Topic Introduction

# Stimulating Neurons with Heterologously Expressed Light-Gated Ion Channels

J. Simon Wiegert, Christine E. Gee, and Thomas G. Oertner[1]

*Institute for Synaptic Physiology, Center for Molecular Neurobiology (ZMNH), 20251 Hamburg, Germany*

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

[1]Correspondence: thomas.oertner@zmnh.uni-hamburg.de

From the Ion Channels collection, edited by Paul J. Kammermeier, Ian Duguid, and Stephan Brenowitz.


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.

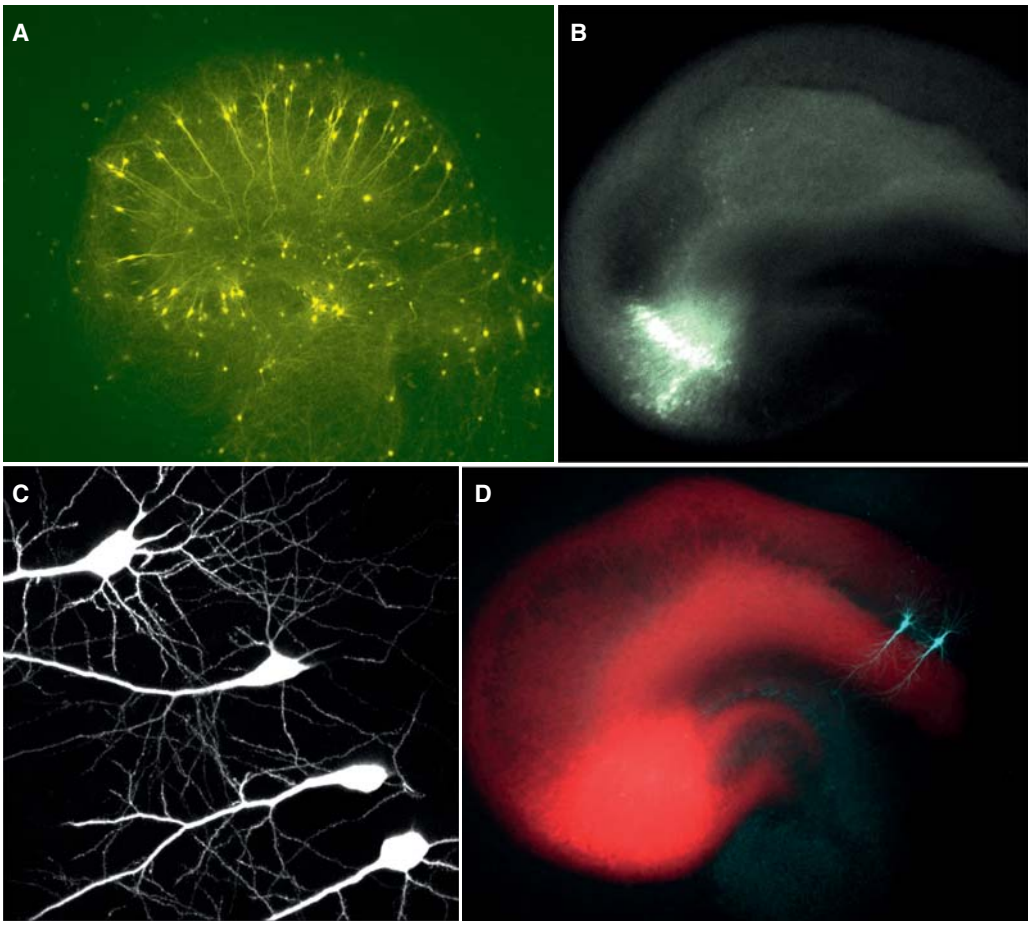


**FIGURE 1.** Outcome of different gene delivery methods. (*A*) Result of biolistic transfection (BioRad gene gun) of a fluorescent protein driven by the neuron-specific promoter synapsin-1. A sparse, random set of pyramidal cells expresses the fluorescent construct. (*B*) Result of local pressure injection 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

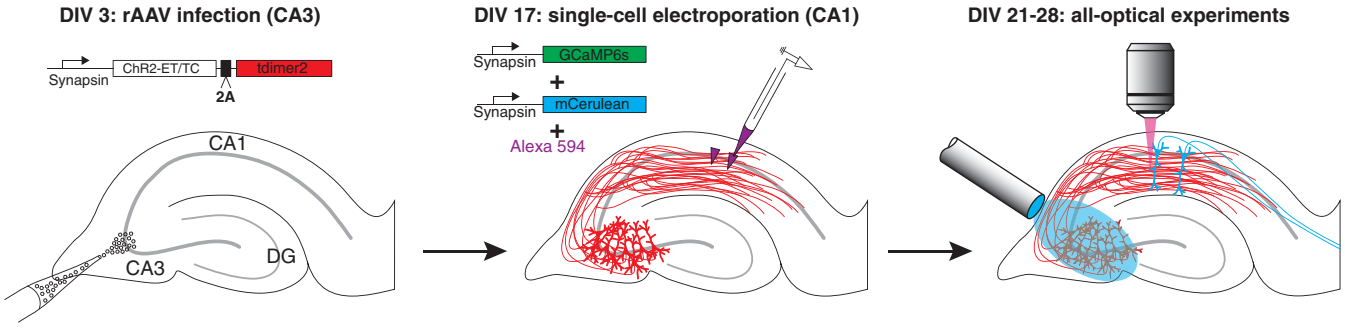


**FIGURE 2.** Sample timeline of an optogenetic experiment. Example time course for combining rAAV transduction and single-cell electroporation for optogenetic stimulation and readout (adapted from Wiegert and Oertner 2013). At DIV3, CA3 cells are transduced with rAAV2/7 encoding ChR2(ET/TC)-2A-tdimer2. 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




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

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

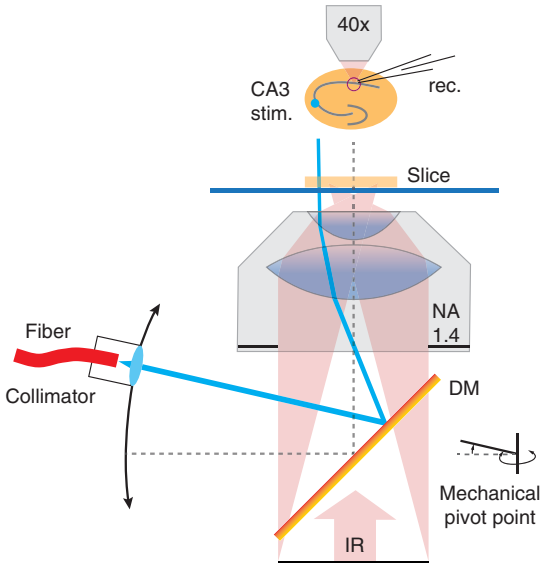


**FIGURE 3.** Beam steering through condenser for off-center light stimulation. An optical fiber collimator is mounted on a swing arm to point a laser beam at variable angles through the back aperture of a high-NA (numerical aperture) condenser (1.4 NA, oil immersion). Different angles correspond to 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.



## 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.

Berndt A, Schoenenberger P, Mattis J, Tye KM, Deisseroth K, Hegemann P, Oertner TG. 2011. High-efficiency channelrhodopsins for fast neuronal stimulation at low light levels. *Proc Natl Acad Sci* **108:** 7595–7600.

Berndt A, Lee SY, Ramakrishnan C, Deisseroth K. 2014. Structure-guided transformation of channelrhodopsin into a light-activated chloride channel. *Science* **344:** 420–424.

Boyden ES, Zhang F, Bamberg E, Nagel G, Deisseroth K. 2005. Millisecond-timescale, genetically targeted optical control of neural activity. *Nat Neurosci* **8:** 1263–1268.

Burger C, Gorbatyuk OS, Velardo MJ, Peden CS, Williams P, Zolotukhin S, Reier PJ, Mandel RJ, Muzyczka N. 2004. Recombinant AAV viral vectors pseudotyped with viral capsids from serotypes 1, 2, and 5 display differential efficiency and cell tropism after delivery to different regions of the central nervous system. *Mol Ther* **10:** 302–317.

De Roo M, Klauser P, Mendez P, Poglia L, Muller D. 2008. Activity-dependent PSD formation and stabilization of newly formed spines in hippocampal slice cultures. *Cereb Cortex* **18:** 151–161.

De Simoni A, Griesinger CB, Edwards FA. 2003. Development of rat CA1 neurones in acute versus organotypic slices: Role of experience in synaptic morphology and activity. *J Physiol* **550:** 135–147.

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.

Gogolla N, Galimberti I, DePaola V, Caroni P. 2006. Preparation of organotypic hippocampal slice cultures for long-term live imaging. *Nat Protoc* **1:** 1165–1171.

Govorunova EG, Sineshchekov OA, Janz R, Liu X, Spudich JL. 2015. Natural light-gated anion channels: A family of microbial rhodopsins for advanced optogenetics. *Science* **349:** 647–650.

Hochbaum DR, Zhao Y, Farhi SL, Klapoetke N, Werley CA, Kapoor V, Zou P, Kralj JM, Maclaurin D, Smedemark-Margulies N, et al. 2014. All-optical electrophysiology in mammalian neurons using engineered microbial rhodopsins. *Nat Methods* **11:** 825–833.

Holbro N, Grunditz A, Oertner TG. 2009. Differential distribution of endoplasmic reticulum controls metabotropic signaling and plasticity at hippocampal synapses. *Proc Natl Acad Sci* **106:** 15055–15060.

Klapoetke NC, Murata Y, Kim SS, Pulver SR, Birdsey-Benson A, Cho YK, Morimoto TK, Chuong AS, Carpenter EJ, Tian Z, et al. 2014. Independent optical excitation of distinct neural populations. *Nat Methods* **11:** 338–346.

Lin JY, Knutsen PM, Muller A, Kleinfeld D, Tsien RY. 2013. ReaChR: A red-shifted variant of channelrhodopsin enables deep transcranial optogenetic excitation. *Nat Neurosci* **16:** 1499–1508.

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.

Mattis J, Tye KM, Ferenczi EA, Ramakrishnan C, O'Shea DJ, Prakash R, Gunaydin LA, Hyun M, Fenno LE, Gradinaru V, et al. 2012. Principles



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

for applying optogenetic tools derived from direct comparative analysis of microbial opsins. *Nat Methods* **9:** 159–172.

Nagel G, Ollig D, Fuhrmann M, Kateriya S, Musti AM, Bamberg E, Hegemann P. 2002. Channelrhodopsin-1: A light-gated proton channel in green algae. *Science* **296:** 2395–2398.

Nagel G, Szellas T, Huhn W, Kateriya S, Adeishvili N, Berthold P, Ollig D, Hegemann P, Bamberg E. 2003. Channelrhodopsin-2, a directly light-gated cation-selective membrane channel. *Proc Natl Acad Sci* **100:** 13940–13945.

Nagel G, Brauner M, Liewald JF, Adeishvili N, Bamberg E, Gottschalk A. 2005. Light activation of channelrhodopsin-2 in excitable cells of *Caenorhabditis elegans* triggers rapid behavioral responses. *Curr Biol* **15:** 2279–2284.

Prigge M, Schneider F, Tsunoda SP, Shilyansky C, Wietek J, Deisseroth K, Hegemann P. 2012. Color-tuned channelrhodopsins for multiwavelength optogenetics. *J Biol Chem* **287:** 31804–31812.

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

Rose T, Schoenenberger P, Jezek K, Oertner TG. 2013. Developmental refinement of vesicle cycling at Schaffer collateral synapses. *Neuron* **77:** 1109–1121.

Schoenenberger P, Gerosa D, Oertner TG. 2009. Temporal control of immediate early gene induction by light. *PLoS One* **4:** e8185.

Stoppini L, Buchs PA, Muller D. 1991. A simple method for organotypic cultures of nervous tissue. *J Neurosci Methods* **37:** 173–182.

Thomas A, Kim DS, Fields RL, Chin H, Gainer H. 1998. Quantitative analysis of gene expression in organotypic slice-explant cultures by particle-mediated gene transfer. *J Neurosci Methods* **84:** 181–191.

Wiegert JS, Oertner TG. 2013. Long-term depression triggers the selective elimination of weakly integrated synapses. *Proc Natl Acad Sci* **110:** E4510–E4519.

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

Wiegert JS, Gee CE, Oertner TG. 2016b. Single-cell electroporation of neurons. *Cold Spring Harb Protoc* doi: 10.1101/pdb.prot094904.

Wietek J, Wiegert JS, Adeishvili N, Schneider F, Watanabe H, Tsunoda SP, Vogt A, Elstner M, Oertner TG, Hegemann P. 2014. Conversion of channelrhodopsin into a light-gated chloride channel. *Science* **344:** 409–412.

Wietek J, Beltramo R, Scanziani M, Hegemann P, Oertner TG, Wiegert SJ. 2015. An improved chloride-conducting channelrhodopsin for light-induced inhibition of neuronal activity in vivo. *Sci Reo* **5:** 14807.



# Stimulating Neurons with Heterologously Expressed Light-Gated Ion Channels

J. Simon Wiegert, Christine E. Gee and Thomas G. Oertner

*Cold Spring Harb Protoc;* doi: 10.1101/pdb.top089714