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Illuminating the Undergraduate Behavioral Neuroscience Laboratory: A Guide for the *in vivo* Application of Optogenetics in Mammalian Model Organisms

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Optogenetics is a technology that is growing rapidly in neuroscience, establishing itself as a fundamental investigative tool. As this tool is increasingly utilized across the neuroscience community and is one of the primary research techniques being presented at neuroscience conferences and in journals, we believe that it is important that this technology is introduced into the undergraduate neuroscience research laboratory. While there has been a significant body of work concentrated to deploy optogenetics in invertebrate model organisms, little to no work has focused on brining this technology to mammalian model organisms in undergraduate neuroscience laboratories. The establishment of *in vivo* optogenetics could provide for high-impact independent research projects for upper-level undergraduate students. Here we review the considerations for establishing *in vivo* optogenetics with the use of rodents in an undergraduate laboratory setting and provide some cost-saving guidelines to assist in making optogenetic technologies financially accessible. We discuss opsin selection, cell-specific opsin expression strategies, species selection, experimental design, selection of light delivery systems, and the construction of implantable optical fibers for the application of *in vivo* optogenetics in rodents.

Key words: adeno-associated virus (AAV); Arduino™ microcontroller boards; behavioral assays; budget; channelrhodopsin-2 (ChR2); Cre-driver transgenic lines; halorhodopsin (NpHR); in vivo optogenetics; lasers; light emitting diodes (LEDs); mammals; opsin; optical fiber; undergraduate research

The use of optogenetics for the manipulation of neural circuits is now a widely-used tool employed in neuroscience to study the functional relationship between circuits and behavior. Most commonly, optogenetics involves the application of light to genetically identified neurons expressing light-sensitive ion channel or pump proteins for the purpose of driving or silencing the activity of those cells. During exposure to a specific wavelength of light these light-sensitive proteins, or opsins, transport ions across the lipid membranes of cells in which they are genetically expressed (Boyden et al., 2005). Optogenetic tools have been employed in the study of diverse neurological and psychiatric disease models, including Parkinson's disease (Kravitz et al., 2010), epilepsy (Paz and Huguenard, 2015), drug addiction (Cao et al., 2011), and mood disorders (McDevitt et al., 2014) to name a few. Further, these tools enable the functional assessment of specific neural circuits, revealing how different populations of neurons contribute to synaptic plasticity and behavior (Aravanis et al., 2007; Mathur et al., 2013; Zhang et al., 2010).

Optogenetics is growing rapidly and, following its establishment as a diverse and potent investigative tool for neuroscientists, we believe that it would be advantageous for this technology to be introduced to the undergraduate neuroscience laboratory. There has been a significant body of work concentrated to deploy optogenetics into undergraduate neuroscience programs, focusing primarily on the use of invertebrate model organisms (especially Drosophila; see Hornstein et al., 2009; Pulver et al., 2011; Pulver and Berni, 2012; Titlow et al., 2015). However, there has been very little focus, if any at all, to make optogenetics in mammalian model organisms (particularly rodents) accessible to undergraduates. Numerous undergraduate research programs are equipped to investigate rodents and, therefore, there is a need for guidelines and resources to help those undergraduate programs that want to establish optogenetics in mammals. Establishing optogenetics in mammalian model organisms can be expensive and confusing, making it initially inaccessible and daunting to some undergraduate research programs. Here we review the general considerations required for establishing *in vivo* optogenetics in mammals in the undergraduate research laboratory (see Table 1) and provide some cost-saving guidelines to assist in making optogenetic tools financially accessible to undergraduate research programs with small budgets.

Major Considerations for Establishing *in vivo* Optogenetics
1. Opsin selection
2. Cell-specific opsin expression strategy
3. Species and behavioral paradigm selection
4. Experimental design and controls
5. Light sources
6. Construction of implantable optical fibers

Table 1. Important considerations for establishing *in vivo* optogenetics in the undergraduate research laboratory.

OPSIN SELECTION

Last year was the 10-year anniversary of the Boyden et al., (2005) paper that introduced the use of channelrhodopsin in mammalian neurons (Adamantidis et al., 2015). In this paper, the team led by Karl Deisseroth expressed the light-

sensitive microbial protein, channelrhodopsin-2 (ChR2), in mammalian neurons and showed that these neurons could be activated in a temporally precise and reliable manner when exposed to pulses of blue light. Significant expansions of the optogenetic toolbox over the last decade have produced a number of novel opsin variants providing greater flexibility in experimental design and more refined manipulations. Systematic comparative reviews of the currently available opsins are available and serve as excellent resources for the selection of opsins for *in vivo* optogenetics (Mattis et al., 2012; Tye and Deisseroth, 2012; Adamantidis et al., 2014;).

With the undergraduate research laboratory in mind, the currently available opsins likely to be utilized for *in vivo* application are (ChR2) and halorhodopsin (NpHR; see Figure 1). ChR2 is a light-activated cation channel that fluxes cations into the cell and, therefore, depolarizes neurons with millisecond precision. Thus, ChR2 drives precisely timed action potentials (Yizhar et al., 2011). Further, ChR2 is able to transduce trains of millisecond-duration light flashes into defined action potential spike trains up to 30-50 Hz and is maximally activated by blue light at 470nm (Boyden et al., 2005; Zhang et al., 2006). Conversely, NpHR, a chloride ion pump maximally activated by yellow light at 580nm, pumps chloride ions into the cell to hyperpolarize and, therefore, inhibit neuronal firing (Zhang et al., 2011). Because of the sufficient spectral separation of light required to activate these opsins, ChR2 and NpHR can be simultaneously expressed in the same neuron to enable bipotential optogenetic control of neural activity (Zhang et al., 2011).

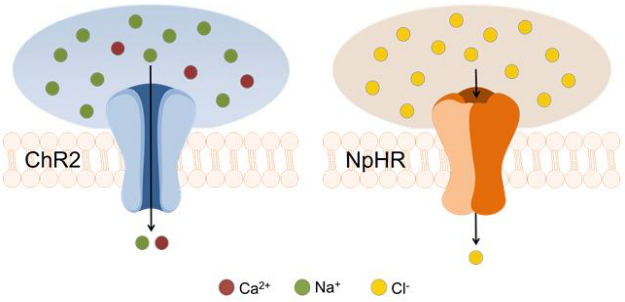

Figure 1. Two major classes of opsins commonly used in *in vivo* optogenetic experiments. Upon expression in mammalian neurons, (1) channelrhodopsin-2 (ChR2), fluxes cations into the cell in response to blue light (470nm), giving rise to neuronal depolarization and action potential generation; (2) halorhodopsin (NpHR), pumps chloride into the cell in response to yellow light (580nm), giving rise to neuronal hyperpolarization and, therefore, suppression of native action potential generation.

CELL-SPECIFIC OPSIN EXPRESSION STRATEGIES

Opsin genes can be selectively expressed in specific classes of genetically defined neurons in the mammalian brain using a variety of developed targeted expression approaches (Zhang et al., 2010). One of the more widely used and commercially available targeted expression systems involves Cre-recombinase (Cre)-lox technology combined with an adeno-associated virus (AAV) (Madisen et al., 2012). Although under normal conditions AAV vectors transduce neurons ubiquitously and, therefore, express genes in all neurons that are transduced, cell-specific promoters can be used to effectively restrict gene expression to specific, genetically defined neurons. In the Cre-dependent AAV expression systems, AAV expression vectors carry transgene cassettes with the opsin gene of choice and only expresses genes in the proper orientation in the presence of Cre. This system capitalizes on the numerous cell-specific Cre-driver transgenic mouse lines that are rapidly increasing in number through the availability of the Allen Brain Institute for Brain Science, the Gene Expression Nervous System Atlas (GENSAT) project, Jackson Laboratory and other individual investigators, with over 280 genetically defined classes of neurons targetable (Gong et al., 2007; Gerfen et al., 2013).

Essentially, if one wishes to express ChR2 in a specific class of neurons, expression of ChR2 in other classes of neurons in the same brain nucleus is unwanted. One such method allowing for high expression levels while simultaneously minimizing expression in the non-targeted surrounding cells is the double-floxed inverse open reading frame (DIO) strategy devised by Karl Deisseroth's group (see Figure 2; Livet et al., 2007; Sohal et al., 2009). The DIO strategy mediates cell-specific opsin expression through the use of a transgene that is initially inverted and, therefore, is an inactive protein upon expression. Expression of the transgene is accomplished using two pairs of incompatible *lox* sixes that flank the transgene; the presence of Cre in the desired class of neuron facilitates the serial recombination between the *lox* sites, reorienting the transgene into the correct orientation allowing for successful transgene expression in the Cre expressing cells only. Put simply, AAV vectors with *lox* sites that flank the transgene (i.e., ChR2) will only successfully express the transgene in cells that express Cre. For example, transgenic lines that express Cre under the choline acetyltransferase promoter will allow for transgene (ChR2) expression specific to acetylcholine neurons.

With smaller undergraduate research budgets in mind, purchasing commercially available Cre-driver transgenic mouse lines can be costly. However, this is an initial investment as breeding pairs can be established and continued at the expense of institutional husbandry. Alternatively, it is not uncommon for undergraduate programs to conduct collaborative projects with local graduate research institutions. In these cases, the necessary transgenic mouse line can be transferred to the undergraduate research facilities following the required institutional animal transfer procedures. The use of transgenic mouse lines requires the genotyping of each individual experimental subject to ensure the subjects are positive for the Cre-driver. Tissue samples can be sent out to biotechnology companies for genotyping or a more economic alternative is to genotype in the research laboratory using basic molecular biology techniques and commercially available DNA extraction and polymerase chain reaction kits.

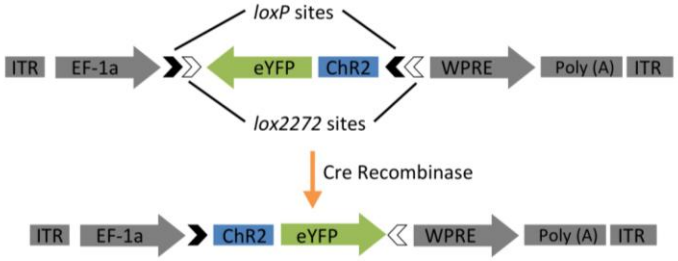

Figure 2. Schematic of the double-floxed inverse open reading frame (DIO), Cre recombinase-dependent adeno associated virus (AAV) vector system expressing channelrhodopsin-2 (ChR2) and the enhanced yellow fluorescent protein (eYPF) under the control of the EF-1a promoter. Cre-recombinase is expressed in a given population of neurons under the control of a gene specific promoter. The AAV construct contains a double l*ox*-flanked (floxed) inverted open reading frame of ChR2 and eYFP, thus the eYFP-ChR2 gene starts in an inverted, inactive orientation. In cells where Cre-recombinase is expressed, Cre-recombinase mediates a serial recombination between the *lox* sites resulting in the inversion of the transgene into the active, fixed orientation. This allows for transgene expression in the Cre-recombinase expressing cells only. [ITR, inverted terminal repeat; WPRE, woodchuck hepatitis virus posttranscriptional regulatory element]

High titer AAV-based vectors can be obtained through a number of virus production facilities (e.g., Penn Vector Core, University of Pennsylvania, Philadelphia, Pennsylvania; Viral Vector Core; and University of North Carolina, Chapel Hill, North Carolina). These facilities provide materials to academic and other non-profit organizations under a Material Transfer Agreement (MTA). As such, undergraduate institutions will be required to complete an MTA with a small one-time fee in order to purchase AAV vectors from these virus production facilities (0.1ml aliquot of AAV costs ~$250, enough for approximately 30 bilateral injections). AAV vectors can be safely used in the undergraduate research laboratory as AAV vectors are classified as Biosafety Level 1 (BSL 1) agents. Moreover, no specific safety precautions are needed other than standard surgical personal protective equipment (Dismuke et al., 2013). Our research group delivers AAV through stereotaxic injection according to the procedures outlined by Zhang et al. (2010). Before the start of any experimentation all procedures must be approved by the relevant institutional animal care and use committees. In general for the Cre-dependent AAV expression system in rodents, opsin gene expression reaches functional levels within 2-3 weeks following AAV transfection (Zhang et al., 2010). In addition to injecting AAV expressing opsin proteins into experimental rats or mice, control animals injected with AAV expressing control fluorescent proteins should be used to ensure that any behavioral outcomes observed are not due to AAV transfection, or simple light delivery, to the brain. In summary, the use of Cre-dependent AAV vector systems with a DIO scheme coupled with Cre-driver transgenic animal lines to achieve cell-specific opsin expression are recommended. To ensure that cell-specific opsin expression is achieved, post-hoc immunohistochemistry for proteins expressed in the specific targeted cells of interest should be performed.

SPECIES AND BEHAVIORAL PARADIGM SELECTION

Mice make excellent subjects for *in vivo* optogenetic manipulations combined with behavioral paradigms; there are numerous transgenic mouse lines for cell-specific opsin expression. Additionally, mice carry relatively little weight, which make them unlikely to damage or destroy costly optical fiber patch cables. Rats are generally the preferred experimental subjects in the undergraduate neuroscience laboratory, but the application of optogenetic techniques in the rat has not been as accessible as in the mouse due to relative genetic intractability. However, there are a few recently available tools and techniques suited for optogenetics in rats (Zalocusky and Deisseroth, 2013). Recently, Cre-driver rat lines have emerged (Witten et al., 2011); one of these transgenic lines expresses Cre under the tyrosine hydroxylase promoter allowing for dopamine neuron-specific and norepinephrine neuron-specific targeting. Another of these transgenic lines expresses Cre under the choline acetyltransferase promoter, allowing for acetylcholine neuron-specific targeting (Witten et al., 2011).

Diverse animal behavior rigs can be outfitted for use with optogenetic equipment, including the forced swim test (Warden et al., 2012), operant behavior paradigms (Witten et al., 2011) and measured behavior in open fields or mazes, which can be analyzed with commercially available software for video recording and real-time tracking (Kravitz et al., 2010). Generally, any traditional behavioral paradigm already available to your undergraduate research program may be utilized, including the light-dark exploration test, social interaction test, novel object recognition test, accelerating rotarod test, pre-pulse inhibition, and various measures of motor function. As an example, an early application of *in vivo* optogenetics in the field of addiction demonstrated that the activation of dopamine neurons in the ventral tegmental area utilizing ChR2 in freely behaving mice was capable of eliciting conditioned placement preference (Tsai et al., 2009). Essential, yet simple, experiments like these can be easily incorporated into neuroscience undergraduate laboratory courses to help introduce this rapidly growing technology to undergraduate students.

EXPERIMENTAL DESIGN

It is important to understand that control experiments are required to ensure that the observed effects are due to the recruitment of the specific opsins in the particular neuronal cell type. First, the expression alone of any foreign protein in the brain can cause alterations in the function of host cells, consequently leading to possible alterations in behavior; opsins are no exception to this confound. As such, fluorescent proteins (XFPs) are most often utilized as control proteins, since opsin are almost always co-expressed with XFPs (Yizhar et al., 2011). Second, light-on and light-off paradigms intrinsically allow for within subject designs (Zaocusky and Deisseroth, 2013). Third,

prolonged light delivery can cause an increase in temperature in the brain, conceivably altering changes in brain physiology and behavior (Yizhar et al., 2011). Finally, the sensory perception of the light may also cause alterations in behavior. As such, it is important to include an experimental cohort in which no opsin is expressed but all other manipulations are performed, including viral transduction (with XFP expression), optical fiber implantation and light delivery (Yizhar et al., 2011).

LIGHT DELIVERY SYSTEMS

In a typical optogenetic system arrangement, a computer or stand-alone light source modulator is used to control the light source, which is connected to an optical fiber patch cable. The patch cable connects to the optical fiber stub that is chronically implanted into the animal. Generally, optical commutators are used to allow the animal to freely rotate in the chosen behavioral apparatus without damaging the patch cables. While lasers have been the most popular light source for the application of optogenetics, light emitting diodes (LEDs) are gaining attention, as they are cheaper and smaller (Grossman et al., 2010). It is important, however, that when using LEDs as the light source for optogenetics that you utilize optical fibers with a high numerical aperture (NA; optical fiber with a 0.66 NA is recommended) to increase the efficiency of light transmittance coupled from the light source into the optical fiber. One advantage of utilizing laser light sources is they have a higher light output power than LEDs and, as such, the difficulty of coupling light from the source into the optical fiber with high efficiency is not as much of a concern. As such, choosing between LEDs and laser light sources for the application of optogenetics is not always a straightforward decision and consider the pros and cons of each. With smaller undergraduate research budgets in concern, purchasing light sources will be the largest investment for the establishment of optogenetics in the research laboratory (~$2,000-3,500); our research group currently utilizes an LED light source (see Recommended Reagents and Equipment in Supplementary Tables). In order to transmit light from the source to the implantable optical fiber stubs, it is necessary to purchase patch cables (or construct them in the research laboratory according to the protocol of Sparta et al., 2011).

ChR2 is able to transduce trains of millisecond-duration light flashes into defined action potential spike trains up to a frequency of 30-50 Hz (Boyden et al., 2005; Zhang et al., 2006). To generate light pulses at a desired frequency, pulse train generators that output TTL signals to the light source can be purchased. However, they are relatively expensive (~$1,500). Another popular and inexpensive strategy for controlling the output of your light source is an Arduino™ microcontroller board (Inagaki et al., 2014). These microcontroller boards are small programmable boards that simply plug into the computer over USB connections and are easily programmed to deliver digital TTL pulses to your light source. Arduino™ microcontroller boards are incredibly easy to use with the available open source Arduino™ software and tutorials online even without any previous computer programming instruction or background. Additionally, they can be used to simultaneously control behavioral apparati and stimuli just as easily.

CONSTRUCTION OF IMPLANTABLE OPTICAL FIBERS

The majority of investigations that combine behavioral tasks with *in vivo* optogenetic manipulations are generally based on experiments that utilize guide cannulae, through which the optical fiber is inserted directly prior to the start of behavioral testing (Kravitz et al., 2010; Tye et al., 2011). While popular, limitations to this method exist that could be costly to undergraduate research laboratories. Particularly, repeated acute implantation of these optical fibers over several sessions can cause the optical fiber to break inside the guide cannula or cause tissue damage. Conversely, implantable, permanently indwelling optical fibers in brain tissue that are affixed to the skull can be used. These implantable optical fibers reduce cost by reducing the likelihood of tissue and optical fiber damage (decreasing animal numbers) and can be easily constructed in the laboratory (Sparta et al., 2011).

Implantable optical fibers are commercially available, such as the optogenetic stimulation system fiber stub implants offered by Plexon Inc. (http://www.plexon.com/). However, these systems are expensive for smaller undergraduate research budgets (available from ~$35-45 per implantable optical fiber) and may not meet the specific requirements for all optogenetic experiments. As such, a popular and affordable approach is to construct implantable optical fibers in the research laboratory. When following the procedures outlined by Sparta et al. (2011), implantable optical fibers for ~$6-9 per implant can easily be manufactured. Although the techniques outlined in the protocol are for the construction of implantable optical fibers for mice, they can be easily adapted for rats by increasing the diameter of the optical fibers and ferrules used. We use 200-µm multimode optical fibers with a NA of 0.66 for the use with an LED light source (see *Recommended Reagents and Equipment in Supplementary Tables*). Further, if you choose to do so, the procedures outline how to construct patch cables, further helping to save costs. Note, it is important that the two optical fibers at the coupling junction (patch cable-to-optical fiber stub implant) match in diameter and NA in order to minimize the loss of light at the ferrule interface.

For all experiments, the light output of the implanted optical fibers is measured before and after experimentation. For neurons expressing ChR2 at typical experimental expression levels, light power densities of 2-5 mW/mm2 with a wavelength from 465-475 nm is sufficient to stimulate an action potential (Boyden et al., 2005). The predicted light power density with distance from optic fiber tip in mammalian brain tissue from constructed optic fiber implants can be calculated using open source optogenetic software based on direct measurements in mammalian brain tissue (http://www.optogenetic.org/calc). When

comparing the light output of optical fibers before and after implantation, data from animals that show a decrease in light output greater than 30% should be excluded from data analysis.

AN OPTOGENTIC BASED UNDERGRADUATE RESEARCH PROJECT

Here we outlined the considerations for establishing *in vivo* optogenetics with the use of mammalian organisms in an undergraduate laboratory setting. We constructed this guide through the experiences of establishing *in vivo* optogenetics in the Undergraduate Neuroscience Program at St. Mary's College of Maryland, Maryland, USA, throughout the course of an undergraduate senior research project. This project focused on optogenetically targeting a specific class of interneurons in the striatum (primary input nucleus of the basal ganglia) and identifying their effects on locomotor activity in a Parkinson's disease mouse model. Specifically, we virally expressed ChR2 in these striatal interneurons and measured locomotor activity during their activation via blue light stimulation (see Kravitz et al., 2010 for a similar study that optogenetically targets specific classes of striatal neurons and investigates their effects on locomotor activity). To measure locomotor activity we utilized the open field and rotarod behavioral paradigms. It is worth mentioning that these techniques were established by a fourth-year undergraduate into a behavioral neuroscience laboratory already equipped for stereotaxic survival surgery on rodents and with a working Institutional Animal Care and Use Committee familiar with the use of mammals for teaching and research purposes.

The initial Establishment of *in vivo* optogenetics into an undergraduate neuroscience research program may seem daunting; however, if you utilize the resources we outlined and start with small pilot experiments, it can be achieved. While the upfront costs may be initially expensive to smaller undergraduate research programs, once established, *in vivo* optogenetics can be relatively inexpensive. We have found establishing *in vivo* optogenetics to be beneficial to our students, providing significant exposure to this rapidly growing and potent investigative tool. As such, we confidently feel our students are prepared to conduct optogenetics based research in mammalian model organisms in their future scientific endeavors, setting them apart from other neuroscience undergraduates when applying for research positions and to graduate programs.

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