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Optogenetic stimulation and recording of primary cultured neurons with spatiotemporal control

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Abstract

We studied a network of cortical neurons in culture and developed an innovative optical device to stimulate optogenetically a large neuronal population with both spatial and temporal precision. We first describe how to culture primary neurons expressing channelrhodopsin. We then detail the optogenetic setup based on the workings of a fast Digital Light Processing (DLP) projector. The setup is able to stimulate 10 to ~100 neurons with independent trains of light pulses that evoked action potentials with high temporal resolution. During photostimulation, network activity was monitored using patch-clamp recordings of up to 4 neurons. The experiment is ideally suited to study recurrent network dynamics or biological processes such as plasticity or homeostasis in a network of neurons when a sub-population is activated by distinct stimuli whose characteristics (correlation, rate, and, size) were finely controlled.

Keywords

Primary culture of neurons, optogenetics, patterned optical stimulation.

Background

Optogenetics provide a mean to control neuronal activity with millisecond precision. However, neurons are often activated simultaneously either by flashes of light that activate the whole population synchronously or by a light whose intensity is temporally modulated (Boyden, Zhang et al. 2005). Yet, several methods exist to modulate the light spatially and have been used to uncage glutamate (Nawrot, Schnepel et al. 2009) or activate channelrhodopsin (ChR2) expressing neurons (Guo, Hart et al. 2009) (for review of available methods see (Anselmi, Banerjee et al. 2015)).

A first possibility is to use a laser and move its beam quickly over different locations. For example, uncaging glutamate at different dendritic locations has been achieved by deflecting a laser beam with acousto-optic deflectors (Shoham, O'Connor et al. 2005). This strategy is likely viable only if we modulate the light intensity sufficiently slowly over a limited area. Alternatively, a spatial pattern of light can be achieved using phase or intensity light modulators. Holographic technique based on phase modulation permits to obtain an image in three dimensions with a good spatial precision but patterns can be displayed at a rate of only 100 Hz (Papagiakoumou, Anselmi et al. 2010). If a two dimensional pattern is sufficient, intensity modulation can simply be obtained by placing a projector or an array of LEDs in the conjugated plane of the sample (Farah, Reutsky et al. 2007, Guo, Hart et al. 2009). This technique has the advantages of being easy to implement, can target many regions of interest simultaneously and has the fastest temporal resolution.

Here we took advantage of a fast video projector based on the workings of a Digital Micromirror Device (DMD). A LED light source is split by an array of micromirrors that can be controlled with sub millisecond precision in order to display any arbitrary pattern of light (Barral and Reyes 2016). An image of the projector is focalized to the sample plane via a pair of lenses and the microscope objective. The DMD technology offers an unprecedented temporal precision that enables to display patterns at 1.44 kHz and even faster DMDs are now available. In our settings, the resulting pixel size (2.2 μm \times 1.1 μm) was sufficiently small to stimulate single neurons.

To stimulate a single neuron, we selected a region of interest of ~30 μm \times 30 μm , centered at the soma of the neuron of interest and sent a 5 ms pulse of blue light. By designing patterns that are projected onto the

sample, we could target independently and simultaneously a large number of neurons (10 to 100 neurons). Stimulated neurons were both excitatory and inhibitory (expression of ChR2 under the Synapsin promoter) and were activated by Poisson spike trains. The rate and correlation of the spike stimuli were controlled by the experimenter (see (Barral and Reyes 2016)). By recording from neurons that expressed ChR2, we verified that stimulated neurons responded faithfully to the light pulses. We then recorded concurrently the membrane potentials of up to 4 neurons in cell-attached and in whole-cell configurations to isolate the postsynaptic inputs and the spiking activity, respectively.

Material and Reagents

For the neuronal culture

1. Poly-L-lysine hydrobromide - mol wt 70,000-150,000 (Sigma, P1274)
2. Sodium tetraborate decahydrate $\geq 99.5\%$ (Sigma, B9876-500G)
3. Boric acid (cell culture tested) (Sigma, B9645-500G)
4. Agar (Sigma, A1296-100G)
5. Papain from Carica papaya - 10 ml 100 mg (Roche, 10108014001)
6. L-Cysteine hydrochloride monohydrate (Sigma, C7880-500MG)
7. DNase I grade II, from bovine pancreas - 100 mg (Roche, 10104159001)
8. Neurobasal Medium (1X) (Thermofisher, 21103049)
9. B-27 Serum-Free Supplement (50X), liquid (Thermofisher, 17504044)
10. GlutaMAX™ Supplement (Thermofisher, 35050061)
11. Sodium pyruvate solution (Sigma, S8636-100ML)
12. HBSS (10X), no calcium, no magnesium, no phenol red (Thermofisher, 14185052)
13. Penicillin-Streptomycin (10,000 U/mL) (Thermofisher, 15140122)
14. HEPES (Thermofisher, 15630080)
15. Albumin from bovine serum (powder, suitable for cell culture, $\approx 96\%$) (Sigma, A9418-5G)
16. Trypsin inhibitor from chicken egg white (Sigma, T9253-500MG)
17. Trypan Blue solution (0.4%, liquid, sterile-filtered, suitable for cell culture) (Sigma, T8154-20ML)
18. AAV virus for ChR2 expression (University of North Carolina Vector Core Services, AAV2-hSyn-hChR2(H134R)-mCherry)

For electrophysiology recordings

1. Borosilicate glass capillaries (1.5 OD) (WPI, 1B150F-4)
2. Artificial cerebrospinal fluid (aCSF, see recipe)
3. Intracellular solution (see recipe)

Equipment

For the neuronal culture

1. Cell culture incubator (Thermofisher, ThermoForma Series Model 3110)
2. Round coverslips, 25 mm diameter, German Glass (Electron Microscopy Science, 72196-25)
3. Vibratome slicer (Leica, VT1200S)
4. Siliconized Low-Retention Microcentrifuge Tubes (1.5mL) (Fisher, 2-681-331)
5. Low-Retention Pipet Tips 200 μ L (Fisher, 02-717-165)
6. Low-Retention Pipet Tips 1000 μ L (Fisher, 02-717-166)
7. Disposable Petri Dishes (35x10 mm) (BD Falcon, 08-757-100A)
8. Stericup-GP sterile vacuum filtration system, 0.22 μ m, polyethersulfone (EMD Millipore, SCGPU05RE)
9. Syringe Filters; MCE membrane; Pore Size: 0.22 μ m (EMD Millipore, SLGS033SS)
10. Sterile transfer pipets (Fisher, 13-711-20)

11. Conical Sterile Polypropylene Centrifuge Tubes (ThermoFisher, 339650)
12. Dumont #5 Forceps (Fine Science Tools, 11251-30)
13. Dumont #7 Forceps (Fine Science Tools, 11271-30)
14. Extra Fine Bonn Scissors (straight) (Fine Science Tools, 14084-08)

For the electrophysiology setup

1. Upright water immersion microscope with fluorescence (Olympus, BX51)
2. Fluorescence filter set for mCherry (Chroma, 41002C TRITC)
3. CCD camera for fluorescence imaging (C8484, Hamamatsu)
4. CCD camera for IR imaging (Olympus, OLY-150 IR)
5. Micromanipulator (Luigs & Neumann, SM6 set)
6. Patch-clamp amplifiers (Dagan, BVC-700A)
7. 18-bit interface card (PCI-6289, National Instruments)
8. Flaming/Brown micropipette puller (Sutter Instruments, Model P-97)

For the optogenetic setup

1. DLP projector (Texas Instrument, DLP LightCrafter)
2. Aluminum Breadboard, 150 x 300 x 12.7 mm, Double Density, M6 Thread (Thorlabs, MB1530/M)
3. Ø1" Achromatic Doublet, SM1-Threaded Mount, f=35 mm, ARC: 400-700 nm (thorlabs, AC254-035-A-ML)
4. Ø2" Achromatic Doublet, SM2-Threaded Mount, f=200 mm, ARC: 400-700 nm (Thorlabs, AC508-200-A-ML)
5. Ø2" (Ø50.8 mm) Protected Silver Mirror, 0.47" (12.0 mm) Thick (Thorlabs, PF20-03-P01)
6. Kinematic Mount for Ø2" Optics (Thorlabs, KM200)
7. U-DP; Dual port intermediate tube (Olympus, U-IT140)
8. U-EPA2; Eyepoint adjuster, BX2, Raises eyepoint 30MM (Olympus, U-IT101)
9. U-MF2; Empty mirror cube, Narrow style (Olympus, U-M610)
10. 510 nm beamsplitter (BS, Part Size: 25.5mm x 36mm x 1mm) (Chroma, T510LPXRXT)
11. 50/50 beamsplitter (BS, Part Size: 25.5mm x 36mm x 1mm) (Chroma, 21000 50/50 Beamsplitter)
12. EVGA GeForce GT 520 graphic card with a mini HDMI connector (EVGA, 01G-P3-1526-KR)
13. Light-to-voltage optical sensors (Texas Advanced Optoelectronics Solutions Inc., TSL13T)

Procedure

A. Primary cultures of neurons expressing channelrhodopsin

Protocol for the culture of primary neurons was originally described by (Brewer, Torricelli et al. 1993) and was further modified by (Hilgenberg and Smith 2007) and (Barral and Reyes 2016).

Coating of the coverslips (prepare the day before culturing under sterile conditions)

1. Clean the coverslips in 3M HCl or concentrated nitric acid (70% wt/wt) for at least 24 hrs.
2. Rinse the coverslips 3X in distilled H₂O, clean them with 70% ethanol and let dry under the tissue culture hood.
3. Coat cleaned coverslips with 0.1 mL of PLL solution for 12 hrs.
4. Rinse 3X in distilled H₂O and let dry.

Dissociation and culture of primary neurons

1. Cut 5-6 600 µm coronal slices of cortex from postnatal (P0-P1) mice in cold dissection solution.
2. In the meantime, prepare the papain solution (see recipe).
3. Dissect out the cortex and remove meninges using forceps. Cut the cortical layer in small pieces of 0.5-1 mm in length. Transfer the brain pieces in clean dissection solution.

4. From this step on, the procedure has to be performed under a tissue culture hood. Filter the papain solution in a petri dish and incubate the brain pieces for 20 minutes at 37°C.
5. In the meantime, dilute the DNase/Mg solution in 10 mL and prepare 4 conical tubes as follow:
 - 1: 4 mL of DNase/Mg solution
 - 2: 2 mL of DNase/Mg solution + 200 µL of Trypsin inhibitor solution
 - 3: 2 mL of DNase/Mg solution + 60 µL of Trypsin inhibitor solution
 - 4: 2 mL of DNase/Mg solution + 30 µL of Trypsin inhibitor solution
6. Using the transfer pipet, transfer the tissue in tube 1 and let the pieces settle down. Repeat with tubes 2-4.
7. Transfer the pieces in about 0.6 mL of equilibrated culture medium in a low retention tube. Pipet 4-5 times with a P1000 low retention pipet and let the large pieces settle down. Transfer the pellet to a second low retention tube containing about 0.6 mL of culture medium. Pipet 4-5 times with a P200 low retention pipet and pool the two tubes.
8. Dilute 10 µL of cell suspension in 10 µL of 0.4% trypan blue and estimate the number of viable cells using a hemacytometer. This is useful to control the final density of neurons.
9. Plate $0.2-3 \times 10^6$ cells on each coverslip, resulting in a density of $\sim 50-1,000$ cells/mm² at the time of experiment. Let settle for 1 hour (in the incubator) for cells to adhere before flooding the petri dish with 3 mL of equilibrated culture medium. Maintain cultures at 37°C and 5% CO₂.
10. Exchange one third of the medium every 2-3 days. Cultures can be maintained in these conditions for up to 3 weeks.

Expression of channelrhodopsin

1. The virus was produced at 3×10^{12} cfu/mL by the University of North Carolina Vector Core Services using plasmid generously provided by Karl Deisseroth (Stanford University).
2. Infect each petri dish with 1 µL of virus at 3 days *in vitro*.
3. Experiments can be performed between 14-21 DIV, when neuronal characteristics and network connectivity were stable and expression of ChR2 was sufficient to enable reliable photostimulation.

B. Building and calibrating the photostimulation setup

The setup for optical stimulation using a video projector was originally described in (Stirman, Crane et al. 2012) and further modified in (Barral and Reyes 2016). The reader will find much details of the procedure to combine a video projector with the microscope in (Stirman, Crane et al. 2011, Stirman, Crane et al. 2012). The general idea is to focus the image of the projector in the same plane as the sample. Because our microscope was mounted on a movable platform, the photostimulation setup had to be physically connected to the microscope. We describe here the procedure to achieve this but alternative solutions are available and might be more appropriate depending on the setup configuration. Here, we attached a breadboard onto the fluorescent path of the microscope to build the photostimulation light path. We chose the breadboard small enough to limit weight on the microscope but it forced us to use an additional mirror. If possible, try to avoid this mirror by having a longer straight path to project the light coming from the projector.

Building the stimulation setup

1. Attach the dichroic mirror DM1 to the cube and place the eyepoint adjuster and the dual port above the fluorescent path and below the camera tube lens of the microscope (Fig. 1).
2. Fix the aluminum breadboard on the fluorescent tube and define the optical path. Make use of a mirror if necessary.
3. Place the Ø2" lens that will play the role of the tube lens (Fig. 1B, PTL) as close as possible to the dichroic mirror and evaluate the position of the primary image plane.

- Place the DLP projector and the Ø1" zoom lens (Fig. 1B, ZL) to form an image of the projector exactly at this location. It might be useful to mount the projector tube length on a movable track in order to finely adjust its position when the microscope objective is changed for example.
- It is likely that the collimated beam will be larger than the dual port entry of the microscope. Install the photodiode there to collect some of this light from the projector that will be used as a trigger to synchronize photostimulation and electrophysiological recordings. If this is not the case, place a 10/90 beamsplitter in-between the projector tube lens and the dichroic mirror to collect some light from the projector.

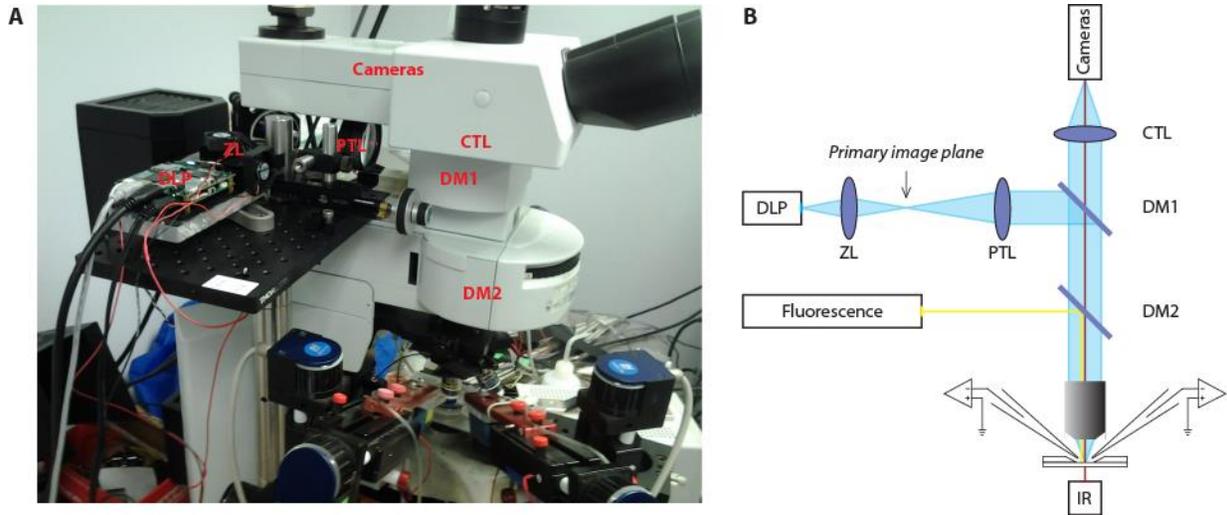


Figure 1: Photostimulation setup. **A.** Picture of the photostimulation setup. **B.** Schematic of the light path. DLP, Digital Light Processing projector; ZL, zoom lens; PTL, projector tube lens; CTL, camera tube lens; DM, dichroic mirror.

Calibrating the setup

Brightness and pixel size

- Project a full-on pattern and measure the total light flux using a light power meter.
- Measure the area of projection using a calibrated camera to deduce the resulting pixel size and the light intensity in mW/mm^2 . With the maximum current to the blue LED, we achieved $15 \text{ mW}/\text{mm}^2$.

Contrast ratio

Here we want to measure the light intensity at the point of stimulation and at other locations to estimate the contrast ratio. This measure is important because it provides an estimate of the light intensity that a non-stimulated neuron receives during stimulation of ChR2 expressing neurons.

- Place a 50/50 beamsplitter at the place of DM1 and place a 100% reflecting mirror at the sample plane.
- Use a camera to measure the average pixel brightness of a white and a black test pattern. Estimate the contrast ratio using the full-on full-off method measurements as a ratio of white to black. We measured 815:1. Use the faintest LED current for this procedure to avoid saturation of the camera.
- Project an ANSI checkerboard pattern composed of 16 rectangles, eight white and eight black. Measure the ANSI contrast ratio as the quotient of the averaged white pixels to the black pixels. We measured 21:1, compared to the 43:1 value that was provided by the manufacturer of the

projector. It gives a lower bound of the contrast ratio since a large amount of pixels (half of them) are ON in this configuration.

4. To measure the real contrast ratio during experiment, namely to estimate background illumination of the system, we measured the contrast ratio when a single region of interest was illuminated or when 20 areas were simultaneously illuminated. We found the respective values of 700:1 and 170:1. During an actual experiment with a light intensity of 10 mW/mm^2 , the background light intensity is therefore between 10 and $50 \text{ }\mu\text{W/mm}^2$, which would give rise to photocurrent of about 8-32 pA. This current is not sufficient to evoke spikes but can however elicit postsynaptic-like potentials on the order of 0.1 to 1 mV. However, the largest estimate of background stimulation is only relevant when neurons are activated simultaneously.

Relation between projector and camera pixels

1. Project a pattern of 4x4 white dots evenly spaced on a black background.
2. Measure their positions on the camera.
3. Use interpolations techniques to map every pixel of the camera to every pixel of the projector.
4. This procedure can be achieved either by placing a mirror at the sample plane. One can also simply look at the reflection of the projector on the glass coverslip of the sample, which is useful if one want to run this calibration during an actual experiment.
5. Draw an ROI, project and image it to assess the quality of the calibration.

Interfacing the projector and the computer

The projector was controlled by the computer through a USB-based connection. One display mode ('pattern sequence' mode) enabled displaying a limited number single-bit pattern at a rate up to 4 kHz. With the DLP lightcrafter, only 96 patterns can be stored. In this configuration, the predefined patterns were loaded into the remote memory of the projector and displayed using an external analog trigger. More recent projectors can have additional memory but it will never be sufficient to display patterns at high rate for more than few seconds. Additionally, the time to transfer data to the projector can be prohibitive.

To increase the number of patterns, an alternative method ('HDMI video' mode) was used to stream the images through a high speed HDMI connection. Here, images were sent continuously from the computer to the projector via the graphic card using the HDMI port. We used the 'monochrome 1-bit per pixel' video mode of the projector. This mode allows bypassing any video processing algorithms. A 24-bits RGB image is then considered as a succession of 24 single-bit planes. The resulting time resolution becomes $24 \times 60 \text{ Hz} = 1,440 \text{ Hz}$ for a single-bit image, but without any limitation on the number of displayed patterns.

Synchronizing accurately the display of each frame with the acquisition of electrophysiological signals was a complicated task because it depends on the precise time at which the computer sends an image to the projector. Since the trigger present on the video projector was already used as an input trigger for the 'pattern sequence' mode, we chose to measure directly the light intensity at the output of the projector using a photodiode. Because, the collimated beam was slightly larger than the entry port of the microscope, we could place the photodiode at the edge of the aperture to capture light that did not enter the microscope. Due to hardware specifications, there is a small $135 \text{ }\mu\text{s}$ delay for every 24-bits plane (i.e. every 16.7 ms) where the image becomes completely dark. We took advantage of this delay, which serves as an accurate trigger for synchronizing photostimulation and electrophysiological recordings offline.

1. Install the graphic card, install the Texas Instrument GUI, and connect the projector using the USB and the HDMI connector.
2. Define the projector as an external display.
3. Build a sequence of stimulation and measure the light intensity. Repeat several times.
4. Realign the temporal trace to synchronize photostimulation and electrophysiological recordings.

C. Independent stimulation of neurons using patterned illumination

1. Take a fluorescent image of the neuronal culture (Fig. 2).
2. Define regions of interest (ROIs) onto neurons that will be activated.

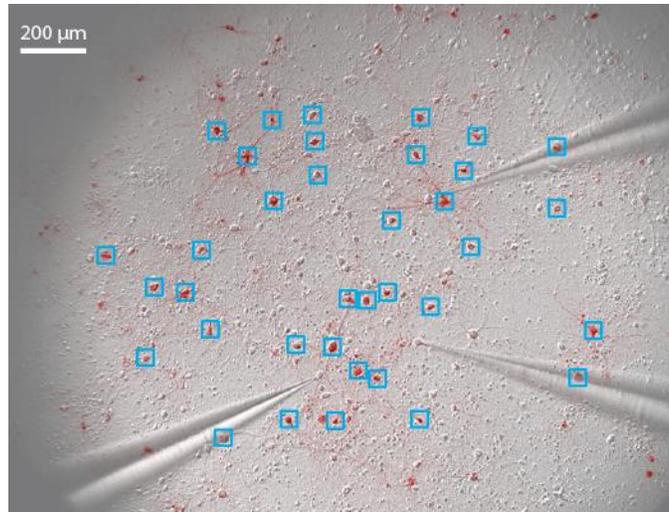


Figure 2: Pattern stimulation of the neuronal culture. 36 regions of interest (ROIs) are drawn around stimulated neurons (blue squares). 3 patch-clamp electrodes record spikes and/or subthreshold membrane potential of non-stimulated neurons.

3. Using a custom-built interface (Labview or Matlab are suitable for this task), build a temporal sequence of images in order to stimulate each ROI with the appropriate temporal pattern.
4. Compile a movie from this sequence of images and display it through the projector (see Supplementary Movie). Remember to save the movie without any compression since it would destroy the desired pattern sequence.
5. Record both the electrophysiological signal and the light intensity from the photodiode. Here, signals were filtered at 5 kHz and digitized at 25 kHz using an 18-bit interface card.
6. Realign and analyze data offline.

Data analysis

A. Control experiment

To assess the effectiveness of the photostimulation setup in evoking spikes, we stimulated ChR2-positive neurons. We verified that spikes were evoked reliably when a single neuron was stimulated (Fig. 3A). Then we stimulated 11 neurons simultaneously and recorded spikes from 2 of them (Fig. 3B). Whereas one neuron responded only when stimulated by the light pulses, the other neuron also displayed action potentials in response to the stimulation of neighboring neurons, meaning that it was an integrant component of the recurrent network.

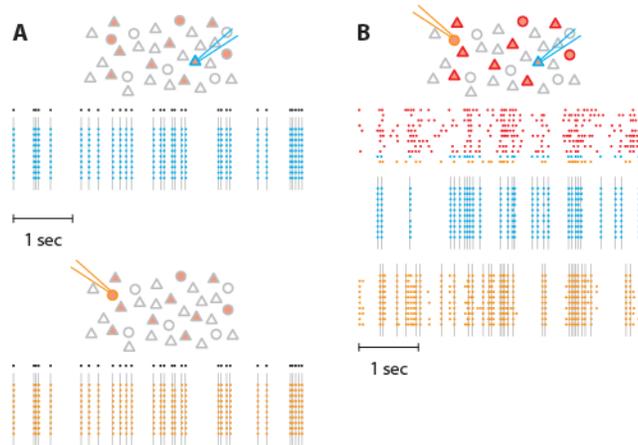


Figure 3: In the following schematics, neurons expressing ChR2 are shown in light red. Recorded neurons are designated by the recording pipets. Light-stimulated neurons have colored contours. **A.** A train of action potentials (black dots) was applied to two different neurons expressing ChR2 (blue and orange) independently (top and bottom, respectively). Several trials were realized to confirm the faithful stimulation of neurons of interest. Colored dots denote recorded action potential and grey lines the light flashes. No action potentials were observed in the non-stimulated neuron. **B.** Then, we stimulated 11 neurons in the network. We generated 11 spike trains (top) that were applied to neurons expressing ChR2, including the two previous neurons. Here, we used correlated spike trains for the stimulus (the average correlation between spike trains was $C = 0.5$). The blue and the orange neurons were recorded simultaneously.

B. Network stimulation

The final experiment consists in stimulating the selected neurons by a controlled stimulus (Fig. 4A). Here, the firing rate of the input was fixed at 5 Hz and the spike trains were not correlated. These quantities can be varied at will by the experimenter. Neuronal activity in non-stimulated neurons was monitored either in cell-attached (Fig. 4B) or in whole-cell (Fig. 4C) configuration to isolate the postsynaptic inputs and the spiking activity, respectively. Cell-attached recordings (Fig. 4B) showed that some cells fired robustly (neurons 1, 2) while little activity was evoked in others (neurons 3, 4). Subsequent whole-cell recordings in the same neurons also showed that large voltage transients evoked reliable action potentials across trials. The amplitudes were much larger than the unitary EPSPs and were mostly likely due to synchronous recurrent activity (Barral and Reyes 2016).

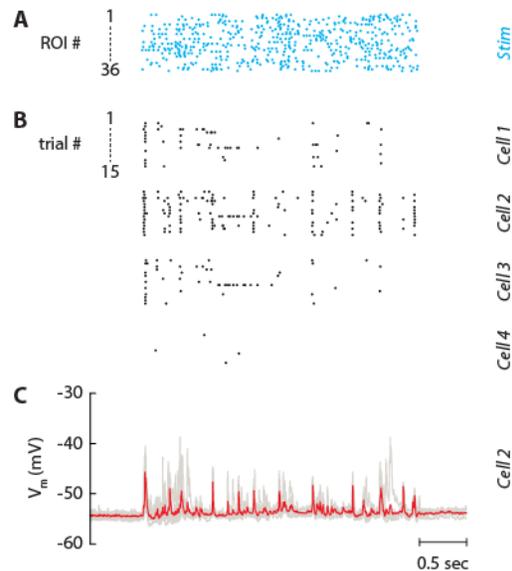


Figure 4: Independent stimulation of the neuronal network. **A.** Spike trains which are used for stimulating the 36 selected neurons in Fig. 2. Each line is a train of light pulses applied to a given ROI. **B.** Raster plot of four neurons recorded simultaneously. Each line represents a given repetition of the same stimulus. **C.** Corresponding membrane potential of Cell 2. Each repetition is drawn as a grey line and the average is displayed in red. The patch pipet contained the Na^+ channel blocker QX-314 to block spikes and isolate postsynaptic potentials.

Recipes

Poly-L-Lysine (1X, 0.1 mg/mL PLL, 20 aliquots of 0.5 mL)

- Make 100 mL of boric acid / sodium tetraborate solution (0.1 M, pH 8.4, 0.95 g sodium tetraborate decahydrate in 100 mL; add boric acid to borax solution until desired pH is reached (0.1 M: about 0.61 g of boric acid).
- Dissolve 1 mg of PLL in 10 mL boric acid / sodium tetraborate solution.
- Filter through 0.2 μm , make 0.5 mL aliquots and store at -20°C for up to 6 months.

Dissection solution (1X, CMF-HBSS/HEPES with antibiotics, 1L)

Ca^{2+} and Mg^{2+} free Hank's balanced salt solution containing 1 mM pyruvate, 15 mM HEPES, 10 mM NaHCO_3 and antibiotics

- Mix
 - 100 ml of 10X CMF-HBSS
 - 3.9 g HEPES (15 mM final)
 - 0.84 g NaHCO_3 (10 mM final)
 - 10 ml 100x sodium pyruvate (1 mM final)
 - 10 ml 100x penicillin/streptomycin (100 U/ml penicillin/100 μg /ml streptomycin final)
 - 800 mL of distilled H_2O
- Adjust pH to 7.2 with 1 N NaOH and add H_2O to 1 L.
- Filter sterilize through 0.2 μm sterile filter, store up to 1 month at 4°C if the pH remains stable.

Papain solution (1X, 15 U/mL papain, 100 U/mL DNase, 2 mL)

- a. Add 30 units papain (i.e. 100 μ L) to 1.8 ml of dissection solution and warm up to 30-32°C for 20 minutes to clear the solution before use.
- b. Add 0.2 mL of DNase/L-cystein solution (containing 200 U of DNase and 0.4 mg of L-cysteine).
- c. Adjust pH to 7.4 with about 5 μ l of 0.1 N NaOH.
- d. Filter through 0.2 μ m and use it right away.

DNase/L-cysteine solution for papain solution (20X, 100 U/mL DNase final, 1.8 mM L-cystein final, 10 aliquots of 0.2 mL)

- a. Dissolve 2,000 U (1 mg) of DNase and 6.4 mg of L-cysteine in 2 mL of dissection solution.
- b. Make 0.2 mL aliquots and store at -20°C for up to 6 months.

DNase/Mg solution for dissociation (50X, 20 U/mL DNase final, 10 aliquots of 0.2 mL)

- a. Dissolve 2,000 U (1 mg) of DNase, 81.3 mg of MgCl₂ (2 mM final) and 14.7 mg CaCl₂ (1 mM final) in 2 ml of dissection solution.
- b. Filter through 0.2 μ m, make 0.2 mL aliquots and store at -20°C for up to 6 months.

Trypsin inhibitor solution (100 mg/mL BSA, 40 mg/mL trypsin inhibitor)

- a. In 3 ml of dissection solution (or CMF-HBSS), dissolve 0.3 g BSA (Bovine Albumin. Sigma A9418) and 0.12 g of Trypsin Inhibitor (Sigma T9253).
- b. Adjust pH to 7.4 with 1 N NaOH.
- c. Filter through 0.2 μ m, make 0.3 mL aliquots and store at -20°C for up to 6 months.

Culture medium (NB/B27 Medium)

- a. Mix
 - 485 ml of NBM
 - 10 mL of B27
 - 1.25 mL Glutamax
 - 5 ml 100X penicillin/streptomycin (100 U/ml penicillin/100 μ g/ml streptomycin final)
- b. Filter through 0.2 μ m filter, aliquot in 50 mL and store at 4°C.

Artificial cerebrospinal fluid (1X)

In mM: 125 NaCl, 10 NaHCO₃, 25 D-glucose, 2.5 KCl, 2 CaCl₂, 1.25 NaH₂PO₄, 1 MgCl₂, and 10 HEPES.

Intracellular solution (1X)

In mM: 130 K-gluconate, 10 HEPES, 10 phosphocreatine, 5 KCl, 1 MgCl₂, 4 ATP-Mg and 0.3mM GTP.

Notes

The protocol to culture primary cortical neurons can work equally on hippocampal neurons.

Add 5 μ M Ara-C (Sigma, C6645) or 5 μ M 5-Fluoro-2'-deoxyuridine (Sigma, F0503) on day 3-5 if glial cells show excessive proliferation. However, it is usually not necessary since Neurobasal culture medium does not promote glia cell proliferation and it might increase cell death.

We described here a setup using a 460 nm light source to stimulate ChR2-expressing neurons. The setup can easily be modified to use another LED of the projector. Alternatively, the light engine of the projector can be dismantled and an external light source can be used to enlighten the digital micromirror device.

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