Video Article

External Excitation of Neurons Using Electric and Magnetic Fields in One- and Two-dimensional Cultures

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Abstract

A neuron will fire an action potential when its membrane potential exceeds a certain threshold. In typical activity of the brain, this occurs as a result of chemical inputs to its synapses. However, neurons can also be excited by an imposed electric field. In particular, recent clinical applications activate neurons by creating an electric field externally. It is therefore of interest to investigate how the neuron responds to the external field and what causes the action potential. Fortunately, precise and controlled application of an external electric field is possible for embryonic neuronal cells that are excised, dissociated and grown in cultures. This allows the investigation of these questions in a highly reproducible system.

In this paper some of the techniques used for controlled application of external electric field on neuronal cultures are reviewed. The networks can be either one dimensional, *i.e.* patterned in linear forms or allowed to grow on the whole plane of the substrate, and thus two dimensional. Furthermore, the excitation can be created by the direct application of electric field via electrodes immersed in the fluid (bath electrodes) or by inducing the electric field using the remote creation of magnetic pulses.

Video Link

The video component of this article can be found at <https://www.jove.com/video/54357/>

Introduction

The interaction between neurons and external electric fields has fundamental implications as well as practical ones. While it is known since the times of Volta that an externally applied electric field can excite tissue, the mechanisms responsible for the production of a resultant action potential in neurons are only recently starting to be unraveled^{1,2,3,4}. This includes finding answers to questions regarding the mechanism that causes depolarization of membrane potential, the role of membrane properties and of ion channels, and even the region in the neuron that responds to the electric field^{2,5}. Therapeutic neurostimulation^{6,7,8,9,10} methodologies are particularly dependent on this information, which can be crucial for targeting the afflicted areas and for understanding the outcome of the therapy. Such understanding can also help in developing treatment protocols and new approaches for stimulation of different areas in the brain.

Measuring the interaction within the *in vivo* brain adds an important component to this understanding, but is hampered by the imprecision and low controllability of measurements within the skull. In contrast, measurements in cultures can easily be performed in high volume with high precision, excellent signal to noise performance and a high degree of reproducibility and of control. Using cultures a large variety of neuronal properties of collective network behavior can be elucidated^{11,12,13,14,15,16}. Similarly, this well controlled system is expected to be highly efficient in elucidating the mechanism by which other stimulation methods work, for example how channel opening during optical stimulation in optogenetically active neurons^{17,18,19} is responsible for creating action potential.

Here the focus is on describing the development and understanding of tools that can efficiently excite the neuron via an external electric field. In this paper we describe the preparation of two-dimensional and one-dimensional patterned hippocampal cultures, stimulation using different configurations and orientation of a directly applied electric field by bath electrodes, and finally stimulation of two-dimensional and patterned one-dimensional cultures by a time-varying magnetic field, which induces an electric field^{5,20,21}.

Protocol

Ethics Statement: Procedures involving animal handling were done in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) of the Weizmann Institute of Science, and the appropriate Israeli law. The Weizmann Institute is accredited by the

Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC). The Weizmann Institutional Animal Care and Use Committee approved this study, conducted with hippocampal neurons.

1. Preparation of Two-dimensional (2D) and One-dimensional (1D) Hippocampal Cultures

1. Preparation of coverslips for 2D cultures.
 1. Prepare plating medium (PM) composed of: 0.9 mL minimum essential media (MEM)+3G, 0.05 mL fetal calf serum (FCS), 0.05 mL heat inactivated horse serum (HI HS) and 1 μ L of B27 supplement. Note: MEM+3G contains for every 500 mL of MEM x 1, 1 mL of gentamycin, 5 mL of stabilized L-glutamine 100x (see **Table of Materials/Reagents**) and 5 mL of 60% D-(+)-glucose.
 2. Prepare borate buffer composed of: 1.9 g borax (sodium tetraborate decahydrate) in 200 mL double distilled water (DDW) (mix at 60 °C) and 1.24 g boric acid in 200 mL DDW. Titrate final solution to pH 8.5 using 1 M HCl. Note: The final solution is 400 mL 0.1 M borate buffer.
 3. Immerse glass coverslips in 65% nitric acid for 2 h. Rinse three times in DDW followed by three rinses with absolute analytical reagent (ABS AR) ethanol.
 4. Pass each coverslip briefly through the flame of a Bunsen burner two or three times for 1 - 2 s, and then leave to incubate overnight in 24 well plates with 1 mL poly-L-lysine 0.01% solution diluted 1:5 in borate buffer (0.1 M, pH 8.4). Then rinse coverslips in DDW three times and leave with 1 mL PM per well in a standard 37 °C, 5% CO₂ incubator overnight.
2. Preparation of coverslips for 1D cultures.
 1. Clean glass coverslips by immersion in a base piranha solution consisting of 75 mL DDW, 25 mL 25% ammonia solution and 25 mL 30% hydrogen peroxide. Place solution with the glass coverslips on a heating plate at ~ 50 °C for 30 min and then dry the glass coverslips with nitrogen.
 2. Coat coverslips first with a thin chrome film (99.999%) of 6 Å thickness followed by a 30 Å layer of gold (99.999%), using either vapor or sputter deposition.
 1. To achieve a sputtering rate of 0.05 – 1 Å/s use a sputtering machine with 2 e-beams and a vacuum system of 260 l/s with target sizes 2" and 4". Use a rotation stage that can go from 0 – 100 rounds per minute (RPM). Use a direct current (DC) sputter power of 0 – 750 Watts.
 2. Use a rotation of 30 rpm and argon 99.999% to get plasma at 10 mTorr pressure in the chamber.
 3. Operate the power supply of the sputtering guns at 40 W DC sputter power for the chrome, leading to ~ 0.12 Å/s coverage rate, and at 10 W DC sputter power for the gold, leading to ~ 0.28 Å/s.
 3. Dissolve 0.1 g 1-octadecanethiol in 100 mL ABS AR ethanol using ultrasound for 30 min. Place Cr-Au coated coverslips for 2 h in this solution, then wash with ethanol ABS AR and dry with nitrogen.
 4. Prepare a solution of 100 mL Dulbecco's phosphate buffered saline (D-PBS) and 3.5 g of a tri-block co-polymer (see **Table of Materials/Reagents**) by stirring for 1 - 2 h at 600 - 700 rpm. Place coverslips in the solution for 1 h. Dry coverslips with nitrogen.
 5. Mechanically etch the desired pattern by scratching the bio-rejection layer²². Do this using a pen plotter, where the pen is replaced by an etching needle. Scratch the pattern through the metal layers to reach the underlying glass. Control this process by a computer to achieve a replicated desired pattern. Patterns formed by this process are demonstrated in **Figure 2** and **Figure 3**.
 6. Prepare a bio-compatible layer of 100 mL D-PBS, 3.5 g tri-block co-polymer, 35.7 μ L/mL fibronectin and 29 μ L/mL laminin.
 1. Sterilize coverslips in ultra-violet light for at least 10 min. Incubate coverslips in the prepared bio-compatible solution overnight. NOTE: The bio-compatible layer will form only where the bio-rejection layer has been etched off in the previous step.
 2. On the next day wash coverslips two times with P-DBS. Incubate coverslips in PM overnight. Coverslips are now ready for cell plating.
3. Perform dissection according to standard procedures that have been published extensively previously^{23,24}.
 1. In brief, extract hippocampus or cortex from rat embryos, typically at day E19, or from mice, typically at day E17^{23,24}.
 2. Dissociate cells first in papain solution for 20 - 30 min, followed by mechanical trituration²⁴ with glass pipettes whose tips are fire polished. NOTE: If the cells come from genetically modified mice then the tissue from each embryo should be maintained in a separate 1.5 mL plastic tube during the entire process.
 3. Count cells with trypan blue before seeding. NOTE: For genetically modified animals the counting should be done separately for each embryo.
 4. For 2D cultures, seed mouse neurons at 750,000 and rat neurons at 850,000 cells per well. For 1D, seed at 650,000 cells per well. Shake plate slightly immediately after seeding to ensure homogeneous coverage of the coverslip.
4. Maintenance of the neuronal cultures.
 1. Prepare changing medium (CM) composed of (per mL): 0.9 mL MEM+3G, 0.1 mL HI HS, 10 μ L 5-fluoro-2'-deoxyuridine (FUDR) with uridine 100x.
 2. Prepare final medium (FM) composed of (per mL): 0.9 mL MEM+3G and 0.1 mL HI HS.
 3. Replace PM with 1-1.5 mL CM after 4 days *in vitro* (DIV). At 6 DIV, replace 50% of the CM with fresh CM. At DIV 8, change the medium to 1.5 mL FM, followed by a 50% change of FM every 2 days. After about one week spontaneous synchronous activity emerges.
5. Imaging of spontaneous or evoked activity in neuronal cultures with fluorescent dyes.
 1. Prepare a solution of 50 μ g calcium sensitive fluorescent dye (see **Table of Materials/Reagents**) in 50 μ L DMSO (dimethyl sulfoxide).
 2. Prepare extracellular recording solution (EM) containing (in mM) 10 HEPES, 4 KCl, 2 CaCl₂, 1 MgCl₂, 139 NaCl, 10 D-glucose, 45 sucrose (pH 7.4).
 3. Incubate neuronal culture in 2 mL EM with 8 μ L of the calcium sensitive fluorescent dye solution for 1 h. Protect from light and gently rotate to ensure homogenous spread of the dye to the cells.
 4. Replace solution with fresh EM prior to imaging. The fluorescent imaging is demonstrated in **Figure 4**.

5. Image in a fluorescence microscope with optical filters for calcium fluorescence imaging (excitation peak at 488 nm, emission peak at 520 nm), using a camera and software capable of quantifying the intensity of any region of interest (ROI) within the field of view of the microscope.

2. Electric Stimulation of Cultures

NOTE: The basic setup for electric stimulation is shown in **Figure 1**. A cover slip on which the neuronal culture has been grown for about 14 days is placed in a Petri dish under a fluorescence microscope. Electrical activity of the neurons is imaged using calcium sensitive dyes while a voltage is applied via two pairs of bath electrodes that are positioned outside the culture. The electrodes are driven by a signal generator whose output is amplified by a dual channel amplifier. Voltage control for stimulation is preferred over the more standard current control^{25,26} because the electric field vectors are determined directly, thus enabling straightforward vector addition and combination. This does require a careful check of the uniformity of the electric field, which can be performed over the whole sample for the case of voltage control. When using voltage control care should be taken to avoid any ground loops and the homogeneity of the electric field should be verified (see 2.2 below).

1. For electric stimulation with a homogeneous electric field use a pair of parallel electrode wires.
 1. Use electrodes made of platinum with a thickness on the order of 0.005" (127 μm). When used with the 13 mm coverslips, ensure that the distance between the two electrodes is around 11 mm, and position the electrodes 1 mm above the culture.
NOTE: To make the electrode holder (**Figures 5A and 6A**) use polytetrafluoroethylene (PTFE). Drill narrow holes through the PTFE to insert the electrodes. The device should be higher than the extracellular solution so that the top end, where the electrodes are exposed, will never come in contact with the solution. For insulation, use epoxy glue on any part of the electrode leads that might be exposed.
 2. Use a square pulse shape with a 50% duty cycle, with no DC component to avoid electrolysis. Vary pulse duration between 10 μs and 4 ms to cause effective stimulation without burning the culture. Ensure that the amplitude is in the order of ± 22 V (see **Figure 5**). The square pulse can be observed on an oscilloscope connected in parallel to the electrodes.
NOTE: For easy programming of any desired waveform, use a commercial waveform editing software (see **Materials list**). Enter graphically the desired waveform and send it to the waveform generator.
2. To test for field homogeneity use a probe electrode. Use a grid of at least 1 mm x 1 mm to allow the probe to be moved in the area between electrodes and measure electric potential.
 1. Measure electric potential. Use $E_x = -\frac{\partial V_x}{\partial x}$ to calculate the electric field. Use one of the electrodes as a reference electrode. Measure the electric field with varying pulse durations between 100 μs to 4 ms (see **Figure 5B** for an example of 100 μs pulse duration) to verify that the field is homogeneous within the range of stimulating durations.
NOTE: See **Figure 5D** for an example of a measured electric field homogeneity when the pulse duration was 1 ms.
3. Use 2 perpendicular pairs of bath electrodes to produce more complicated electric field shapes, and to be able to orient the field in different directions (see **Fig. 6B**). The device with 2 pairs of electrodes will be used, and the signal on each pair of the electrodes will be observed on two separate oscilloscopes.
 1. Position the electrodes 1 mm above the culture and at a distance of 10 - 11 mm from each other. Make sure that both electrode pairs are floating (have no ground connection), and do not have any common ground by measuring the resistance between any set of electrodes and verifying absence of short circuits between any of the electrodes. Verify that all equipment used, which is connected to the electrodes (such as the oscilloscopes, the amplifiers, the signal generators, etc.) is floating with respect to ground by checking that all equipment is floating and that none of the reference electrodes is touching any grounded equipment.
 2. To change field orientation, vary the amplitude of the voltage fed to the two electrode pairs with respect to each other (see **Figure 7A**). For example, for 0° use ± 22 V on the electrode pair which are perpendicular to the pattern and 0 V for the other electrode. For 45° , use ± 15.6 V on both pairs of electrodes with no phase lag, for an amplitude vector sum of $15.6^2 + 15.6^2 = 22^2$.
 3. To apply a rotating field use one single waveform of a sine voltage pulse and one single waveform of a cosine voltage pulse to the two pairs of electrodes to produce a fixed amplitude rotating electric field (see **Figure 6B**).
NOTE: As can be viewed in **Figure 6B**, when using one cycle of a sine wave with ± 22 V in one electrode and one cycle of a cosine wave with ± 22 V in the other electrode, the vector sum is a rotating electric field with the cycle duration same as the sine and cosine waves, and with an amplitude of ± 22 V.
4. To measure and calculate Chronaxie and Rheobase of axons in the neuronal culture vs. dendrites in the same culture perform the following steps.
 1. Use a calcium sensitive fluorescent dye for calcium imaging as described in the **Table of Materials/Reagents** and in 1.5) with a 1D culture patterned on thin (170 μm), long (10 mm) lines. Calcium sensitive dye will be applied to the culture, and incubated for 45 minutes. The dye will be washed by replacing with a fresh recording solution.
 2. Disconnect the network to achieve population statistics of the direct response to electrical stimulation, without the effect of synaptic transmission. To do so, apply a combination of 40 μM of bicuculline, which blocks the inhibitory action of gamma-Aminobutyric acid-A (GABA_A) receptors, 10 μM of 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), to block the α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic (AMPA) and kainate receptors and 20 μM of (2R)-amino-5-phosphonovaleric acid (APV), which blocks the N-methyl-D-aspartate (NMDA) receptors. The blockers will be added to the recording solution.
 3. Apply a square pulse as described in 2.1 and 2.3 with varying time durations between 100 μs and 4 ms. The signal can be observed on the oscilloscope.
 4. Use a fluorescence microscope with an image acquisition program (see 1.5) to monitor the intensity of the calcium transients at several ROIs, each containing a few hundred neurons. Acquire images using a sensitive EMCCD camera (see Materials list), capable of an image acquisition rate of at least 20 frames per second. The change in light intensity is proportional to the amount of neurons which were stimulated. Estimate the fraction of stimulated neurons using the relative change in intensity.

5. For each pulse duration (100 μ s to 4 ms), change the amplitude of the voltage applied to the electrodes starting at a voltage where no change in intensity is seen (a few Volts), to the voltage where the changes in intensity due to the applied voltage has saturated (Up to $\pm$ 22 V).
NOTE: The intensity change will be distributed as a cumulative Gaussian⁵ with respect to the applied voltage for stimulation for each time duration of the voltage pulse.
6. Fit a cumulative Gaussian distribution for the intensity vs. the applied voltage for each duration and extract the Gaussian mean from this fit.
NOTE: This mean is the representative voltage to which the neurons responded.
7. At the end of this process obtain for each stimulation duration a mean voltage to which the neurons responded. Use these pairs of durations and strengths to plot the Strength-Duration curves (see **Figure 7**).

3. Magnetic Stimulation of Cultures

Note: The basic setup for magnetic stimulation is shown in **Figure 2**. On top right is shown an inverted fluorescence microscope that is used to image calcium sensitive dyes in the neurons. The magnetic coil (blue circles) is positioned about 5 mm concentrically above a neuronal ring culture, (blue outline). A pickup coil (red circle) on the circumference of the Petri dish monitors the voltage induced by the magnetic pulse. On the top left is shown the measured dynamics of the magnetic stimulator (MS) coil with a capacitor voltage load of 5,000 kV, as integrated from the pickup coil. The induced electric field (calculated for a ring radius of 14 mm) is depicted in green while the magnetic field is depicted in blue. On the bottom are shown images of the neuronal culture. At bottom left is a bright field image of a patterned 24-mm coverslip. The white areas are the neurons. The photographed pattern consists of concentric ring cultures with different radii. At the bottom right is a zoom onto a short segment of the rings, showing individual neurons. For a scale, the rings' width is about 200 μ m.

1. Grow the neurons in a circular ring pattern (etched as described in 1.2.5) for 1D culture stimulation. Use a calcium sensitive fluorescent dye for calcium imaging (as described in section 1.5) with a 1D culture patterned on thin (170 μ m), long (10 mm) lines.
 1. Use a circular magnetic coil and position a Petri dish approximately 5 mm below and concentric with the coil. Use a custom coil of approximately 30-mm (inner diameter, 46-mm outer diameter) coil with an inductance of $L = 90$ mH driven by a homemade or commercial MS loaded with a maximal voltage of 5 kV.
 2. Discharge a high voltage and current through a conducting coil using a high-current-high-voltage switch to magnetically stimulate neuronal cultures. The magnetic stimulator (MS) can be built as described in²¹ using large capacitors, on the order of 100 mF, to obtain a high voltage of 1 - 5 kV. Alternatively use a commercially available MS (see **Table of Materials/Reagents**).
NOTE: Use a 0.254 mm thick and 6.35 mm wide polyester-coated rectangular copper wire to fabricate a homemade coil²¹. Turn wires on custom made frames, insulated with glass fibers and cast in epoxy (see **Table of Materials/Reagents**). Alternatively use commercially available coils (see **Table of Materials/Reagents**).
2. Use rotating magnetic fields to stimulate 2D cultures.
3. Now, use the rotating magnetic fields to stimulate 2D cultures. Fire the TMS with no culture in the dish, at different intensities, to show the linear correlation of the coil reading with the intensities.
4. Next, while recording calcium transients with a neuronal culture, start firing the TMS at increasing intensities while recording both calcium transients and the coil. At first, network bursts observed as large calcium transients, should not synchronize with pick up coil TMS spikes. Continuing to increase intensity, at some point, the calcium transients start to become synchronized with the TMS spikes. Alternatively, or in addition, after achieving a synchronized response, start decreasing the intensities until synchronization is abolished, to determine the TMS threshold.
NOTE: Conditions should be maintained strictly fixed for each of the setups, using the exact volume every time, the same vessels and exact coil positions and orientations.
 1. Mount the pickup coil on the base of the recording dish so that it is in a plane parallel to the neuronal culture and in a fixed position with respect to the culture.
NOTE: This ensures that the dependence of the positioning of the pickup coil with respect to the magnet is faithful to the position of the culture and that any discrepancies in the positioning will be negligible on the pickup coil readings.
 1. Use two independent coils that are positioned perpendicular to each other (**Figure 8B**) to generate a rotating magnetic and induced electric field. Connect each coil to its own MS. Ensure that the stimulators discharge similar currents at a phase lag of 90 degrees (**Figure 10A**), resulting in a rotating magnetic field which scans 270 degrees of the real space at a maximum field of $\sim$ 270 V/m (**Figure 10B**).
 2. Position the neuronal culture inside a spherical glass container filled with the external recording solution (EM).
 3. Monitor stimulation (change in fluorescence intensity of the neurons) with the camera as described in step 2.4.4.
 4. In the case of the cross coils (**Figure 8B**), position the pickup coil parallel and at a specific distance below the neuronal culture. Carefully maintain this configuration throughout the experiments.
5. Calculate analytically the electric field for 1D configuration by $E_{max} = k_1 B r$, where E_{max} is the maximum amplitude of the induced electric field and is directed along the tangent of the rings with radius r . B is the amplitude of the magnetic pulse and k_1 is a dimensional proportionality constant that can be measured using the pickup coil (**Figure 8A**).
6. Use a numerical simulation package (see Material list) to numerically simulate the electric field²¹.

Representative Results

The protocol presented allows for easy patterning of neuronal cultures. When it is combined with several methods we developed for stimulation, it enables to make measurements of some intrinsic neuron properties such as Chronaxie and Rheobase⁵, to compare properties of healthy and diseased neurons²⁷, to find optimal ways to stimulate cultures as a function of their structure and many more novel approaches. Some examples are presented in the next figures.

Figure 3 shows the 1D patterned configurations that are etched into the bio-rejection layer. Thin lines of about 170 μm width are typically inscribed that can, for example, be concentric rings with varying radii (**Figure 3A**) or parallel lines, typically $\sim 11\text{ mm}$ long (**Figure 3B**).

The top panel of **Figure 4** shows a typical picture of fluorescent activity in the culture, taken with a charge-coupled device (CCD) and showing an image of fluorescently labeled neurons in a 2D culture. In this example three different ROIs are shown, marked in black, green and red. Integrated fluorescence intensity traces are shown in the bottom panel of **Figure 4**, measured in these three different ROIs (traces are in the corresponding color of the ROIs). As the inset shows, within the 200 ms resolution of the frame acquisition time at which this particular data was taken, all the neurons in the three different ROIs burst simultaneously.

The basic apparatus used for stimulation by a uni-directional, constant electric field is shown in **Figure 5A**. The electrode wires are immersed in the recording medium, about 1 mm above the neuronal culture. The basic pulse shape used for electric stimulation is shown in **Figure 5B**. When this voltage pulse is applied it creates a constant electric field that flips its orientation by 180° after half of the cycle. The change in field direction after half a cycle is used to avoid electrolysis and damage to the electrodes. Typical voltages applied to the electrodes are $\pm 22\text{ V}$, and the typical pulse duration for stimulation of 2D cultures is in the order of 100 - 500 μs .

To control for any anisotropy in the growth of neurites, we verified that stimulation of 2D cultures is isotropic. By manually rotating the electrodes in all 360° at 15° resolution (**Figure 5C**) it is seen that there is no preference of orientation for the stimulation. Each color in **Figure 5C** represents the stimulation of a different culture. The distance from the origin represents the minimal duration needed for stimulation with a constant amplitude. It is evident that, to a good approximation, 2D cultures do not have a preferred orientation for electric stimulation.

Since the single pair of electrodes is limited in having one defined direction for the field (though it can be flipped), we developed an apparatus for electric stimulation with two pairs of electrodes (**Figure 6A**). A schematic of the culture (grey circular background, darker spots are the cell bodies) and of the electrodes (parallel thick lines) is shown in **Figure 6B**. When the waveforms, shown as blue and green inserts in **Figure 6B**, are cosine and sine waves and are applied together then a rotating E vector field is produced. When the voltage pulses are square, have different amplitudes and have zero phase lag between them then they create a constant field with the desired orientation determined by the relative amplitude between the two pairs of electrodes. This electric rotation is an obvious improvement over the manual, mechanical rotation that was used to obtain the angular distribution of stimulation strengths in **Figure 5C**. Of course, as **Figure 6C** shows, it is also possible to use this configuration to excite only one fixed direction, possibly as a control, by activating only one pair of electrodes.

These configurations were used to excite 2D neuronal cultures with either a rotating field or a fixed-angle field, with the same root mean square (RMS) voltage and then to compare the pulse duration needed to excite the culture when the amplitude is fixed. We found that when using a rotating electric field with a constant amplitude of $\pm 22\text{ V}$, an average duration of $150 \pm 14\text{ }\mu\text{s}$ was needed to achieve stimulation, while with a single direction electric field an average duration of $290 \pm 30\text{ }\mu\text{s}$ was needed. The ratio between the durations needed for exciting the culture with a rotating versus a fixed-angle field is therefore 0.53 ± 0.02 .

Since in a 2D culture the axons extend in all directions, the rotating field is able to excite many of the axons. In contrast, with the single orientation field there are only few axons oriented in that specific angle and axonal excitation is not achieved. When the duration is increased, there is the possibility of exciting other parts of the neuron as well, in particular the dendrites. This is further explained below in describing the Chronaxie measurements. The fact that much shorter durations are needed for excitation of the same culture when using a rotating field is of high importance when using external fields for brain stimulation, since there are often technical limitations on the pulse durations.

An important point is that in a connected network (no synaptic blockers applied) excitation is initiated by a small percent of the neurons, which then excite the rest of the network via synaptic connections. This means that a small number of neurons dominate the measurement. To obtain reproducible and quantitative measurements it was necessary to monitor the full population statistics in the network, which was disconnected by using synaptic blockers. The schematics for the configuration used to monitor the population response during axonal excitations vs. dendritic ones are shown in **Figure 7**. In **Figure 7A** a 1D disconnected network is stimulated with the electric field either along the line of the culture (at 0° for axonal stimulation) or perpendicular to the line of the culture (at 90° for dendritic stimulation) by two pairs of electrodes. As the voltage is increased, more neurons will be stimulated, and this is reflected by the fluorescence intensity, which is proportional to the number of neurons stimulated (see **Figure 7B**). Increasing voltage amplitudes are used to gradually stimulate the entire culture. Each neuron has a minimal threshold voltage (for a specific pulse duration) that it will respond to, and by the law of large numbers, the distribution of these thresholds is expected to be Gaussian. Therefore, if we look at the number of neurons that responded and plot it against the voltage applied, the distribution will be a cumulative Gaussian distribution, which is an Error function (erf)⁵. The number of neurons that respond to a specific amplitude of electric field has approximately a Gaussian distribution, and therefore the fluorescence is well described by its integral — an Error function of the amplitude of the stimulating field (**Figure 7C**).

Figure 7C is used to extract one parameter, the expectation (mean) of the distribution. This is done by fitting a cumulative Gaussian distribution and obtaining the best fit. This expectation is the applied voltage to which 50% of the cells will respond. This process is repeated for several pulse durations (ranging from 100 μ s to 4 ms). The mean voltage to which the culture responded is plotted vs. the pulse duration to obtain the Strength-Duration curve. Two sets of measurements were performed. In the first the field was parallel to the pattern, and in the second it was perpendicular to it. It has been previously shown^{15,23} that axons align with pattern, while dendrites grow in all directions. This gives two different Strength-Duration curves, which are very useful for obtaining a clear difference between the axonal and dendritic excitation. Full separation to dendritic and axonal contributions is achieved by the known fact that the axonal time constant for excitation is much shorter than dendritic ones^{2,3,4}.

The Strength-Duration curves are then fit to the Chronaxie decay equation $V_0 = \frac{V_{th}}{1 - 2^{-t/C}}$, where V_{th} is the Rheobase voltage and C is the Chronaxie. At pulse durations of $t < 1$ ms it is the axons that are excited first and cause the neuron to fire, having a lower value for excitation voltage in the Strength-Duration curve. Axonal Chronaxie was calculated to be 110 μ s. In striking contrast, for excitation at long durations ($t > 1$ ms) the dendritic compartment is the source of excitation for the neuron with a dendritic Chronaxie calculated at 900 μ s.

The principles underlying stimulation of 2D and 1D cultures with a circular coil are described in **Figure 8**. To obtain a better understanding of the physical situation, numerical simulations of the magnetic and induced electric fields are produced using the COMSOL package. To stimulate 1D cultures a circular magnetic coil is used, positioned concentrically above the Petri dish. Neurons are grown in circular rings on a round coverslip that is placed inside the Petri dish for recording. In this case the induced electric field can be calculated analytically and is equal to $E_{max} = k_1 B r$, where E_{max} is the maximum amplitude of the induced electric field and is directed along the tangent of the rings with radius r . B is the amplitude of the magnetic pulse and k_1 is a dimensional proportionality constant that can be measured using a pickup coil.

The COMSOL numerical calculation of the magnetic field created by the coil in the 1D culture is shown in the top panel of **Figure 8A** (red streamlines). In the bottom panel of **Figure 8A** the calculation is presented for the induced electric field. Stimulation of a 2D culture with a crossed coil configuration is shown in **Figure 8B**. To stimulate 2D cultures a cross coil was used that produces rotating magnetic fields, positioned with a 2D neuronal culture inside it, placed in a spherical glass container that is filled with recording medium (EM). In this case the induced electric field can no longer be calculated analytically. The cross coil is depicted in the top panel of **Figure 8B**, with the spherical container placed inside the two coils and the glass coverslip supporting the 2D culture placed in the bottom of the container. For scale, the inner diameter of the inner coil is 65 mm. Numerical simulations using COMSOL with the Eddy Currents 3D model are shown in the bottom panel, portraying the electric field induced in the inner surface of the spherical container. A bright field microscope image of a glass coverslip with typical 1D neuronal cultures used for stimulation is shown in **Figure 8C**. The white lines show neurons on the patterned lines, which are oriented both tangentially and radially for experimental comparison and control on the effect of directionality.

Geometry of the 1D culture plays a big role in determining whether a culture will fire in response to magnetic stimulation. Only 22% of the 1D ring cultures responded to magnetic stimulation. The successful excitation rate strongly depended on the culture's linear length, and as **Figure 9A** shows, more than 60% of cultures that are longer than 80 mm respond to magnetic stimulation. This implies that special conditions are necessary for magnetic response. To investigate this geometric dependence cultures were grown in configurations that are parts of rings - having the same radius but different lengths by patterning them on arcs rather than on complete circles (see **Figure 8C**).

A comprehensive investigation of the magnetic field thresholds as a function of the radius of 1D ring cultures is shown in **Figure 9B**. White circles represent experimental measurements of stimulation thresholds, while the color coding denotes the calculated probability to measure an excitation threshold. A stimulation threshold is defined as the weakest field that still elicits a response for a given neuronal culture.

Figure 9B emphasizes the trend that larger rings have lower stimulation thresholds, with a clear inverse correlation between the radius of the rings and the stimulation threshold. For example, the average 14 mm ring responds to magnetic pulses with amplitude of 1.5 T while the average 7 mm ring only responds to 3 T or more. This is expected from the theoretical calculation of the induced electric field, which is depicted as the color coded predicted probability to fire. Indeed, the electric field induced by 3 T at a radius of 7 mm is equal to that induced by 1.5 T at twice the radius. In summary, the average electric field threshold is 301 ± 128 (standard deviation) V/m, independent of the radius of the ring cultures.

In contrast to the single circular coil, which could not elicit response in 2D cultures, 15 of 30 2D cultures that were stimulated by rotating field magnetic stimulation responded with bursting neuronal excitation. The two independent coils that are positioned perpendicular to each other (**Figure 8B**) generate rotating magnetic and induced electric fields. Each coil is connected to its own MS, both of which discharge similar currents at a phase lag of 90 degrees. The amplitude of the electric field induced by each of the two coils in the cross coil configuration is plotted in **Figure 10A**, and the same traces are represented in **Figure 10B** by a polar representation of the direction and amplitude of the superimposed electric field of both coils. The resulting induced electric field scans 270 degrees of the real space at a maximum field of ~ 270 V/m.

Figure 1: Schematic of the Setup Used for Electrical Stimulation of Neuronal Cultures. The desired signal is produced by a signal generator with two outputs that have no common ground. These signals are amplified to give an output voltage of up to ± 30 V. The electric signals are then fed through two separate pairs of electrodes, stimulating a neuronal culture in two orthogonal and independent directions. Stimulation of neurons can be viewed and monitored by calcium dyes. [Please click here to view a larger version of this figure.](#)

Figure 2: Schematic of the Setup Used for Magnetic Stimulation of Neuronal Cultures. **A.** At top is shown the magnetic coil (blue circles), which is located 5 mm concentrically above the neuronal ring culture, placed in a Petri dish (blue outline). A pickup coil (red circle) positioned on the circumference of the Petri dish measures the voltage induced by the magnetic pulse. At bottom the measured dynamics of the magnetic stimulator coil is shown (using an MS capacitor voltage load of 5,000 kV), as integrated from the pickup coil. Induced electric field (calculated for a ring radius of 14 mm) is depicted in green while the magnetic field is depicted in blue **B.** An inverted microscope images fluorescent dyes sensitive to calcium transients of neurons reacting to magnetic pulses. **C.** Neurons grown on a pattern of concentric rings, used for an effective stimulation by the ring magnetic stimulator. **D.** Bright field microscope image of neurons grown on one line of the pattern. [Please click here to view a larger version of this figure.](#)

Figure 3: Examples of Patterns of 1D Neuronal Cultures Used for Orienting the Electric Field with the Direction of Axonal Growth. **A.** circular pattern is used for the circular magnetic coil, when the induced electric field has a circular orientation. **B.** Line patterns are used when the induced or direct electric field has a single orientation.

Figure 4: Example Traces of Calcium Transients Imaged During Synchronous Network Bursts. **A.** An image of neurons that were dyed previous to the experiment with a calcium dye. **B.** Traces of intensity vs. time of the ROIs in A with the color of the trace representing the color of the border of the ROI in A. A large increase in intensity synchronized within the three ROIs represents a network burst. [Please click here to view a larger version of this figure.](#)

Figure 5: Basic Setup for Electric Stimulation, using One Pair of Parallel Bath Electrodes to Determine that 2D Cultures Have no Preferred Orientation of Electric Stimulation. **A.** Apparatus used for culture stimulation. The electric field is produced by applying voltage to the platinum wire electrodes. The distance between the two wires is 13 mm. **B.** An example of a voltage signal to be applied on the electrodes in A. The bipolar shape of the pulse helps prevent electrolysis of the recording solution at the electrodes. **C.** When stimulating a 2D culture in different rotations of the electrodes, there is isotropy of the neuronal response. **D.** An example of the electric field measurement using the probe described in step 2.2. The electric field is uniform to an error of up to 10%. This figure has been modified from ⁵. [Please click here to view a larger version of this figure.](#)

Figure 6: Electric Stimulation with Two Pairs of Electrodes Allows for Rotation of the Electric Field and for Stimulation in any Desired Angle. **A.** To produce more complicated shapes of the electric field, two independent electrode pairs are needed. **B.** Applying a cosine voltage shaped pulse to one electrode and a sine pulse to the second electrode creates a rotating electric field with constant amplitude. **C.** Applying a square voltage pulse to one pair of the electrodes creates a unidirectional uniform electric field. This figure has been modified from ⁵. [Please click here to view a larger version of this figure.](#)

Figure 7: Network Disconnection by Blocking Synaptic Inputs (as Described in 2.4.2) Enables Observation of the Total Population of Single Cells that are Stimulated by a Given Electric Field. **A.** By applying different amplitudes of voltage pulses to the two pairs of electrodes, the electric field can be oriented to every angle without the need to manually turn the culture. **B.** Example recordings of calcium transients with electric stimulation at different applied voltages. Four traces are shown, slightly shifted vertically to allow viewing. Voltage values are written beneath the blue traces. **C.** When applying a constant duration of electric field, the number of neurons that will fire in response to the electric field has a cumulative distribution function (CDF) that is a cumulative Gaussian distribution (or an erf function) as a function of the amplitude of the voltage (which is proportional to the field strength). **D.** An example of a Strength-Duration curve obtained while employing this protocol. This figure has been modified from ⁵. [Please click here to view a larger version of this figure.](#)

Figure 8: Magnetic Coil Configuration and Calculation of the Induced Electric Field. **A.** At top is a circular coil (blue circles) is positioned 5 mm above one-dimensional neuronal ring cultures (blue disk). The rings are parallel to and concentric with the coil, which creates a magnetic pulse that is oriented along the red lines. By Faraday's law, the induced electric field lies on planes that are parallel to the coil along rings concentric with it. At bottom is a horizontal cross-section along the plane of the ring cultures. The relative value of the electric field is color-coded, with direction depicted by arrows. Larger rings enclose a larger area of flux and therefore the electric field induced there is higher. **B.** Image of the cross-coil magnetic stimulator (top) and a simulation of the electric field that is induced by it. **C.** Pattern used to grow neurons which can be stimulated with either a circular electric field or a radial electric field. This figure has been modified from ^{21,28}. [Please click here to view a larger version of this figure.](#)

Figure 9: Response of Ring Cultures to Magnetic Field. **A.** The success rate of stimulation grows with the radius of the culture. The error bars represent the standard error (SE). **B.** The relation between ring size and magnetic field strength is depicted. The probability to excite the culture is color coded. Indeed most successful excitations of cultures lie in the overlap between the experimentally accessible phase space (white rectangle) and the high probability region (red). This figure has been modified from ²¹. [Please click here to view a larger version of this figure.](#)

Figure 10: Rotating Magnetic Field Setup Field. **A.** In order to induce a rotating electric field when stimulating with the cross-coil magnetic stimulator, a phase shift of 90 degrees is needed between the two coils. Shown is the electric field induced in a pickup coil positioned on 2 neighboring wings of the clover leaf coil. The coils were driven separately by 2 commercial stimulators. **B.** A reconstruction, using the curves shown in A, of the resultant electric field amplitude and direction during a pulse of the clover leaf coil. This figure has been modified from ^{21,28}. [Please click here to view a larger version of this figure.](#)

Discussion

1D patterning is an important tool that can be used for a variety of applications. For example, we have used 1D patterning for creating logic gates from neuronal cultures ²⁹ and more recently to measure the Chronaxie and Rheobase of rat hippocampal neurons ⁵, and the slowing down of signal propagation velocity of firing activity in Down syndrome hippocampal neurons compared to the wild type (WT) hippocampal neurons ²⁷. The suggested protocol for 1D patterning is robust and it is easy to form any desired pattern. We suggest to observe the 1D culture before measurements to be aware of any possible disconnections of the network, which may affect its function.

Chemical patterning of neuronal configurations has a distinguished history ^{30,31,32}. We found that our approach yields similar results to chemical patterning, yet they are more robust and easier to attain. Recently the option for microfluidic patterning of neurons has become an interesting alternative to the approach we offer, with comparable simplicity and ease of use ^{33,34}. The microfluidic approach is being used in our lab in parallel with the etching method described here.

As we showed ⁵, 2D cultures have no preferred orientation and their stimulation is isotropic and does not depend on the orientation of the field applied. Shorter durations are needed for stimulation of 2D cultures when the field is rotating. In contrast, 1D cultures are very dependent on the orientation of the field. When the field is oriented with the pattern (and therefore with the direction of axonal growth), the duration of a fixed-amplitude field needed for stimulation is much shorter than when the field is oriented perpendicular to the pattern. Similarly, if the duration is kept constant then the field needed to excite the culture is smaller if the field and pattern are parallel. When stimulating perpendicularly, the field pulse needs to be much longer (in the order of a millisecond when the network is disconnected), and then mainly the dendrites are stimulated. When electric fields (either direct or induced by a magnetic field) are used for brain stimulation, this is of extreme importance. If the brain area targeted is known to have bundles of axons, these axons can be excited by orienting the field in their direction and then need less power or a shorter duration of the field for stimulation. If the brain region targeted does not have preferred orientation, then a rotating field is more efficient for stimulation. If dendrites are the target of the stimulation, longer pulses are more effective.

Magnetic fields can stimulate neuronal activity when a magnetic pulse induces an electric field that excites neurons. Because currently achievable magnetic pulses are microseconds in duration, dendrites, whose response time is of the order of milliseconds, are not expected to be sensitive to magnetic stimulation. In contrast, axons, whose response time is faster, are the target of stimulation^{2,3}. The response of axons to electric excitation is maximal when they are parallel to the electric field and diminishes when they are perpendicular to it^{1,35}; therefore, in magnetic excitation, we strive to set up systems where the induced electric field orients parallel to the targeted axons. Therefore, when stimulating a 1D ring, the field should be oriented parallel to the axons, and the size of the ring determines the threshold for stimulation. Similar for the direct stimulation by bath electrodes, to stimulate a 2D culture, a rotating induced electric field is much more efficient.

The combination of patterning of neurons and of external excitation with electric or magnetic field is a potent technology with many possible future applications. Chronaxie and Rheobase, as well as threshold for activation and activation propagation dynamics and velocities are all parameters that are telling of a neuronal culture's intrinsic properties. In particular, therapeutic treatments and the effect of drugs or pharmacological treatment can be immediately tested directly on the neurons. This would allow an efficient survey of both disease model neurons and of protocols for TMS stimulation. On a more conceptual direction, the ability to stimulate neuronal cultures precisely leads to novel possibilities regarding the information processing aspects of neural configurations, and allow us to envisage micro printed circuitry that combines electronic stimulation with living neuronal networks to provide new computational devices and novel brain interfacing devices.

Disclosures

The authors declare that they have no competing financial interests.

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References

- Nagarajan, S. S., Durand, D. M., & Warman, E. N. Effects of induced electric fields on finite neuronal structures: a simulation study. *IEEE Trans Biomed Eng.* **40** (11), 1175-1188 (1993).
- Nowak, L. G., & Bullier, J. Axons, but not cell bodies, are activated by electrical stimulation in cortical gray matter. II. Evidence from selective inactivation of cell bodies and axon initial segments. *Exp Brain Res.* **118** (4), 489-500 (1998).
- Ranck, J. B., Jr. Which elements are excited in electrical stimulation of mammalian central nervous system: a review. *Brain Res.* **98** (3), 417-440 (1975).
- Rattay, F. The basic mechanism for the electrical stimulation of the nervous system. *Neuroscience.* **89** (2), 335-346 (1999).
- Stern, S., Agudelo-Toro, A., Rotem, A., Moses, E., & Neef, A. Chronaxie Measurements in Patterned Neuronal Cultures from Rat Hippocampus. *PLoS One.* **10** (7), e0132577 (2015).
- Brunelin, J. *et al.* Examining transcranial direct-current stimulation (tDCS) as a treatment for hallucinations in schizophrenia. *Am J Psychiatry.* **169** (7), 719-724 (2012).
- Crucchi, G. *et al.* EFNS guidelines on neurostimulation therapy for neuropathic pain. *Eur J Neurol.* **14** (9), 952-970 (2007).
- Kennedy, S. H. *et al.* Canadian Network for Mood and Anxiety Treatments (CANMAT) Clinical guidelines for the management of major depressive disorder in adults. IV. Neurostimulation therapies. *J Affect Disord.* **117** Suppl 1 S44-53 (2009).
- Minzenberg, M. J., & Carter, C. S. Developing treatments for impaired cognition in schizophrenia. *Trends Cogn Sci.* **16** (1), 35-42 (2012).
- Vaidya, N. A., Mahabeshwarkar, A. R., & Shahid, R. Continuation and maintenance ECT in treatment-resistant bipolar disorder. *J ECT.* **19** (1), 10-16 (2003).
- Bartlett, W. P., & Banker, G. A. An electron microscopic study of the development of axons and dendrites by hippocampal neurons in culture. II. Synaptic relationships. *J Neurosci.* **4** (8), 1954-1965 (1984).
- Bartlett, W. P., & Banker, G. A. An electron microscopic study of the development of axons and dendrites by hippocampal neurons in culture. I. Cells which develop without intercellular contacts. *J Neurosci.* **4** (8), 1944-1953 (1984).
- Beggs, J. M., & Plenz, D. Neuronal avalanches in neocortical circuits. *J Neurosci.* **23** (35), 11167-11177 (2003).
- Breskin, I., Soriano, J., Moses, E., & Tlusty, T. Percolation in living neural networks. *Phys Rev Lett.* **97** (18), 188102 (2006).
- Feinerman, O., & Moses, E. Transport of information along unidimensional layered networks of dissociated hippocampal neurons and implications for rate coding. *J Neurosci.* **26** (17), 4526-4534 (2006).
- Soriano, J., Rodriguez Martinez, M., Tlusty, T., & Moses, E. Development of input connections in neural cultures. *Proc Natl Acad Sci U S A.* **105** (37), 13758-13763 (2008).
- Deisseroth, K. Optogenetics. *Nat Methods.* **8** (1), 26-29 (2011).
- Fenno, L., Yizhar, O., & Deisseroth, K. The development and application of optogenetics. *Annu Rev Neurosci.* **34** 389-412 (2011).
- Williams, S. C., & Deisseroth, K. Optogenetics. *Proc Natl Acad Sci U S A.* **110** (41), 16287 (2013).
- Rotem, A., & Moses, E. Magnetic stimulation of curved nerves. *IEEE Trans Biomed Eng.* **53** (3), 414-420 (2006).
- Rotem, A., & Moses, E. Magnetic stimulation of one-dimensional neuronal cultures. *Biophys J.* **94** (12), 5065-5078 (2008).
- Feinerman, O., & Moses, E. A picoliter 'fountain-pen' using co-axial dual pipettes. *J Neurosci Methods.* **127** (1), 75-84 (2003).
- Feinerman, O., Segal, M., & Moses, E. Signal propagation along unidimensional neuronal networks. *J Neurophysiol.* **94** (5), 3406-3416 (2005).
- Papa, M., Bundman, M. C., Greenberger, V., & Segal, M. Morphological analysis of dendritic spine development in primary cultures of hippocampal neurons. *J Neurosci.* **15** (1 Pt 1), 1-11 (1995).

25. Bikson, M. *et al.* Effects of uniform extracellular DC electric fields on excitability in rat hippocampal slices in vitro. *J Physiol.* **557** (Pt 1), 175-190 (2004).
26. Rahman, A. *et al.* Cellular effects of acute direct current stimulation: somatic and synaptic terminal effects. *J Physiol.* **591** (10), 2563-2578 (2013).
27. Stern, S., Segal, M., & Moses, E. Involvement of Potassium and Cation Channels in Hippocampal Abnormalities of Embryonic Ts65Dn and Tc1 Trisomic Mice. *EBioMedicine.* **2** (9), 1048-1062 (2015).
28. Rotem, A. *et al.* Solving the orientation specific constraints in transcranial magnetic stimulation by rotating fields. *PLoS One.* **9** (2), e86794 (2014).
29. Feinerman, O., Rotem, A., & Moses, E. Reliable neuronal logic devices from patterned hippocampal cultures. *Nat Phys.* **4** (12), 967-973 (2008).
30. Kleinfeld, D., Kahler, K. H., & Hockberger, P. E. Controlled outgrowth of dissociated neurons on patterned substrates. *J Neurosci.* **8** (11), 4098-4120 (1988).
31. Bugnicourt, G., Brocard, J., Nicolas, A., & Villard, C. Nanoscale surface topography reshapes neuronal growth in culture. *Langmuir.* **30** (15), 4441-4449 (2014).
32. Roth, S. *et al.* Neuronal architectures with axo-dendritic polarity above silicon nanowires. *Small.* **8** (5), 671-675 (2012).
33. Peyrin, J. M. *et al.* Axon diodes for the reconstruction of oriented neuronal networks in microfluidic chambers. *Lab Chip.* **11** (21), 3663-3673 (2011).
34. Renault, R. *et al.* Combining microfluidics, optogenetics and calcium imaging to study neuronal communication in vitro. *PLoS One.* **10** (4), e0120680 (2015).
35. Roth, B. J., & Basser, P. J. A model of the stimulation of a nerve fiber by electromagnetic induction. *IEEE Trans Biomed Eng.* **37** (6), 588-597 (1990).