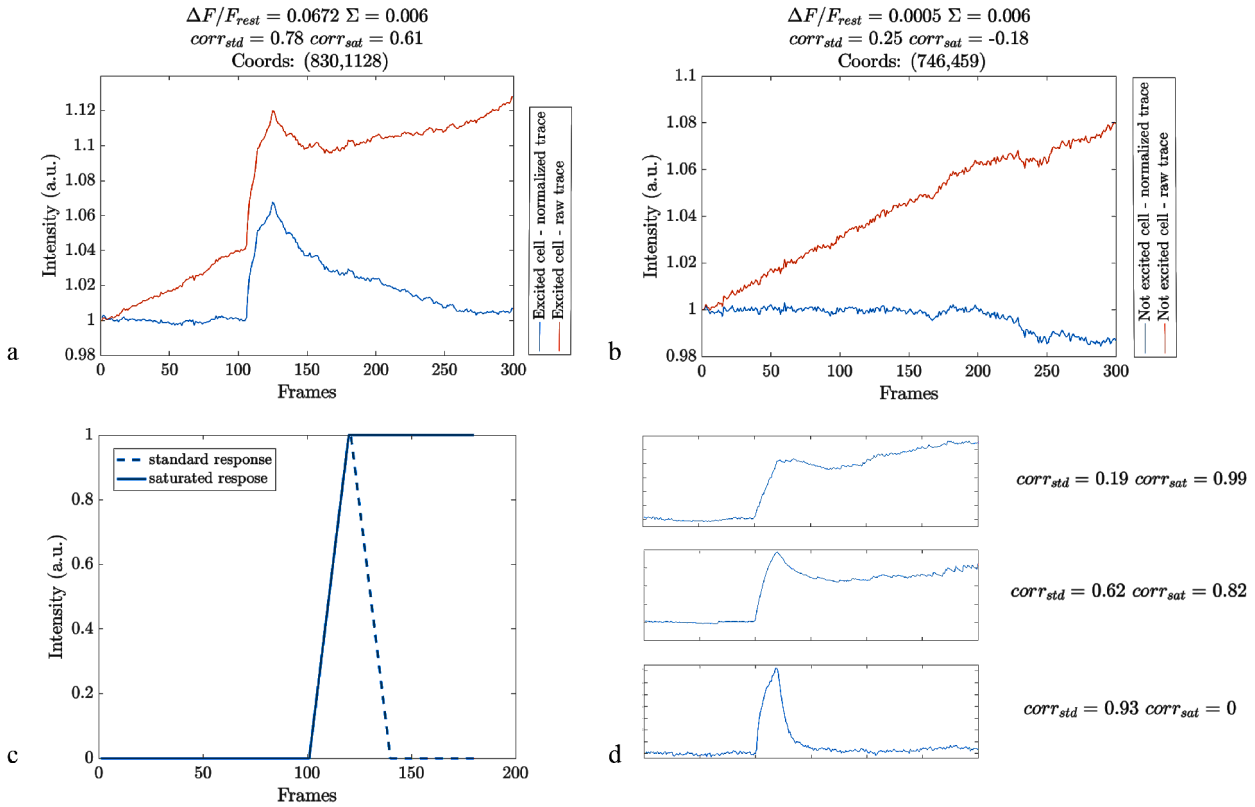

Figure 4. The raw (red line) and normalized (blue line) traces from a responding cell in the field of view (a) and one from a not responding cell (b). (c) Reference traces of a standard (dashed line) and of a saturated (continuous line) response. (d) Examples of standard or saturated traces, with on the right the computed $corr_{std}$ and $corr_{sat}$ values.

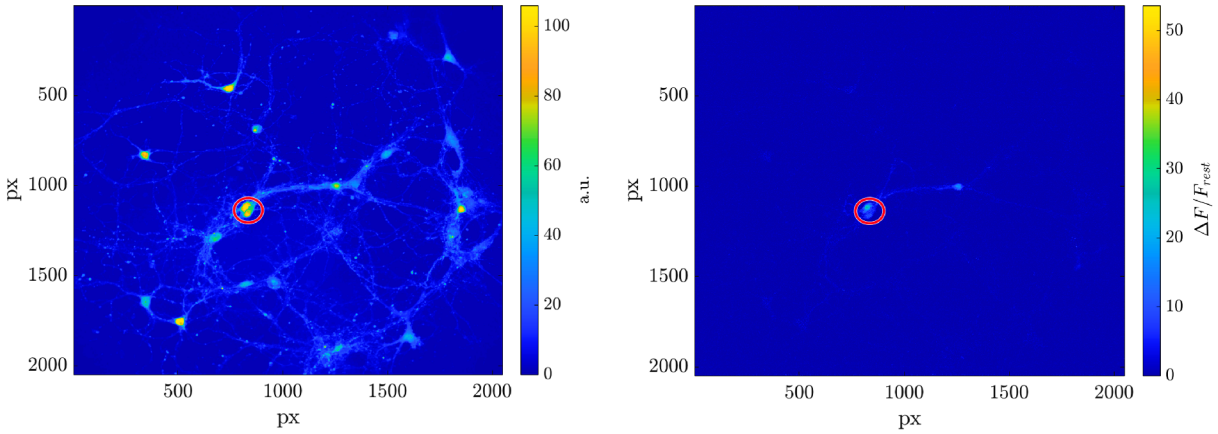

Figure 5. (left) Level of YFP indicator (i.e. the level of infection with ChR2); (right) response intensity ($\Delta F/F_{rest}$) obtained from the traces of each pixel.

engram in the neuronal culture. The cells can then be fixed and different morphological and molecular studies can be performed.

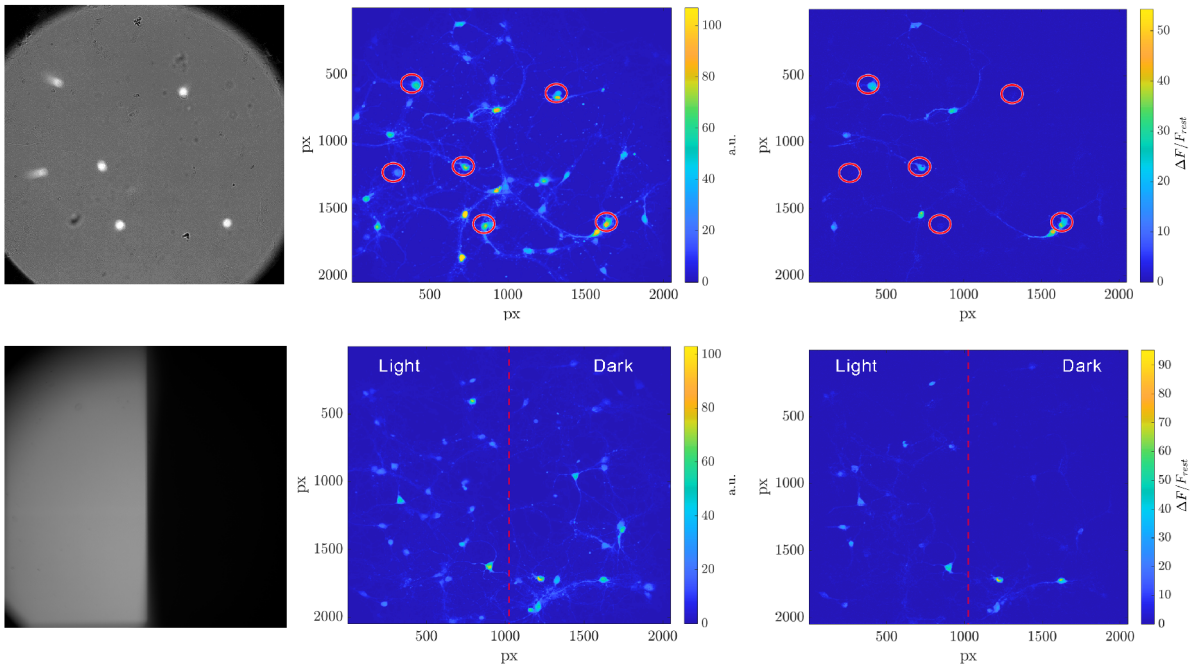

Figure 6. In figure are shown one example of 6 spots excitation (top row) and one of half field illumination (bottom row). From left to right are represented the pattern of illumination used, the level of infection of the cells with ChR2, and the pixel by pixel analyses.

5. CHIP

The second approach presented in this work is a photonic chip, properly designed to illuminate single neurons plated on its surface. In photonic chips, light travels inside *waveguides*, which are layers of material with high refractive index, embedded in low refractive index material which we refer as *cladding*. We design custom scatterer arrays that deflect the light travelling in the waveguides out of the surface of the chip. The scatterers consist in aperiodic gratings whose design was performed with a *FEM enhanced transfer matrix method* 50. If we consider the grating as a sequence of holes in a waveguide, this method consists in simulating with a Finite Element Method (FEM) simulator the scattering of a single hole with different geometric characteristics and find the optimal sequence of holes with a particle swarm optimization on a transfer matrix description of the system; the optimization is performed in order to get a desired interference pattern between the light scattered by all the holes. In particular, in this application the aim is to get a specific light distribution at a certain height above the grating, collecting the maximum intensity in the desired place. At the same time around and above the area where the desired distribution is formed, the light intensity should be low enough to not excite other sample parts that can possibly be hit by the light. We designed grating scatterers able to get 10 mW/mm^2 intensity on an area comparable to the neuronal body, a rounded shape with about 10 μm diameter, with a gaussian distribution. This spot is formed at a distance of 5 μm from the waveguide plane. This distance was chosen considering that the SiO_2 cladding layer on the top of the grating was 2μm and that a neuronal body is about 10μm thick: therefore, every distance from the grating plane between 2μm and 12μm would ensure the cell excitation. In figure 7a is shown the scattering profile of the optimized gratings and the integral of the scattered field along the 10 μm thickness of a neuron, in all the x domain. From this integral profile we can get the effective length of the area that will excite the neuron: the continuous vertical lines are located at 50 % of the maximum intensity, while the dashed ones are located at 20 %. Changing the input power of the laser, it can be chosen the effective area of excitation of the scatterer. In figure 7b is shown the spot created on the surface of the chip: it can be seen that the maximum intensity is confined in an about 10x10 μm^2 region. This image is obtained by the multiplication of the fields propagating along z and x directions, independently obtained: the field along x results from a FEM based mode solver, considering a waveguide with the same materials and

a rectangular cross section 8 × 0.15 μm^2. The drawing in figure 7c shows how the system works: if we plate neurons on the surface of the chip and if some of them grow near enough to the grating position, they can be excited by the scattered light. In photo 7d is shown the behaviour of one matrix of the chip: it can be seen that the spots are spatially well confined and, if looked under the microscope, these spots are about 10μm large, as shown in figure 8. In the photo, the chip was cut and the alignment to the chip was performed in butt coupling for the sake of simplicity during the chip characterization. The coupling during the experiments with neurons is performed by using grating couplers and a fiber array, as will be discussed later. The grating coupling was designed in order to not have straight light that could hit other neurons in the culture.

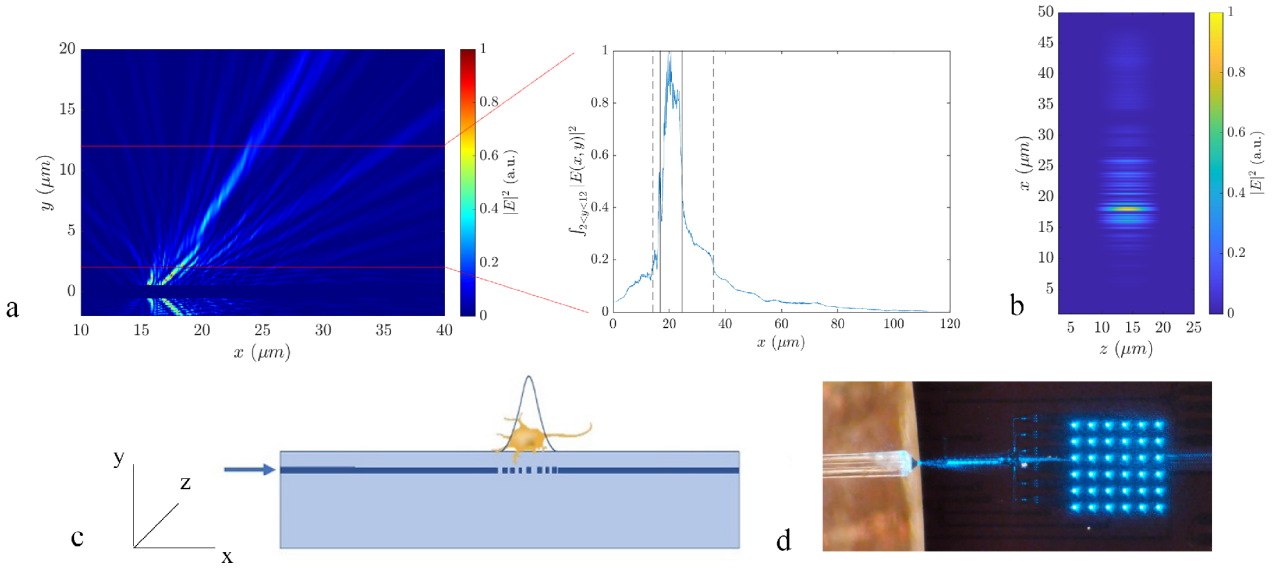

Figure 7. (a) Cross section of the light scattered from the optimized grating, considering water over y=2μm. On the right is represented the integral of the $|E(x,y)|^2$ in the range 2< y <12 μm, plotted along x; the continuous vertical lines are located at 50 % of the maximum intensity, while the dashed ones are located at 20 %. (b) The simulated spot created on the surface of the chip (y = 2 μm). (c) Sketch of the system: if a neuron grows on one of the scatterers, it can be excited by the scattered light. (d) Photo of a matrix of scatterers realized in the photonic chip. In this photo, the chip was cut and the alignment to the chip was performed in butt coupling for the sake of simplicity during the chip characterization.

The scatters are arranged in matrices and can be switched on simultaneously or singularly. The designed chip has a 5.3x4.7 mm^2 area and was designed in order to work at 488 nm, with SiN waveguides, embedded in SiO_2. Light is coupled inside the chip through a fiber array that directs light to grating couplers as shown in figure 8. In order to plate neurons on the surface of the photonic chip and let them grow until the desired age, the chip must be put inside a dish in order to contain culture medium. Moreover, the whole system must have the proper dimensions to be used under the microscope in order to be able to excite the neurons with the chip and simultaneously monitor possible fluorescence indicators inside the cells. Thus, a proper design of the system was performed, in order to be as versatile as possible. In figure 8 are shown in detail all the steps followed to create the final system. First of all the fiber array is aligned to the chip with the help of a teflon fork, mounted on a 3 axis stage. The presence of a waveguide that connects the first and last grating of the chip helps the correct alignment. The coupling is performed putting a small drop of water on the surface of the chip: indeed, the presence of air or water between the chip and the fiber array changes the coupling angle. Since the system will be water immersed for the experiments with neurons, it is important to ensure the coupling with water. Once the chip and the fiber array are aligned, they are glued with Attack (ethyl cyanoacrylate): this adhesive was chosen because it has low viscosity (20-80 cps), good tensile strength (2700 psi), a refractive index near to the water one (1.4), and good biocompatibility. The fork is then detached and the chip with the fiber array are inserted in a petri dish, with a fissure on one side, in order to let the optical fibers to lay othside the petri. The fissure is then sealed with Elastosil E43 silicone. All this system is then fixed on a custom platform compatible with the microscope sample holder, in order to be able to perform optical monitoring and measurements of neuronal