Stimulating Neurons with Heterologously Expressed Light-Gated Ion Channels

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Heterologous expression of ion channels that can be directly gated by light has made it possible to stimulate almost any excitable cell with light. Optogenetic stimulation has been particularly powerful in the neurosciences, as it allows the activation of specific, genetically defined neurons with precise timing. Organotypic hippocampal slice cultures are a favored preparation for optogenetic experiments. They can be cultured for many weeks and, after transfection with optogenetic actuators and sensors, allow the study of individual synapses or small networks. The absence of any electrodes allows multiple imaging sessions over the course of several days and even chronic stimulation inside the incubator. These timescales are not accessible in electrophysiological experiments. Here, we introduce the production of organotypic hippocampal slice cultures and their transduction or transfection with optogenetic tools. We then discuss the options for light stimulation.

OPTOGENETIC TOOLS: DESIGNER CHANNELRHODOPSINS

Channelrhodopsin-2 (ChR2) from the unicellular alga *Chlamydomonas reinhardtii* and its homologs are proteins with seven transmembrane domains. The hydrophobic core regions of ChRs create a binding pocket for retinal and show homology with the light-activated proton pump, bacteriorhodopsin. Nagel et al. discovered in 2002 that expression of this core region from ChR1 (amino acids 76–309 out of 712) is sufficient to form a directly light-gated ion channel in eukaryotic cells (Nagel et al. 2002). One year later, they demonstrated that amino acids 1–315 of ChR2 formed a cation channel that could depolarize cells (Nagel et al. 2003). As a first engineering step, they replaced the carboxy-terminal tail of ChR2 with yellow fluorescent protein (YFP) to identify transfected neurons by their fluorescence (Boyden et al. 2005). Based on the sequence homology with bacteriorhodopsin, they then introduced a point mutation (H134R) that increased stationary photocurrents. This version was used for the first in vivo optogenetic experiments (Nagel et al. 2005).

The structural homology of ChRs to bacteriorhodopsin inspired several laboratories to introduce point mutations at other critical residues (most of which are close to the retinal-binding pocket) to generate novel optogenetic tools with altered properties. It was soon discovered that mutations that accelerate the closing kinetics of channels generally result in smaller photocurrents, whereas "slow" mutants are much more light-sensitive than wild-type ChR2 because, even at low photon flux, a large fraction of channels accumulate in the open state (Berndt et al. 2011; Mattis et al. 2012). An important achievement was the generation of a red-shifted ChR by combining elements from ChR2 and ChR1 to form chimeric ChRs (Prigge et al. 2012; Lin et al. 2013). An even more red-shifted variant with fast

Cite this introduction as *Cold Spring Harb Protoc*; doi:10.1101/pdb.top089714

kinetics and large currents (ChrimsonR) was discovered in a recent genetic screen (Klapoetke et al. 2014). It should be noted that even the most blue- and red-shifted ChRs known today have considerable spectral overlap, meaning that blue light will activate all known ChRs. However, by using wavelengths at the extreme ends of the action spectra (UV/red), two neuronal populations can be activated independently. We have successfully achieved independent activation of neurons expressing the highly potent CheRiff as a UV-sensitive ChR and ChrimsonR as a red-light-sensitive ChR (Hochbaum et al. 2014; Klapoetke et al. 2014).

ChR2 is a nonspecific cation channel with a reversal potential in neurons of ~0 mV; therefore, brief ChR2 activation resembles excitatory synaptic input. Mutating the central gate region of ChR2 has resulted in light-gated Cl^- channels that clamp neurons close to their resting membrane potential and may, therefore, be useful for inhibiting neurons (Berndt et al. 2014; Wietek et al. 2015). Natural anion-conducting ChRs with similar favorable properties have recently been found in cryptophyte algae (Govorunova et al. 2015).

HIPPOCAMPAL SLICE CULTURE PREPARATION

Mammalian neurons can be cultured directly on glass coverslips where they form two-dimensional networks that are spontaneously active. Activity in these dissociated cultures is characterized by highly synchronized bursting patterns that do not resemble natural activity in the brain. As a more physiological alternative, entire slices of brain tissue can be cultured for months under interface conditions on sterile tissue culture inserts. Hippocampal slice cultures establish a pattern of connectivity that resembles in many ways the situation in vivo, including maturation of synaptic properties (De Simoni et al. 2003; Rose et al. 2013). This preparation also combines very good optical and electrophysiological accessibility and can be maintained for several weeks—long enough to address fundamental questions about synaptic plasticity and long-term dynamics of network connectivity (De Roo et al. 2008; Wiegert and Oertner 2013). Furthermore, organotypic slice cultures have proven to be an ideal preparation for testing and characterizing novel optogenetic tools (Schoenenberger et al. 2009; Berndt et al. 2011; Wietek et al. 2014).

Sterility during the preparation, maintenance and experimental interrogation of slice cultures is essential, especially for long-term experiments. The initial description of hippocampal slice cultures (Stoppini et al. 1991) and a more recent protocol (Gogolla et al. 2006) recommend the use of antibiotics or antimycotics to prevent microbial infection. Antibiotics, however, are known to affect many properties of neurons (Amonn et al. 1978; Llobet et al. 2015) and may introduce artifacts. If sterile condtions are maintained during production, maintenance ("feeding"), and handling (e.g., transfection and imaging) of the cultures, antimicrobial drugs can and should be avoided (see Protocol: **Preparation of Slice Cultures from Rodent Hippocampus** [Gee et al. 2016]; Protocol: **Viral Vector–Based Transduction of Slice Cultures** [Wiegert et al. 2016a]).

INTRODUCTION OF TRANSGENES INTO NEURONS OF SLICE CULTURES

Several different delivery methods are available for the introduction of transgenes into cells of organotypic slice cultures, such as biolistic transfection, electroporation, and viral vector–based transduction. These methods give different patterns of transgene expression and, therefore, a suitable delivery method has to be chosen depending on the goals of an experiment. Biolisitic transfection via gene gun is a fast method to achieve a random pattern of transfected neurons (Fig. 1A; Thomas et al. 1998). Drawbacks include poor control of transfection density, resulting in many untransfected cultures, as well as nonspecific tissue damage from the pressure wave and gold particle impact. Transfected cultures with the ideal "Golgi"-like transfection pattern are rare. The impact of gold particles sometimes induces a plasma bridge between two cells, resulting in neurons with two nuclei and pyramidal neurons with two apical dendrites.
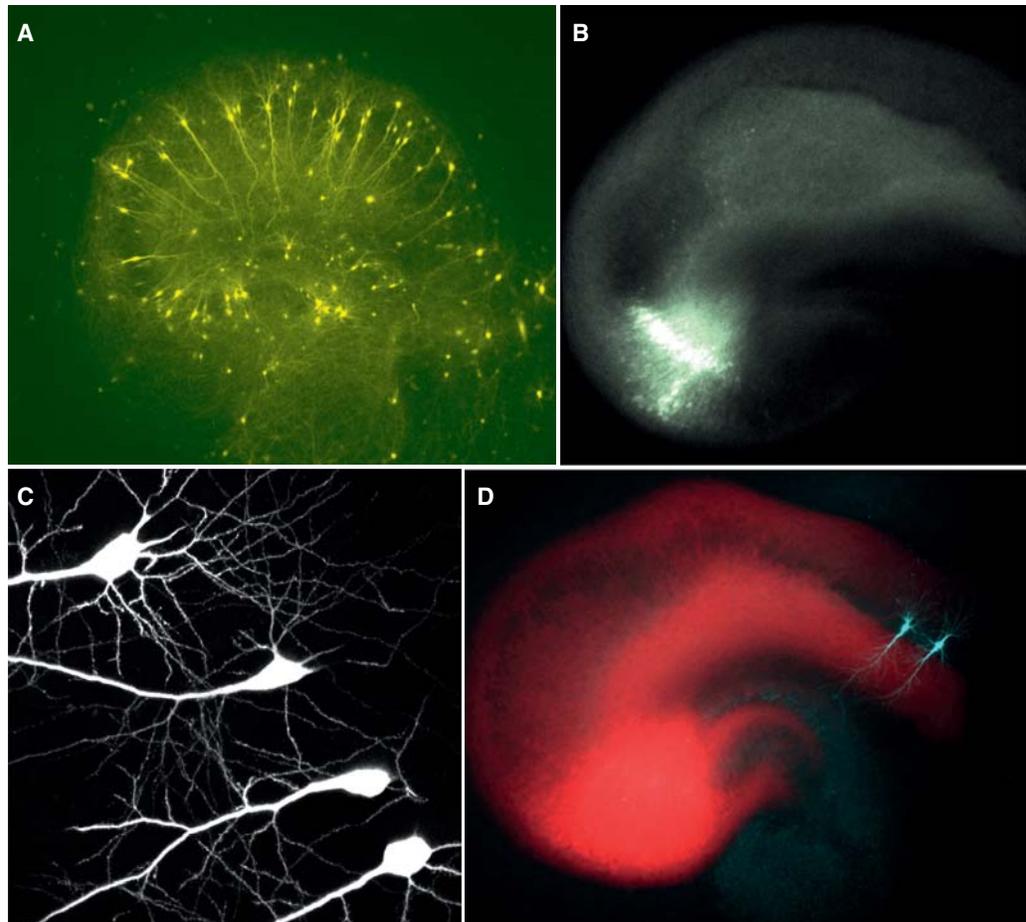
FIGURE 1. Outcome of different gene delivery methods. (*A*) Result of biolistic transfection (BioRad gene gun) of a fluorescent protein driven by the neuron-specific promoter synapsin-1. A sparse, random set of pyramidal cells expresses the fluorescent construct. (*B*) Result of local pressure injection of recombinant adeno-associated virus (rAAV) into area CA3. A dense cluster of neurons expresses the construct at high levels. The axonal projection of these neurons is seen as a faint glow. (*C*) Single-cell electroporation allows the transfection of individual neurons under differential interference contrast (DIC) or Dodt contrast. Electroporation is labor intensive but highly reproducible. (*D*) Combining rAAV injection into CA3 (red) and single-cell electroporation of CA1 pyramidal cells (cyan) allows the expression of different constructs in pre- and postsynaptic cells. (*A*, Adapted from Holbro et al. 2009; *C*,*D*, adapted from Wiegert and Oertner 2013.)

To produce a small cluster of transgene-expressing neurons, local injection of recombinant adeno-associated virus (rAAV) can be performed (Fig. 1B; Burger et al. 2004; Protocol: **Viral Vector–Based Transduction of Slice Cultures** [Wiegert et al. 2016a]). Transgene expression in individual cells varies with the distance from the injection site, indicating that varying numbers of rAAV particles are taken up by neurons. The serotype of the virus and the transgene promoter also determine levels of expression. After local injection into hippocampal slice cultures, AAV2/9 or AAV2/10 vectors and the synapsin-1 promoter rapidly produce high levels of protein expression in neurons.

If locally defined expression is the goal, single-cell electroporation produces exactly the desired pattern of transfection but is labor intensive (Fig. 1C; Rathenberg et al. 2003; Protocol: **Single-Cell Electroporation of Neurons** [Wiegert et al. 2016b]). The level of expression can even be controlled by the concentration of DNA in the electroporation pipette, and reliable cotransfection of several plasmids (e.g., ChR2, CFP, and synaptophysin-RFP) at defined ratios is possible. Single-cell electroporation can be combined with viral transduction to express different optogenetic constructs in pre- and postsynaptic cells (Fig. 1D). We routinely combine local rAAV transduction of CA3 neurons with single-cell electroporation of CA1 pyramidal neurons to investigate synaptic plasticity at individual
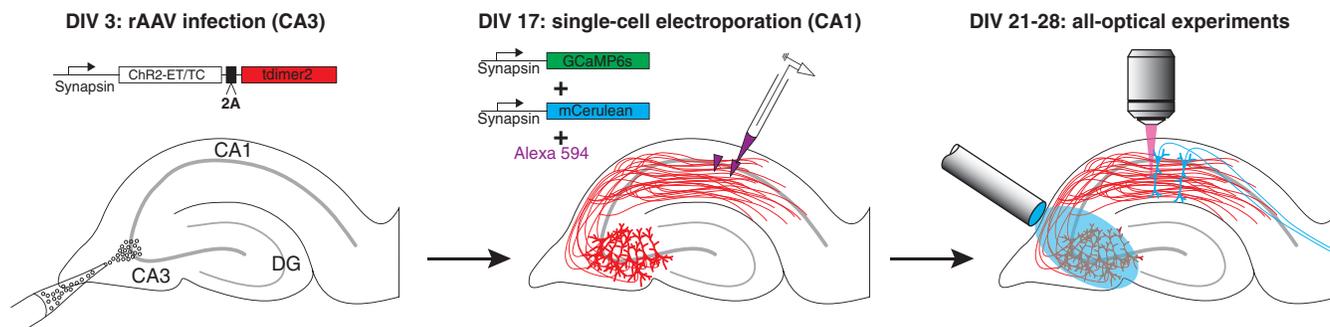
DIV 3: rAAV infection (CA3)   **DIV 17: single-cell electroporation (CA1)**   **DIV 21-28: all-optical experiments**

FIGURE 2. Sample timeline of an optogenetic experiment. Example time course for combining rAAV transduction and single-cell electroporation for optogenetic stimulation and readout (adapted from Wiegert and Oertner 2013). At DIV3, CA3 cells are transduced with rAAV2/7 encoding ChR2(ET/TC)-2A-tdimer2. Two weeks after rAAV infection (DIV 17), ChR2 (ET/TC) and tdimer2 have reached stable expression levels. CA1 pyramidal neurons are then transfected with GCaMP6s and mCerulean via single-cell electroporation. Alexa Fluor 594 is included in the pipette to immediately visualize successfully electroporated cells. At DIV 21, expression levels are suitable for optical synaptic plasticity experiments.

Schaffer collateral synapses over many days (Fig. 2; Wiegert and Oertner 2013). Presynaptic ChR2 can be coexpressed with a red label for presynaptic morphology, whereas postsynaptic CA1 neurons can coexpress a green-fluorescent calcium indicator (i.e., GCaMP6) together with the blue mCerulean, which serves as a postsynaptic morphology marker.

OPTOGENETIC STIMULATION OF TRANSFECTED SLICE CULTURES

Optogenetic tools that have been introduced into cells of slice culture can be stimulated with light in a very controlled fashion. Depending on the type of experiment, different illumination methods are available. To calibrate the properties of optogenetic tools and to determine the optimal transfection conditions, it is often necessary to perform patch-clamp recordings from the neuron expressing the tool of interest (Berndt et al. 2011; Wietek et al. 2014).

Light Stimulation through the Epifluorescence Pathway

To characterize the properties of a light-gated channel in a single neuron, the simplest method is to use the epifluorescence pathway of the microscope to deliver light to the cells that are under the objective. Several light-emitting diode (LED)-based light engines are commercially available that provide up to 16 different wavelengths. They can be manually controlled through a graphical user interface (GUI), by transistor-transistor logic (TTL) pulses, or via analog outputs of a data acquisition board. Using a high-pressure Hg lamp with a mechanical shutter is not recommended for optogenetic stimulation because the shutter blades heat up tremendously and pulse timing becomes imprecise. Monochromators based on 75-W xenon lamps are also not ideal because the power output is too low to saturate most channelrhodopsins and pumps (or fluorescent indicators).

To optically stimulate a neuron within the field of view (FoV) of a water immersion objective, the epifluorescence condenser of the microscope can be used to deliver light from high-power LED illumination systems (e.g., Lumencor SPECTRA, CoolLED pE-4000, Mightex, Thorlabs) via brief light pulses under TTL control. To avoid vibrating the microscope by cooling fans, a flexible coupling of the light source through a liquid light guide is recommended. Wavelength and intensity of the LED pulses can be chosen to excite commonly used fluorescent proteins and dyes; in this way a single light source can be used for optogenetic stimulation and visualization of labeled neurons. When upgrading an existing microscope from a high-pressure Hg arc lamp to an LED system, it might be necessary to exchange some of the narrow-band excitation filters in the fluorescence filter cubes. For epifluorescence

stimulation with multiple wavelengths combined with IR (infrared)-DIC or two-photon imaging, a short-pass dichroic beamsplitter (e.g., Semrock FF705-Di01-25x36) should be mounted in an empty filter cube. Because the epifluorescence condenser is designed to produce an even illumination across the entire FoV, the local intensity can be determined by measuring total light power under the objective and dividing this value by the illuminated area of the objective (see below). Intensities of 10–50 mW/mm^2 are necessary to drive some light-gated channels and pumps to saturation. On the other hand, some optogenetic tools are fully saturated at light levels several orders of magnitude lower. As most LEDs cannot be dimmed below 1%–5% of their maximum light output, neutral density filters must be inserted into the light path to extend the dynamic range of the light stimulation system downward.

To calibrate light intensity in the specimen plane, total power (mW) under the objective (e.g., Newport 1918-R with detector 818-ST2-UV/DB) should be measured and divided by the illuminated field of the objective (mm^2). The illuminated field of an objective is typically larger than its field of view. The illuminated field can be measured by focusing the objective on a calibration slide (e.g., Thorlabs R1L3S1P 10-mm Stage Micrometer) and shining light through the objective (i.e., turn on the epifluorescence light source). A mirror inserted at 45° under the condenser and a focusing lens ($f = 100$ mm) is used to project an image of the calibration slide on a screen mounted sideways. The diameter of the circular illuminated field can be directly read out from the projected image and is used to calculate the illuminated area.

For experiments combining optogenetic stimulation with patch-clamp recordings or imaging, an upright microscope with motorized stage should be used. When slices will be repeatedly stimulated and imaged over multiple days, the chamber and objectives should be sterilized by wiping with 70% ethanol. To avoid constant perfusion and bubbling with oxycarbon (carbogen), sterile HEPES-buffered solution rather than bicarbonate-buffered artificial cerebral spinal fluid can be used. Cultures can easily tolerate 30- to 60-min imaging sessions under a water immersion objective (25°C–30°C) without perfusion as long as the pH and osmolality of the imaging solution is maintained (Wiegert and Oertner 2013).

Light Stimulation through Oblique Light Fiber

When synapses and networks are under investigation (e.g., the CA3–CA1 connection in the hippocampus), the cell bodies of presynaptic neurons are often far outside the field of view of a water immersion objective that is being used to visualize the postsynaptic neurons (Wiegert and Oertner 2013). To stimulate neurons far away from the optical axis of the microscope, a blunt light fiber can be positioned using a micromanipulator just above the area to be stimulated (e.g., CA3). A relatively large-diameter fiber (typically 400 µm) is required to provide enough light from high-powered LEDs (see above) to drive action potentials in ChR2-expressing neurons. Because of the shallow angle of illumination and the divergent cone of light, all parts of the slice culture are illuminated at different intensities. Although this style of light delivery is flexible and economical, it is not possible to exactly reproduce the local light intensity at all cells of interest or to completely avoid illuminating the tissue under the objective.

Off-Center Illumination through Condenser

Certain optogenetic experiments require the illumination of CA3 pyramidal neurons at different wavelengths or with timing different from CA1 illumination. For these experiments, high local light intensities have to be generated at positions up to 1.5 mm off the optical axis with minimal stray light to other parts of the culture. To selectively stimulate neurons far away from the optical axis of the microscope, we steer a collimated laser beam through the oil immersion condenser (Fig. 3). This strategy produces a very restricted (Gaussian) pencil of light with minimal stray light to other areas of the culture, such as the area under the objective, but requires a laser as light source. Measures must also be taken to ensure there is no possibility of accidently looking into the laser through the eyepieces. This could be a notch filter inserted below the tube lens or an interlock switch that enables observation
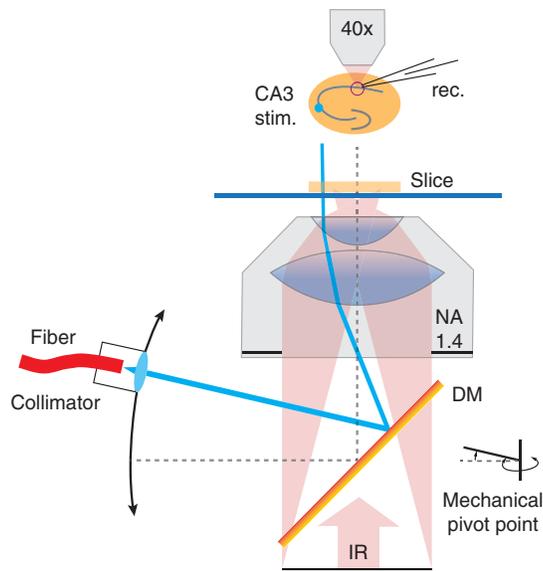
FIGURE 3. Beam steering through condenser for off-center light stimulation. An optical fiber collimator is mounted on a swing arm to point a laser beam at variable angles through the back aperture of a high-NA (numerical aperture) condenser (1.4 NA, oil immersion). Different angles correspond to different positions of the laser spot in the slice. A long-pass dichroic mirror (DM) mounted below the condenser allows simultaneous visualization of the tissue by infrared (IR) DIC or Dodt contrast for patch clamp experiments.

of the laser only with the camera. Because of their high output power, diode-pumped solid-state (DPSS) and diode lasers are well suited for such focal illumination. For two-color stimulation (e.g., 405 and 594 nm), a laser combiner (Omicron LightHub) coupled to a multimode fiber can be used. The far end of the SMA fiber-optic patch cable is connected to a collimator (Thorlabs). The collimator is mounted on a swing arm (lockable, with counter weight) to point the laser beam at different angles through the center of the back aperture of the condenser (Fig. 3). Using this system, high local intensities can be realized away from the optical axis with little scattered light in other hippocampal areas. It is readily combined with patch-camp recordings and optical stimulation of neurons close to the optical axis through the epifluorescence pathway (see above). This simple mechanical system can be refined for multispot illumination, using galvanometric scan mirrors and a scan lens for remote control of the laser spot position.

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Preparation of Slice Cultures from Rodent Hippocampus

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This protocol describes the preparation of hippocampal slice cultures from rat or mouse pups using sterile conditions that do not require the use of antibiotics or antimycotics. Combining very good optical and electrophysiological accessibility with a lifetime approaching that of the intact animal, many fundamental questions about synaptic plasticity and long-term dynamics of network connectivity can be addressed with this preparation.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous material used in this protocol.

RECIPES: Please see the end of this protocol for recipes indicated by <R>. Additional recipes can be found online at http://cshprotocols.cshlp.org/site/recipes.

Reagents

Animals (rat [P4–P6] or mouse [P5–P8] pups)
Appropriate anesthesia according to local guidelines
Dissection solution (sterile; 50 mL is sufficient for approximately 10 pups) <R>
Slice culture medium (sterile; 50 mL is sufficient for approximately 10 pups) <R>

Equipment

95% O_2/5% CO_2 regulator and tubing
Beaker (glass; 500-mL)
Cell Saver Tips (1000-μL) (BIOzym 693000)
Culture plates (six-well; e.g., Corning 3516 or Sarstedt 83.1839)
Dissection stereomicroscope with cold platform (e.g., ice-cold metal plate or glass Petri dish filled with ice, prewiped with 70% ethanol)
Filter paper or Whatman paper (sterile, 6-cm diameter; MACHEREY-NAGEL 431005)
Fine paintbrush (e.g., Ted Pella 11810/11812)
Hot bead sterilizer (e.g., Fine Science Tools 18000-45)
Incubator (37°C/5% CO_2 with rapid humidity recovery, copper chamber recommended; e.g., Heracell 150i/160i, Thermo Scientific)
Large shallow ice bucket containing ice scattered with NaCl

Cite this protocol as *Cold Spring Harb Protoc*; doi:10.1101/pdb.prot094888

Millicell Cell Culture Insert (30-mm, hydrophilic PTFEm [polytetrafluoroethylene], 0.4-µm;
   Millipore PICMORG50)
Paper towels (sterile)
Pasteur pipettes (glass, 9″, sterilized; e.g., Carl Roth 4518.1)
Pasteur pipettes (plastic disposable, sterile; e.g., Sarstedt 86.1171.010)
Pasteur pipettes with tips broken off (glass, sterilized)

> *Wear goggles and wrap pipettes before breaking to prevent injury.*

Pipette bulbs (two, rubber, 2-mL; e.g., Sigma-Aldrich Z111597)
Pipettor (1000-µL; BIOzym 655070)
Razor blade (two-sided; Fine Science Tools 10050-00)
Small ice bucket containing ice scattered with NaCl
Spray bottle containing 70% ethanol
Stainless steel tray
Sterilized dissection tools
   Bone curette (small, back thinned on sharpening stone; DO608R Lucas sharp spoon, Aesculap)
   Coarse forceps (e.g., Fine Science Tools 11002-16)
   Fine scissors (Carl Roth LT28.1)
   Forceps (two, No. 5 Dumont [fine]; WPI 500342)
   Large scissors (ET140R, Aesculap or Fine Science Tools)
   Scalpel handle and blades (#3 and #10) (e.g., Fine Science Tools 10003-12/10010-00)
   Small spatula or spoon
Tissue Chopper (McIlwain type 10180, Ted Pella)
Tissue culture dishes (60-mm, sterile; Sarstedt 83.1801)
Tissue culture hood
Tubes (sterile, 50-mL)

METHOD

The preparation of slice cultures that are to be made and maintained without antibiotics requires stringent sterility be maintained at all times. Antibiotics affect a myriad of cellular properties (Amonn et al. 1978; Llobet et al. 2015); therefore, it is essential to avoid their use. Aseptic techniques must therefore be strictly observed at all steps and everything coming into contact with brain tissue must be sterile. For a culture to be maintained it must remain uncontaminated. Normally experiments should be performed within 6 wk of culture preparation, but slice cultures can be successfully maintained for more than a year.

Preparation

1. Put a 1-mL slice of culture medium in each well of the six-well plates. Using sterile forceps place one membrane insert into each well. Put plates into 37°C incubator.

   > *A mouse pup will yield approximately 10 to 15 slices, a rat pup approximately 15 to 20. If two slices are to be placed on each insert, then one six-well plate will hold 12 slice cultures.*

2. Next to the tissue culture hood arrange the following.

   - A stainless steel tray with two 60-mm dishes per animal

     > *One dish should contain a sterile filter paper. Sit the tray in a shallow ice bucket containing ice sprinkled with NaCl. The dishes can be turned upside down and the lids used because the rims are lower.*

   - Hot bead sterilizer and beaker filled with water for rinsing tools

   - Large scissors, fine scissors, coarse forceps, scalpel, and spatula arranged between sterile paper towels

   - Sterile paper towel for placing head

   - Container for body

3. Inside the tissue culture hood arrange the following.

- Tissue chopper set to cut 400-µm slices

    The blade must be cleaned of oil/wax before mounting.

- Dissection stereomicroscope with ice-cold platform
- Small bucket of NaCl-sprinkled ice holding a 50-mL tube containing dissection solution (~5 mL per animal), bubbled with 95% O_2/5% CO_2 through a sterile glass Pasteur pipette

    Place a sterile disposable pipette in the dissection solution. When the bubbled dissection solution has turned from magenta to red/orange in color it is ready to use.

- 1000-µL pipettor with Cell Saver Tip
- Sterile glass Pasteur pipette with bulb
- Sterile transfer pipette, made by breaking the tip from a Pasteur pipette and mounting the bulb on the broken end
- Scalpel, two fine forceps (#5 Dumont), bone curette, arranged on sterile paper towel
- Sterile fine paint brush

4. Spray or wipe down the microscope, tissue chopper, ice bucket, and stainless steel tray with 70% ethanol. Spray the blade and stage (with mounted disks) of the tissue chopper and allow to air dry before beginning the procedure.

Dissection

Wear sterile gloves and a mask. Neurons are sensitive to ethanol, including fumes, so if using ethanol to sterilize surfaces, tools, etc., allow thorough drying to prevent tissue exposure.

5. Immediately before beginning the procedure, place 1–2 mL of the cold, bubbled dissection solution into one of the 60-mm dishes containing the filter paper (this can be done while pups are undergoing anesthesia). Place covered dish back on the chilled tray.

6. Anesthetize pups according to local regulations and decapitate using the large scissors.

    Do not put the head in the dissection solution as this will result in contamination of the slice cultures.

7. Place the head on a sterile paper towel and hold (without squeezing) between the fingers of one hand—it is important to note that this hand is now contaminated. With a scalpel, open the skin from the nose to the base of the skull without cutting through the skull. Pull back the skin with the fingers already holding the head, slide the scalpel under the skin on one side and cut downward through the ears, repeat on the other side, and the skin can now easily be held back leaving the skull exposed. Cut the skull with the fine scissors along the midline. Make small cuts at the most rostral end out toward the sides keeping the orientation of the scissors so that the blade closest to the brain is always the same. Put the scissors into the beaker of water. Take the coarse forceps and pull open/remove the skull. The flaps of skull can be held with the fingers holding the head if necessary. Again be sure to keep the orientation of the forceps constant to avoid contaminating the surface of the brain. Put the forceps into the beaker of water and remove the cover from the chilled 6-cm dish with filter paper. Take the spatula/spoon and slide under the brain flipping it out of the skull onto the chilled wetted filter paper.

8. Put the dish with the brain on an ice-cold platform under the dissection microscope in the hood. Drop some of the cold, bubbled dissection solution onto the brain. While the brain chills for ~1 min, quickly dry off the dissection tools and place the tips into the bead sterilizer for 10 sec to sterilize. Replace and/or clean contaminated glove(s) with 70% ethanol. Ensure gloves are dry before moving close to the tissue to avoid exposing the tissue to ethanol vapor.

9. Under the dissection microscope in the hood, dissect the hippocampi. Holding the cerebellum with one pair of the fine forceps, cut along the midline twice with the scalpel without severing the most caudal part of the hemispheres (Fig. 1A). Using the bone curette fold down one hemisphere, which will expose the hippocampus (Fig. 1B). Cut the ends of the hippocampus with the scalpel
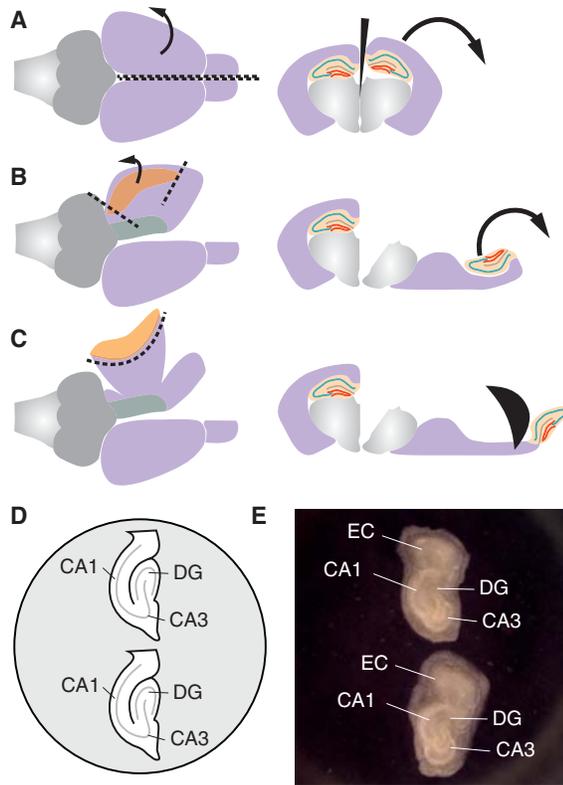
FIGURE 1. Dissecting the hippocampus. (*A*) Cut along the midline with a scalpel, using the cerebellum/brain stem as a handle. (*B*) Fold out the left hemisphere, exposing the hippocampus. Cut free the tips of the hippocampus. (*C*) Flip out the left hippocampus and cut through the entorhinal cortex to completely separate the left hippocampus from the brain. (*D*) Arrangement of slices on a cell culture insert for injection and electroporation. (*E*) Six-week-old Wistar rat hippocampal slice cultures. DG, dentate gyrus; EC entorhinal cortex; CAI and CA3, regions of the hippocampus proper.

and carefully flip out the hippocampus with the curette. Cut away most of the attached cortex (Fig. 1C). Repeat with the other hippocampus.

> *It is often helpful to pull away some of the meninges from the brain with the fine forceps before removing the hippocampi.*

10. Transfer the hippocampi to the stage of the tissue chopper using the transfer pipette. Arrange the hippocampi perpendicular to the blade and remove all surrounding dissection solution with the glass Pasteur pipette. Cut 400-μm thick slices.

> *The sliced hippocampus should remain on the platform. If the slices stick to the blade, there is probably too much dissection solution remaining on the stage.*

11. Take the Teflon chopping disk off the stage and gently rinse the slices into one of the prechilled 60-mm dishes using sterile dissection solution. Quickly clean the chopping disk with 70% ethanol, wipe dry with a sterile paper towel, and replace. If necessary, gently tease apart any slices that are adhering to each other with fine forceps. Do not grasp the slices but slide the forceps between the slices. The slices should be cut through and come apart easily.

12. Transfer the undamaged slices showing nice morphology onto the prewarmed membrane inserts (from Step 1) using the Cell Saver Tip. Two slices are typically placed near the center of each membrane and oriented so that the CA1 regions are pointing to one side using a fine brush (Fig. 1D). This simplifies injection of recombinant adeno-associated virus and electroporation. Carefully suck away any liquid from the top of the membrane using the glass Pasteur pipette and put the slices into the incubator.

> *If slices are to be transfected by gene gun, place three to five slices on each membrane and do not worry about the orientation. From the start of the dissection to placing the slices in the incubator should take 20–30 min per pup. It is more important not to damage the hippocampus and to not contaminate anything that will come in contact with the slices than it is to perform the procedure very quickly.*

13. One or two days after making the cultures, change the medium by aspirating about two-thirds and replacing with 700–800 μL prewarmed fresh medium. Thereafter, change the medium

twice per week (or at least every 5 d) for one to two slices per membrane and at least every 3 d for three to five slices per membrane. Allow 3 d for the slices to firmly adhere to the membrane.

Do not inject recombinant adeno-associated virus or electroporate the cultures within the first 2 d after preparation because they are likely to become detached and are then no longer usable.

RECIPES

Dissection Solution

Reagent	Final concentration	Amount (for 500 mL)
Sucrose (Fluka 84100)	248 mM	40 g
NaHCO$_3$ (Fluka 31437)	26 mM	1.09 g
D-glucose (Fluka 49152)	10 mM	0.9 g
KCl (1 M; Fluka 60129)	4 mM	2 mL
MgCl$_2$ (1 M; Fluka 63020)	5 mM	2.5 mL
CaCl$_2$ (1 M; Fluka 21114)	1 mM	0.5 mL
Phenol red (0.5%; Riedel-De Haen 32662)	0.001%	1 mL
Ultrapure H$_2$O	–	to 500 mL
Kynurenic acid (100 mM; Fluka 61260)	2 mM	1 mL in 50 mL dissection solution

After mixing all ingredients except the kynurenic acid, check the osmolality (it should be 310–320 mOsm/kg) and the color (it should be red-magenta, indicating a pH >8). Filter-sterilize (0.2 µm pore size) and store at 4°C in 50-mL aliquots. Prepare a 100 mM stock solution of kynurenic acid and store at −20°C in 1-mL aliquots. Just before use, thaw and vortex one aliquot of kynurenic acid and add to 50 mL of dissection solution.

Slice Culture Medium

Reagent	Final concentration	Amount (for 500 mL)
MEM (Sigma-Aldrich M7278)		394 mL
Heat-inactivated horse serum[a]	20%	100 mL
L-glutamine (200 mM; Gibco 25030-024)	1 mM	2.5 mL
Insulin (1 mg/mL; Sigma-Aldrich I6634)	0.01 mg/mL	0.5 mL
NaCl (5 M; Sigma-Aldrich S5150)		1.45 mL
MgSO$_4$ (1 M; Fluka 63126)	2 mM	1 mL
CaCl$_2$ (1 M; Fluka 21114)	1.44 mM	0.72 mL
Ascorbic acid (25%; Fluka 11140)	0.00125%	2.4 µL
D-glucose (Fluka 49152)	13 mM	1.16 g

[a]The serum is often a critical factor in slice quality and it is often necessary to test several batches (lots); three products that have been successfully used are Sigma-Aldrich H1138, Gibco 26050070, and Gibco 16050122. Gibco 16050122 must be heat-inactivated for 30 min at 55°C.

After mixing, filter-sterilize (0.2-µm pore size), and store at 4°C in 50-mL aliquots. (The solution should be orange-red [i.e., pH ∼7.3] and osmolality should be ∼320 mOsm/kg.)

REFERENCES

Amonn F, Baumann U, Wiesmann UN, Hofmann K, Herschkowitz N. 1978. Effects of antibiotics on the growth and differentiation in dissociated brain cell cultures. *Neuroscience* **3:** 465–468.

Llobet L, Montoya J, Lopez-Gallardo E, Ruiz-Pesini E. 2015. Side effects of culture media antibiotics on cell differentiation. *Tissue Eng Part C Methods* **21:** 1143–1147.

Single-Cell Electroporation of Neurons

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Single-cell electroporation allows the transfection of a small number of neurons in an organotypic culture with a single plasmid or a defined mixture of plasmids. Desired protein expression levels can vary depending on the experimental goals (e.g., high expression levels are needed for optogenetic experiments); however, when too much protein is expressed, cellular toxicity and cell death may arise. To a large degree, protein expression can be controlled by adjusting the concentration of plasmid DNA in the electroporation pipette. Here, we present a protocol for transfecting individual neurons in hippocampal slice cultures by electroporation. Essentially, a patch-clamp setup is required that includes an upright microscope with infrared differential interference contrast or Dodt contrast with a camera and a specialized amplifier that is able to deliver large-voltage pulses to the electroporation pipette.

Cold Spring Harbor Protocols
www.cshprotocols.org

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous material used in this protocol.

RECIPES: Please see the end of this protocol for recipes indicated by <R>. Additional recipes can be found online at http://cshprotocols.cshlp.org/site/recipes.

Reagents

Alexa Fluor 594 (Invitrogen A33082, or other dye compatible with available filter sets)
Hippocampal slice cultures on inserts (at least 5 d in vitro [DIV], optimally 2 wk)

> *For preparation of hippocampal slice cultures, see Protocol: **Preparation of Slice Cultures from Rodent Hippocampus** (Gee et al. 2016).*

K-gluconate-based intracellular solution <R>
Plasmid DNA in TE buffer (pH 7.4)

> *Prepare using any standard commercial purification kit.*

Saturated $FeCl_3$ solution
Slice culture medium <R>
Slice culture transduction solution (sterile, prewarmed to 37°C) <R>

Equipment

Centrifuge (table-top, refrigerated, e.g., Eppendorf 5415 R)
Culture plates (six-well; e.g., Corning 3516 or Sarsted 83.1839)

Electroporation setup

    20×–40× water immersion objective (20× with a variable magnifier tube before the camera)

    Axoporator 800A with pipette holder (Molecular Devices)

    Epifluorescence illumination and filters (optional: see Step 11)

    Headphones or speakers

    Microscope chamber consisting of glass microscope slide ($70 \times 100 \times 1$ mm) onto which a Teflon ring (inner diameter $\sim$35 mm, $\sim$2 mm high) is affixed with silicone aquarium sealant

    Motorized micromanipulator (e.g., from Luigs & Neumann or Sutter)

    Silver wire ($\sim$0.25 mm diameter)

    Upright microscope with motorized stage, CCD/CMOS (charge-coupled device/complementary metal oxide semiconductor) or video camera and IR-DIC (infrared-differential interference contrast) or Dodt contrast

Forceps (coarse; e.g., Fine Science Tools 11002-16)

Hot bead sterilizer (e.g., Fine Science Tools 18000-45)

Incubator (37°C/5% CO_2 with rapid humidity recovery, copper chamber recommended; e.g., Heracell 150i/160i, Thermo Scientific)

Micropipette puller (e.g., PC-10, Narishige)

Thin-walled borosilicate glass capillaries (WPI TW150F-3)

Tissue culture dishes (60-mm, sterile; Sarstedt 83.1801)

Tissue culture hood

Ultrafree centrifugal filter units (UFC30GV0S, Millipore)

METHOD

Preparation of DNA

1. Dilute Alexa Fluor 594 to a final concentration of 20 μM in K-gluconate-based intracellular solution and filter sterilize the solution through a Millipore Ultrafree centrifugal filter unit by centrifugation at 16,100*g* for several seconds in a table-top centrifuge at 4°C.

2. Remove the filter insert and add plasmid DNA to the desired final concentration. Centrifuge the solution again for 5 min at 16,100*g*, 4°C to pellet debris.

> *One hundred microliters of solution will be enough for more than 50 slices and can be stored between uses at −20°C. It is important that the DNA-containing solution is not passed through the Millipore Ultrafree centrifugal filter unit. The optimal final DNA concentration must be determined empirically and is usually 1–100 ng/μL.*

Single-Cell Electroporation

> *The hippocampal slice cultures used in this protocol must have been prepared under stringent sterile conditions and all steps in this procedure have to be performed under sterile conditions. For precautions to avoid contamination, see Protocol: **Preparation of Slice Cultures from Rodent Hippocampus** (Gee et al. 2016). It is recommended that the electroporation setup be close to the tissue culture hood. Furthermore, construction of a cabinet around the electroporation microscope with a fan and HEPA (high-efficiency particulate arrestance) filter to blow clean air down over the setup will reduce the incidence of contamination to almost never (for further description of this setup, see Protocol: **Viral Vector–Based Transduction of Slice Cultures** [Wiegert et al. 2016]).*

3. Coat the tips of silver wires for the electroporation and grounding electrodes with Cl by bathing them in $FeCl_3$ solution for a few minutes (when scratched) or overnight (when new).

4. Place a fresh six-well plate containing 1 mL of slice culture medium in each well in the incubator for pre-equilibration.

5. Fabricate electroporation pipettes using a micropipette puller to pull thin-walled borosilicate capillaries to a resistance of 10–15 MΩ when filled with the intracellular solution. For each slice to
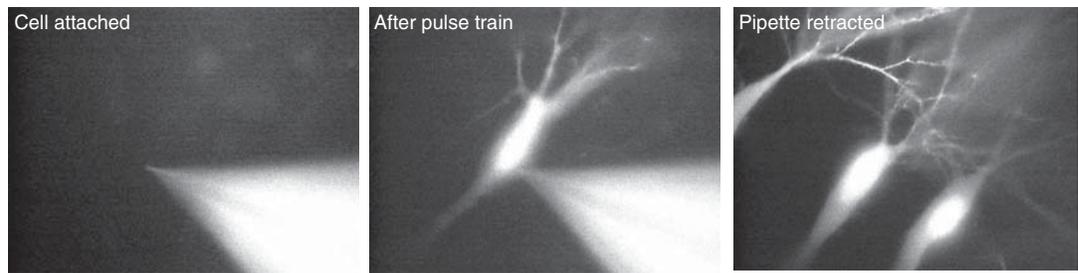
FIGURE 1. Single-cell electroporation. Fluorescence images taken during electroporation procedure. (*Left*) Pipette filled with DNA solution and Alexa Fluor 594 touching target cell. (*Middle*) Immediately after applying pulse train, target cell is filled with DNA solution and Alexa Fluor 594. (*Right*) Successfully electroporated neurons after retraction of the pipette.

be electroporated, back-fill one electroporation pipette with 1.5 μL of DNA/Alexa Fluor 594 solution (from Step 2).

> *Several pipettes can be filled at once and kept upright (tip down) for 1–2 h.*

6. Working in a tissue culture hood, pipette 1 mL of transduction solution prewarmed to 37°C into the microscope chamber. Use sterile forceps to transfer one slice culture insert into the chamber and add another 2 mL of transduction solution on top of the slice cultures. Place forceps into the hot bead sterilizer for ∼10 sec to resterilize between handling of inserts.

7. Cover the microscope chamber with a sterile 60-mm dish and transfer to the microscope.

8. Approach the selected cells with the electroporation pipette while applying positive pressure to the pipette by mouth. Monitor the tip resistance, which should be 10–15 MΩ, by the audio output of the Axoporator 800A amplifier. Make sure the tip is not clogged by monitoring tip resistance and expulsion of Alexa Fluor 594 fluorescence if needed.

9. Move the tip of the electroporation electrode close to a cell of interest while reducing pressure (easily controlled by mouth by blowing less hard). Monitor the tip resistance acoustically.

10. Approach the cell without sealing the electrode with membrane from other cells in the tissue.

11. Lightly touch the plasma membrane, which will cause a rise in pitch and tip resistance.

12. Release the pressure (do not apply suction) and wait for resistance to increase to 25–40 MΩ; however, avoid the formation of a GΩ seal.

13. Apply pulse train (e.g., voltage: −12 V, pulse width: 500 μsec, frequency: 50 Hz, train duration 500 msec; the optimal settings may differ depending on cell type).

14. Slowly retract the pipette and begin applying very light pressure when 2–5 μm away from the soma. Increase pressure at larger distances.

> *Do not apply too much pressure while retracting the pipette, otherwise the cell may rupture. Electroporation success can be instantly evaluated by assessing Alexa Fluor 594 fluorescence in electroporated cells (Fig. 1).*

15. Repeat Steps 8–14 to electroporate more cells.

16. Change the pipette before electroporating the second culture on insert and when the pipette becomes clogged.

17. Cover the chamber with a 60-mm dish and transfer to the tissue culture hood. Aspirate all solution and return the insert to the slice culture medium in the six-well plate (from Step 4).

> *The optimal time between electroporation and starting an experiment has to be determined empirically for each plasmid.*

RELATED INFORMATION

This protocol is based upon the electroporation of single neurons in organotypic cultures described by Rathenberg et al. (2003).

RECIPES

K-Gluconate-Based Intracellular Solution

Reagent	Final concentration	Amount (for 50 mL)
Aldrich K-gluconate (Sigma-Aldrich G4500)	135 mM	1.581 g
Aldrich EGTA (Sigma-Aldrich E0396)	0.2 mM	0.004 g
Aldrich HEPES (Sigma-Aldrich H4034)	10 mM	0.119 g
$MgCl_2$ (1 M; Fluka 63020)	4 mM	0.200 mL
Aldrich Na_2-ATP (Sigma-Aldrich A3377)	4 mM	0.121 g
Aldrich Na-GTP (Sigma-Aldrich G8877)	0.4 mM	0.010 g
Aldrich Na_2-phosphocreatine (Sigma-Aldrich P7936)	10 mM	0.128 g
Aldrich Ascorbate (L-ascorbic acid; Sigma-Aldrich A5960)	3 mM	0.026 g

Adjust pH to 7.2 with KOH, and check osmolality (it should be 290–300 mOsm/kg). After mixing, filter-sterilize (0.2-μm pore size) and divide into 100–500-μL aliquots. Store at −20°C.

Slice Culture Medium

Reagent	Final concentration	Amount (for 500 mL)
MEM (Sigma-Aldrich M7278)		394 mL
Heat-inactivated horse serum[a]	20%	100 mL
L-glutamine (200 mM; Gibco 25030-024)	1 mM	2.5 mL
Insulin (1 mg/mL; Sigma-Aldrich I6634)	0.01 mg/mL	0.5 mL
NaCl (5 M; Sigma-Aldrich S5150)		1.45 mL
$MgSO_4$ (1 M; Fluka 63126)	2 mM	1 mL
$CaCl_2$ (1 M; Fluka 21114)	1.44 mM	0.72 mL
Ascorbic acid (25%; Fluka 11140)	0.00125%	2.4 μL
D-glucose (Fluka 49152)	13 mM	1.16 g

[a]The serum is often a critical factor in slice quality and it is often necessary to test several batches (lots); three products that have been successfully used are Sigma-Aldrich H1138, Gibco 26050070, and Gibco 16050122. Gibco 16050122 must be heat-inactivated for 30 min at 55°C.

After mixing, filter-sterilize (0.2-μm pore size), and store at 4°C in 50-mL aliquots. (The solution should be orange-red [i.e., pH ∼7.3] and osmolality should be ∼320 mOsm/kg.)

Slice Culture Transduction Solution

Reagent	Final concentration	Amount (for 500 mL)
NaCl (Sigma-Aldrich S5150)	145 mM	4.23 g
HEPES (Sigma-Aldrich H4034)	10 mM	1.19 g
D-glucose (Fluka 49152)	25 mM	2.25 g
KCl (1 M; Fluka 60129)	2.5 mM	1.25 mL
$MgCl_2$ (1 M; Fluka 63020)	1 mM	0.5 mL
$CaCl_2$ (1 M; Fluka 21114)	2 mM	1 mL

Adjust pH to 7.4 with NaOH. After mixing all ingredients, check the osmolality—it should be 310–320 mOsm/kg. Filter-sterilize (0.2-μm pore size) and store at 4°C.

REFERENCES

Gee CE, Ohmert I, Wiegert JS, Oertner TG. 2016. Preparation of slice cultures from rodent hippocampus. *Cold Spring Harb Protoc* doi: 10.1101/pdb.prot094888.

Rathenberg J, Nevian T, Witzemann V. 2003. High-efficiency transfection of individual neurons using modified electrophysiology techniques. *J Neurosci Methods* **126:** 91–98.

Wiegert JS, Gee CE, Oertner TG. 2016. Viral vector–based transduction of slice cultures. *Cold Spring Harb Protoc* doi: 10.1101/pdb.prot094896.

Viral Vector-Based Transduction of Slice Cultures

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Transgenes can be introduced into the cells of organotypic slice cultures using different delivery methods, such as biolistic transfection, electroporation, and viral vector-based transduction. These methods produce different patterns of transgene expression. Local injection of recombinant adeno-associated virus (rAAV) produces a small cluster of transgene-expressing neurons around the injection site. Expression in individual cells varies with the distance from the injection site, indicating that many neurons take up several rAAV particles. The serotype and promoter also play a role in transgene expression. Here, we present a protocol for the transduction of previously prepared hippocampal slice cultures with rAVV.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous material used in this protocol.

RECIPES: Please see the end of this protocol for recipes indicated by <R>. Additional recipes can be found online at http://cshprotocols.cshlp.org/site/recipes.

Reagents

Hippocampal slice cultures on inserts (at least 3 d in vitro [DIV])

> *For preparation of hippocampal slice cultures, see Protocol:* **Preparation of Slice Cultures from Rodent Hippocampus** *(Gee et al. 2016).*

Recombinant adeno-associated virus (rAAV) suspension (concentration: 10^{11}–10^{13} GC/mL)
Slice culture medium (sterile) <R>
Slice culture transduction solution (sterile) <R>

Equipment

Compressed/pressurized air (for Picospritzer)
Culture plates (six-well; e.g., Corning 3516 or Sarstedt 83.1839)
Forceps (coarse; e.g., Fine Science Tools 11002-16, sterile)
Forceps (fine, No. 5 Dumont; e.g., WPI 500342, sterile)
Incubator (37°C/5% CO_2 with rapid humidity recovery, copper chamber recommended; e.g., Heracell 150i/160i, Thermo Scientific)
Microelectrode holder (e.g., WPI MPH6S)
Micromanipulator (e.g., LN Junior 3 or 4 axis; Luigs & Neumann or PatchMan, Eppendorf)
Micropipette puller (e.g., Sutter P-1000)

Cite this protocol as *Cold Spring Harb Protoc*; doi:10.1101/pdb.prot094896

Microscope (either an upright microscope with camera, wide-field illumination, and 4× objective or a USB microscope [e.g., dnt DigiMicro Profi] and movable stage)

Picospritzer III with foot switch (Parker Hannafin)

Sharps container

Thin-walled borosilicate glass capillaries with filament (WPI TW150F-3)

Tissue culture hood

METHOD

The hippocampal slice cultures used in this protocol must have been prepared under stringent sterile conditions and all steps in this procedure have to be performed under sterile conditions. For precautions to avoid contamination, see Protocol: **Preparation of Slice Cultures from Rodent Hippocampus** *(Gee et al. 2016). It is recommended that the injection setup be close to the tissue culture hood. Furthermore, construction of a cabinet around the injection microscope with a fan and HEPA (high-efficiency particulate air) filter to blow clean air down over the setup (Fig. 1C) will reduce the incidence of contamination to almost never. A dedicated viral vector injection setup can be constructed using an inexpensive USB microscope and the required ancillary parts. This setup is shown in Figure 1. Viral vector injections can be performed equally well on an upright microscope using wide-field illumination and a low-magnification objective (i.e., a standard patch-clamp setup).*

1. Place a fresh six-well plate containing 1 mL of slice culture medium in each well in the incubator to allow for temperature and pH equilibration.

2. Fabricate injection pipettes using a micropipette puller to pull thin-walled borosilicate capillaries to obtain a long and narrow tip with a shallow taper. Insert an empty injection pipette into the holder attached to the microscope and focus on the tip (Fig. 1A). Break off the tip with fine forceps under visual guidance to achieve a tip diameter of ~10 μm.
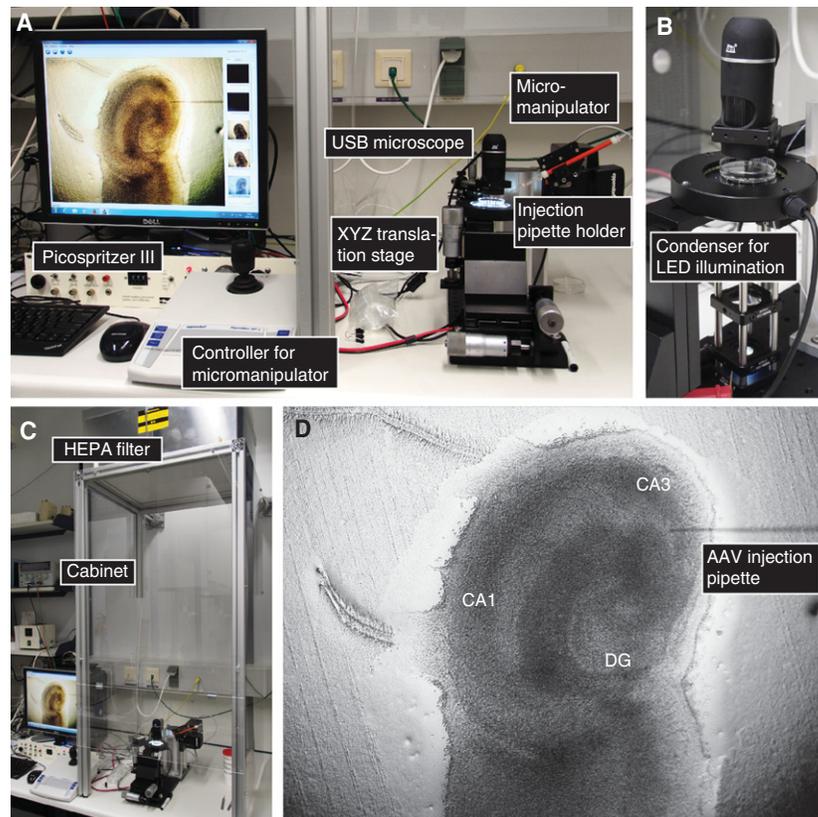

FIGURE 1. Injection of rAAV into CA3. (*A*) Photograph of USB microscope–based injection setup. (*B*) Detail of LED (light-emitting diode) illumination condenser. (*C*) View of setup showing surrounding cabinet. (*D*) Hippocampal slice culture with tip of injection pipette in area CA3.

3. Remove the pipette from the holder and back-fill with 1.2 µL rAAV suspension (this typically lasts for 12 slice cultures). Hold the pipette tip down and wait until all liquid has migrated along the filament into the tip. Set the pressure at the Picospritzer to 1.8 bar (25 p.s.i.) and the pulse duration to 50 msec.

4. Insert the pipette into the holder, focus on the tip, and apply a pressure pulse to test whether the rAAV suspension is expelled from the tip. If the tip is open, a small droplet should emerge. This check is most easily and safely done with a membrane insert already in the chamber (see Step 5). If the tip of the pipette is positioned to just touch the membrane (away from the slice cultures to be injected), then the drop will form on the membrane. The pulse duration/pressure should be adjusted if larger/smaller injection volumes are desired.

   Perform all subsequent steps quickly to avoid degradation of the rAAV at room temperature and to minimize drying out of air-exposed slice cultures.

5. Working in a tissue culture hood, fill the microscope chamber with 800 µL transduction solution prewarmed to 37°C and, using coarse forceps, place an insert with slice cultures into the chamber.

   When using a USB camera setup, the lid of a sterile 35-mm tissue culture dish serves as the chamber. For injecting cultures on the stage of an upright microscope a large glass microscope slide (70 × 100 × 1 mm) onto which a Teflon ring (inner diameter 34 mm, 2 mm high) is fixed with silicone aquarium sealant should be used as the chamber. Before use, the chamber should be wiped with 70% ethanol and rinsed with sterilized transduction solution.

6. Transfer the chamber to the microscope and focus on the slice culture. Bring the pipette tip into view and move tip axially into the cell body layer of CA3 (Fig. 1A,D). Depending on the desired infection density, one to three pressure pulses should be given. If desired, this procedure can be repeated several times at neighboring sites until the whole target area is covered. Axially retract the pipette until it no longer touches the culture and move to the second slice culture on the insert to repeat the injection procedure.

7. Transfer the insert to the six-well plate newly prepared in Step 1. Repeat Steps 5 and 6 until all cultures are injected with viral constructs; the same injection pipette may be used for many slice cultures. When finished, remove the injection pipette immediately from the holder and dispose in a sharps container.

   The ideal time between rAAV infection and when cells may be stimulated with light depends on many factors including the promoter, rAAV serotype, and the construct itself. We recommend testing cultures at various intervals before beginning actual experiments.

RECIPES

Slice Culture Medium

Reagent	Final concentration	Amount (for 500 mL)
MEM (Sigma-Aldrich M7278)		394 mL
Heat-inactivated horse serum[a]	20%	100 mL
L-glutamine (200 mM; Gibco 25030-024)	1 mM	2.5 mL
Insulin (1 mg/mL; Sigma-Aldrich I6634)	0.01 mg/mL	0.5 mL
NaCl (5 M; Sigma-Aldrich S5150)		1.45 mL
MgSO$_4$ (1 M; Fluka 63126)	2 mM	1 mL
CaCl$_2$ (1 M; Fluka 21114)	1.44 mM	0.72 mL
Ascorbic acid (25%; Fluka 11140)	0.00125%	2.4 µL
D-glucose (Fluka 49152)	13 mM	1.16 g

[a]The serum is often a critical factor in slice quality and it is often necessary to test several batches (lots); three products that have been successfully used are Sigma-Aldrich H1138, Gibco 26050070, and Gibco 16050122. Gibco 16050122 must be heat-inactivated for 30 min at 55°C.

After mixing, filter-sterilize (0.2-µm pore size), and store at 4°C in 50-mL aliquots. (The solution should be orange-red [i.e., pH ∼7.3] and osmolality should be ∼320 mOsm/kg.)

Slice Culture Transduction Solution

Reagent	Final concentration	Amount (for 500 mL)
NaCl (Sigma-Aldrich S5150)	145 mM	4.23 g
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D-glucose (Fluka 49152)	25 mM	2.25 g
KCl (1 M; Fluka 60129)	2.5 mM	1.25 mL
MgCl$_2$ (1 M; Fluka 63020)	1 mM	0.5 mL
CaCl$_2$ (1 M; Fluka 21114)	2 mM	1 mL

Adjust pH to 7.4 with NaOH. After mixing all ingredients, check the osmolality—it should be 310–320 mOsm/kg. Filter-sterilize (0.2-μm pore size) and store at 4°C.

REFERENCES

Gee CE, Ohmert I, Wiegert JS, Oertner TG. 2016. Preparation of slice cultures from rodent hippocampus. *Cold Spring Harb Protoc* doi: 10.1101/pdb.prot094888.