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Citation: Chow, Brian Y., Amy S. Chuong, Nathan C. Klapoetke, and Edward S. Boyden. "Synthetic Physiology: Strategies for Adapting Tools from Nature for Genetically Targeted Control of Fast Biological Processes." Methods in Enzymology, Synthetic Biology, Part A., Volume 497, Pages 2-662, (2011).

As Published: http://dx.doi.org/10.1016/b978-0-12-385075-1.00018-4

Publisher: Elsevier

Persistent URL: http://hdl.handle.net/1721.1/103538

Version: Author's final manuscript: final author's manuscript post peer review, without publisher's formatting or copy editing

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Author Manuscript

Methods Enzymol. Author manuscript; available in PMC 2013 January 21.

Published in final edited form as:

Methods Enzymol. 2011 ; 497: 425–443. doi:10.1016/B978-0-12-385075-1.00018-4.

Synthetic Physiology: Strategies for Adapting Tools from Nature for Genetically-Targeted Control of Fast Biological Processes

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Abstract

The life and operation of cells involve many physiological processes that take place over fast timescales of milliseconds to minutes. Genetically-encoded technologies for driving or suppressing specific fast physiological processes in intact cells, perhaps embedded within intact tissues in living organisms, are critical for the ability to understand how these physiological processes contribute to emergent cellular and organismal functions and behaviors. Such “synthetic physiology” tools are often incredibly complex molecular machines, in part because they must operate at high speeds, without causing side effects. We here explore how synthetic physiology molecules can be identified and deployed in cells, and how the physiology of these molecules in cellular contexts can be assessed and optimized. For concreteness, we discuss these methods in the context of the “optogenetic” light-gated ion channels and pumps that we have developed over the past few years as synthetic physiology tools, and widely disseminated for use in neuroscience for probing the role of specific brain cell types in neural computations, behaviors, and pathologies. We anticipate that some of the insights revealed here may be of general value for the field of synthetic physiology, as they raise issues that will be of importance for the development and use of high-performance, high-speed, side-effect free physiological control tools, in heterologous expression systems.

I. Introduction

The life and operation of cells involve many physiological processes that take place over fast timescales of milliseconds to minutes. These physiological changes include variations in cell membrane potential and cellular ionic composition, changes in protein conformation, post-translational modification, localization, and interaction, and other biochemical and mechanical processes, all occurring at length scales ranging from nanometers to meters. Technologies for driving or suppressing specific fast physiological processes in intact cells, perhaps embedded within intact tissues in living organisms, are critical for the ability to understand how those physiological processes contribute to emergent cellular and organismal functions and behaviors. For example, the ability to drive a specific physiological process can reveal precisely which functions that process is sufficient to initiate or sustain, whereas the ability to suppress a specific physiological process can reveal the set of functions for which the process is necessary. Such precision physiological control technologies may, of course, also serve therapeutic purposes if they offer the ability to remedy a pathway thrown into disarray in a disease context, ideally while leaving other pathways unperturbed.

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A diversity of molecular tools have been developed that allow the precision control of physiological processes – including high-specificity pharmacological compounds, caged chemicals that can be activated by pulses of light, and tools whose physiological impact is unleashed by administration of heat or radiofrequency energy. This ongoing effort has led to a number of physiological control tools that are partly or entirely genetically encoded, and therefore easy to use in genetic model organisms in conjunction with commonly-available transgenic strategies, e.g. viruses for delivery to specific mammalian cells embedded within intact organ systems. One might call the set of capabilities opened up by these tools "synthetic physiology," because these tools enable a synthetic approach to studying physiological pathways, with an emphasis on perturbation of specific pathways, to see what their influence is on other pathways. Many of the synthetic physiology tools in widespread use have come to be known as `optogenetic,' because they enable specific physiological processes to be controlled by light, and thus enable temporally and spatially precise control of physiology with microscopes, lasers, and other common laboratory optical equipment, often without the need for exogenous chemical delivery (important for use *in vivo*). Such light-driven tools, or prototype of tools, exist for applications including driving of protein-protein interactions (Levskaya, Weiner et al. 2009; Yazawa, Sadaghiani et al. 2009; Kennedy, Hughes et al. 2010), enzyme activity (Wu, Frey et al. 2009), intracellular signaling (Schroder-Lang, Schwarzel et al. 2007), and many other fast changes (Moglich and Moffat). A widely-used set of optogenetic tools are the microbial rhodopsins, molecules that respond to light by translocating ions from one side of the plasma membrane to the other, thus enabling electrical activation or silencing of electrically excitable cells such as neurons, in response to pulses of light (Boyden, Zhang et al. 2005; Han and Boyden 2007; Zhang, Wang et al. 2007; Gradinaru, Thompson et al. 2008; Chow, Han et al. 2010; Gradinaru, Zhang et al. 2010). For example, channelrhodopsins, microbial opsins from algae, admit cations into cells in response to light, depolarizing the cells; halorhodopsins, opsins from archaea, pump in chloride in response to light, resulting in cellular hyperpolarization; archaerhodopsins and bacteriorhodopsins, also isolated from archaea, pump out protons, also resulting in cellular hyperpolarization. In the mammalian nervous system, these molecules do not require any exogenous chemical supplementation for their operation, and thus can be treated as fully genetically-encoded. The hyperpolarization opsins are used to enable optical silencing of genetically-targeted neurons in order to see what neural dynamics, behaviors, and pathologies they are necessary for, whereas the depolarizing opsins are used to drive neural activity in genetically-targeted neurons, to determine how downstream neural computations and behaviors causally result. Both sets of tools are in widespread use for investigating the roles that specific cells play within the nervous systems of species ranging from *C. elegans* to non-human primate (see the following references for some early papers in the field, (Li, Gutierrez et al. 2005; Nagel, Brauner et al. 2005; Bi, Cui et al. 2006; Ishizuka, Kakuda et al. 2006; Schroll, Riemensperger et al. 2006; Adamantidis, Zhang et al. 2007; Aravanis, Wang et al. 2007; Arenkiel, Peca et al. 2007; Farah, Reutsky et al. 2007; Petreanu, Huber et al. 2007; Wang, Peca et al. 2007; Zhang, Ge et al. 2007; Zhang and Oertner 2007; Alilain, Li et al. 2008; Atasoy, Aponte et al. 2008; Douglass, Kraves et al. 2008; Huber, Petreanu et al. 2008; Lagali, Balya et al. 2008; Liewald, Brauner et al. 2008; Mahoney, Luo et al. 2008; Toni, Laplagne et al. 2008; Zhang, Holbro et al. 2008; Han, Qian et al. 2009)).

Although one of the goals of synthetic biology is to be able to regard such tools as `black box parts' (Endy and Brent 2001; Canton, Labno et al. 2008; Carr and Church 2009), whose internal workings can be hidden beneath an abstraction layer, the genetically-encoded tools in use for synthetic physiology are incredibly complex machines, in part because they must operate at high speeds. To be useful, their inner workings must be sophisticated enough to enable these tools to accomplish their precision functions when activated by an external stimulus such as light, while avoiding undesired side effects. Understanding and engineering these tools requires thinking and working at the level of protein structure and dynamics,

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which means that the ability to systematically engineer synthetic physiology tools is primitive, compared to, say, DNA synthesis or gene engineering, where design principles emerge from systematic application of straightforward considerations of the structure and chemistry of nucleic acids.

As just one example, halorhodopsins are just a few hundred amino acids long, yet these molecules contain an anchor site for a vitamin A-derived chromophore, and upon illumination undergo structural rearrangements through at least seven coupled photointermediates during the process of translocating a chloride ion from one side of the cell membrane to the other. Halorhodopsins also possess alternate photocycles that involve shifts in the spectrum of light responsivity, as well as secondary transport of protons (Hegemann, Oesterbelt et al. 1985; Oesterhelt, Hegemann et al. 1985; Bamberg, Tittor et al. 1993; Han and Boyden 2007). A great many individual amino acids in halorhodopsins, when mutated, impair or otherwise alter opsin function, implying that the operation of these proteins relies upon a great many of the residues remaining intact for structural or protein dynamics reasons (Rudiger, Haupts et al. 1995; Otomo 1996; Rudiger and Oesterhelt 1997; Sato, Kikukawa et al. 2003; Sato, Kikukawa et al. 2003). As one might therefore guess, methodologies for the discovery, characterization, and optimization of synthetic physiology parts represent at this time something of an art form.

The purpose of this article is to present how synthetic physiology molecules can be identified and implemented (Section II, below), how these molecules can be expressed in cells (Section III, below), and how the physiology of these molecules in cellular contexts can be assessed (Section IV, below). For concreteness, we discuss these methods in the context of the "optogenetic" light-gated ion channels and pumps that we have worked on, and widely disseminated, since 2004. During this time we have characterized dozens of gene products of microbial rhodopsin sequence homologs from every kingdom of life, and we have begun to understand the principles governing how to create, express, and analyze them. However, we anticipate that many of the insights revealed here may be of general use for the field of synthetic physiology, as they raise issues that will be of importance for the development and use of high-performance, high-speed, side-effect free physiological control tools. Because of the scope of methodologies involved in the research, the information here provides the reasoning behind our current best practices, as opposed to the laying out of step-by-step protocols. Detailed protocols will be posted as white papers on our website (http://syntheticneurobiology.org) and updated regularly; the goal for this paper is to lay out the principles that guide these protocols.

Synthetic physiology tools, at this point in protein engineering history, heavily rely upon naturally-occuring genetically-encoded proteins as the effectors that perform the actual modulation of physiological functions, although one might imagine that in the future entirely artificial designs might be realized. In the case of microbial opsins, which transport ions into or out of cells in response to light, the light sensor is built into the ion translocation machinery, embedded within the middle of the 7-transmembrane domain protein. (In most other classes of optogenetic synthetic physiology tool, genetically-encoded light sensors are fused to genetically-encoded effectors so as to couple the conformational change of the light sensor under illumination to the physiological function downstream, as reviewed in (Moglich and Moffat).) To date, many opsins have been derived from organisms such as archaea, algae, bacteria, and fungi: there is considerable molecular diversity in such organisms, which provides vast genetically encoded wealth from which one can re-purpose proteins as novel molecular tools or building blocks of of tools, and the proliferation of publically available sequence information, coupled to the rapidly decreasing costs of *de novo* gene synthesis (Carlson 2003; Carr and Church 2009), makes it increasingly easier to mine molecular wealth. Thus, the pipeline for developing opsins as tools begins with

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isolation of gene sequences from genomes, followed by *de novo* gene synthesis, then mutagenesis and/or appending of useful sequences for visualization and improvement of trafficking of opsins, and then finally embedding of the sequence in a transgenic vector (e.g., a viral vector) for heterologous expression in the cells of a target organism.

II. Molecular Design and Construction

Synthetic physiology tools in the opsin space have largely been identified from genomic databases by searching for proteins with similar amino acid sequence homology to previously characterized opsins. Microbial opsins were first discovered by biologists around four decades ago, and many members of this class have been identified at a genomic level over the intervening time, although only a small subset of these molecules have been characterized at a physiological level. *De novo* gene synthesis has proven important for rapid construction of opsin DNA from sequences derived from genomic and transcriptomic databases (Han and Boyden 2007; Chow, Han et al. 2010). One can obtain a gene that is codon-optimized to the target organism (Richardson, Nunley et al. ; Wu, Bashir-Bello et al. 2006; Welch, Govindarajan et al. 2009), important for proper protein expression in cells of the target organism, within a few days to weeks of sequence identification, from a gene synthesis vendor. Codon optimization is very useful for expression of these genes, which are isolated from algae, bacteria, and other non-animal species, in heterologous systems (e.g., animal cells in an organism of interest). During *de novo* gene synthesis, it is possible to eliminate restriction sites within the gene to ease later molecular cloning steps, so that they can be easily engineered to facilitate opsin function, e.g. by enabling fusion of a fluorescent protein tag to the molecule, concatenation of trafficking sequences to the molecule, or addition of a cell type-specific promoter to the gene to delimit the expression to specific cells within the target organism.

After gene synthesis, the next step is to alter the gene as needed, or to append extra sequences, to optimize its function towards a directed physiological control goal. A few opsin crystal structures have been obtained (e.g., (Luecke, Schobert et al. 1999; Luecke, Schobert et al. 1999; Kolbe, Besir et al. 2000; Luecke, Schobert et al. 2001; Enami, Yoshimura et al. 2006; Yoshimura and Kouyama 2008)), and decades of studies have been performed in which specific residues within opsins were mutated, followed by spectroscopic or physiological characterization of the resultant mutated opsins (e.g., (Hackett, Stern et al. 1987; Mogi, Stern et al. 1987; Mogi, Stern et al. 1988; Marinetti, Subramaniam et al. 1989; Mogi, Marti et al. 1989; Mogi, Stern et al. 1989; Otto, Marti et al. 1989; Stern and Khorana 1989; Gilles-Gonzalez, Engelman et al. 1991; Marti, Otto et al. 1991; Subramaniam, Greenhalgh et al. 1992; Greenhalgh, Farrens et al. 1993)). These datasets have proven influential in guiding the strategic engineering of these molecules through site-directed mutagenesis (Berndt, Yizhar et al. 2009; Lin, Lin et al. 2009; Wang, Sugiyama et al. 2009; Chow, Han et al. 2010; Gunaydin, Yizhar et al. 2010), enabling molecules with improved trafficking, or faster or slower kinetics, to be created. In part because many of these opsins are coming online at a rapid rate, antibodies for localizing them in an immunocytochemical fashion are not commonly available. Thus, tagging the proteins with a fluorophore, or with a small epitope to which antibodies already exist, is helpful in order to determine efficiently which cells within the target organism are expressing the opsin. In addition, such tagging yields critical information about the membrane trafficking and localization of opsins within cells – indeed, fluorophore localization to the plasma membrane of opsin-fluorophore fusions has been used to predict photocurrent magnitude, as measured through electrophysiology characterization (Wang, Sugiyama et al. 2009; Chow, Han et al. 2010). We have previously reported a method for quantifying membrane localized proteins in neurons (Chow, Han et al. 2010), based on a Gaussian-blur-based technique developed for the same purpose in HEK293 cells (Wang, Sugiyama et al. 2009). Of course, this method is

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useful for quantifying protein localization in the cells, but it does not provide information on whether the protein is properly folded and functional, within the membrane. Ultimately it is the number of proteins in the membrane that are functional which determines their overall efficacy in physiological control. Since these molecules, when expressed in neurons or other animal cells, are often in a very different lipid environment than the one they evolved to function in, even a properly folded molecule in a lipid membrane may not be fully functional. As a concrete example, photocurrent enhancement of an opsin by appending the flanking sequences of the KiR2.1 protein (as done in (Gradinaru, Zhang et al. 2010)) boosts the membrane expression of opsins as observed through microscopy, but may boost the photocurrent even more than might be expected from the cellular appearance alone; this appearance-current discrepancy may vary from opsin to opsin. Indeed, KiR2.1 sequences may even decrease overall cellular expression for some opsins, even as it might be increasing the amount of properly folded membrane-embedded protein that is functional. Thus, quantitative confocal microscopy must be supplemented by a functional, physiological assay. This theme, that there are few proxies for function in the assessment of synthetic physiology tools, is partly why they are hard to find, engineer, and assess.

It is important to realize that the complexity of these molecules means that even an innocuous change like creating a fusion protein between an opsin and a fluorophore may modulate the function of the opsin. For example, an observation that has been made by several laboratories and requires further investigation is that fluorophore fusions with a target molecule can alter performance of the target molecule, altering viral titer for example, important when viral delivery is the preferred route for transgenically engineering the target organism (Weber, Bartsch et al. 2008). Appending different fluorophores (e.g., EGFP vs. mCherry vs. ECFP) to an opsin can result in different appearances (e.g., due to mCherry's greater tendency to aggregate than EGFP or ECFP) and potentially different levels of current, for a given cell type. In the event that fusion of a fluorophore to a given opsin is undesirable, alternatives exist to directly fusing fluorophores to opsins, while still enabling identification of cells expressing the opsin, including interposing IRES (internal ribosome entry sites) and 2A sequences (`self-cleaving' linkers first identified in foot-and-mouth-disease virus) in between opsins and fluorophores. Protein expression levels for the gene that appears after the IRES is often a small fraction of that of the gene before the IRES (Mizuguchi, Xu et al. 2000; Hennecke, Kwissa et al. 2001; Yu, Zhan et al. 2003; Osti, Marras et al. 2006). 2A sequences in principle yield highly stoichiometric amounts of translated protein, but in reality, different functional levels may be observed for the pre- and post-2A proteins, due to alterations in protein trafficking or function that result from the residual amino acids of the 2A sequence left behind after protein translation (Han, Qian et al. 2009; Tang, Ehrlich et al. 2009).

The use of trafficking sequences, export motifs, and other signal sequences, both natural and designed, is useful for improving the heterologous expression of opsins in the cells of target organisms, since the function of opsins in neurons is primarily achieved when opsins express on the plasma membrane. Practically all opsins come from organisms whose membrane structure and overall cellular architecture is different from neurons. For example, *N. pharaonis* halorhodopsin photocurrents can be enhanced several fold in mammalian cells by appending the N- and C-terminal sequences of the human KiR2.1 potassium channel protein, which are responsible for endoplasmic reticulum-export and Golgi-export (although, see alternative explanations of the role that these KiR2.1 sequences play in boosting cellular expression, above) (Ma, Zerangue et al. 2001; Stockklausner and Klocker 2003; Hofherr, Fakler et al. 2005; Gradinaru, Zhang et al.). The enhancement offered by a given exogenous trafficking sequence is opsin-dependent – for example, appending a trafficking sequence that boosts *N. pharaonis* halorhodopsin expression levels (the ER2 sequence (Gradinaru, Thompson et al. 2008)) has no effect on improving the currents of the *H. sodomense*

archaerhodopsin-3, although adding a different sequence (the Prl sequence, derived from the prolactin secretion targeting sequence) does improve archaerhodopsin-3 expression and photocurrent (Chow, Han et al. 2010). We have found, through experiments with combinatorial addition of N- and C-terminal signal sequences, that adding multiple signal sequences does not necessarily improve expression in a linear way, perhaps owing to interactions between the multiple trafficking mechanisms at play. It should be noted that opsins may also possess intrinsic, even covert, sequences that enable them to be expressed very well on the plasma membrane. For example, the light-driven outward proton pump archaerhodopsin-3 from *H. sodomense* (and, in general, members of the archaerhodopsin class of opsins) naturally expresses rapidly and extremely well on plasma membranes (Chow, Han et al. 2010). Opsin mutagenesis and chimeragenesis has pointed towards candidate amino acids that may play a critical role in opsin trafficking and expression on the plasma membrane (Lin, Lin et al. 2009; Wang, Sugiyama et al. 2009).

III. Transduction of microbial opsins into cells for heterologous expression

The analysis of the potential power of a given microbial opsin to control the voltage or ionic composition of a target cell type (e.g., in a given organism under study), should be performed ideally in the target cell type itself, or in a testbed cell type that is as similar as possible to the target cell type. For example, the trafficking-enhancement and protein folding enhancement sequences described above are derived from specific species, and were optimized in cells from specific species; accordingly, they may not work equally well in species different from the source species, or in cell types greatly different from the cell types used to assess and optimize the sequences. Similarly, the covert trafficking sequences found within opsins may not function equally well in all cell types. As a concrete example, the *H. salinarum* bacteriorhodopsin has long been considered a difficult protein to express in *E. coli* (e.g., (Dunn, Hackett et al. 1987)), but it expresses readily in mammalian neurons, and can mediate biologically meaningful photocurrents (Chow, Han et al. 2010). Similarly, channelrhodopsin-2 does not express well in *E. coli*, but expresses well in mammalian neurons. Conversely, proteorhodopsins from uncultured marine gamma-proteobacteria express and function well in *E.coli*, but do not generate photocurrents in mammalian cells (HEK293 cells or mouse neurons) (Chow, Han et al. 2010), despite a rudimentary degree of expression of the proteorhodopsin protein in these mammalian cells. Thus, reliance on just a single heterologous expression cell type (e.g., *E. coli*, yeast, *Xenopus* oocytes, HEK cells) as the sole testbed for characterizing the physiological function of opsins, may lead to a partial picture of how well the opsins assessed will perform across the broad set of cell targets confronted in biology. Similarly, screening for enhancing mutations, trafficking sequences, or other beneficial modifications, using a single hetereologous expression cell type, may lead to unintentional optimization of the opsin for function in that particular cell type, and potential deoptimization of expression, trafficking, or function in other cell types of interest within the ultimate spectrum of usage of the tool.

If mammalian neurons in the living mouse or rat are the target, then mammalian neurons in primary culture should be at some point used to assess the function of a given opsin (Boyden, Zhang et al. 2005; Chow, Han et al. 2010), although ideally *in vivo* assessment should be performed as well, given the very different state of neurons *in vivo* vs. *in vitro*. It is important to note that different types of neurons, at different ages, may well differ in their level and timecourse of opsin expression and function. We typically utilize mouse hippocampal and cortical primary cultures because they contain representatives of some of the major cell classes in the brain (Boyden, Zhang et al. 2005; Han and Boyden 2007; Chow, Han et al. 2010). However, primary neuron cultures are laborious to prepare and maintain, and so we and others use HEK293 cell lines to perform electrophysiological characterization of opsins (Nagel, Szellas et al. 2003; Lin, Lin et al. 2009; Wang, Sugiyama et al. 2009;

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Chow, Han et al. 2010). HEK cells are more robust, and easier to work with, than neurons, and can be grown for multiple cell division cycles in culture, unlike neuron cultures which do not replicate after plating and differentiation. In addition, HEK cells possess cellular shapes and molecular phenotypes that are somewhat less variable than those of neurons, and possess fewer active conductances than do neurons; both of these features help reduce variability of opsin characterization measurements. Conversely, HEK cells may yield smaller photocurrents than do neurons due to their smaller surface area, and may have limited utility in fully predicting how well a protein will traffic in neurons (and thus, how they will perform as optical modulators of neural physiology) due to their differences from neurons. As a simple example of this latter point, HEK cells do not possess axons or dendrites; some findings have been published claiming that certain opsins preferentially traffic to the synaptic processes of neurons (Li, Gutierrez et al. 2005), and of course, any such effects would not be observable in a HEK cell. However, HEK cells are still extremely useful for performing fast screening assays of whether there is any physiological effect of illuminating a given opsin, and may be particularly useful for characterization of amplitude-normalized features of opsins such as the action spectrum, the plot of the relative photocurrent observed upon delivery of light of different colors.

Transfection is the simplest and fastest way to get DNA that encodes for opsins into cells, for rapid characterization of opsins in a cellular context. For HEK cells, transfection can increase the likelihood of delamination from the substrate; the use of Matrigel to promote cell adhesion to a glass coverslip when plating, as opposed to poly-lysine, is suggested. Well-dissociated HEK cells that are spatially separated from one another are critical for high-quality electrophysiological assays, as HEK cells that grow together can form gap junction-connected syncytia that can preclude accurate electrophysiological analysis of expressed opsins, by compromising voltage-clamp fidelity. In order to improve the quality of HEK cells for physiological assessment, passage the cells for their final plating when they reach medium levels of confluence (~50%); then, during the final plating step, trypsinize the HEK cells, resuspend the cells in serum-free media and pipette the cells against the sidewalls of the dish or flask to break up clumps of cells, perhaps triturating the cells with a fine-gauged sterile needle (e.g., <5 times to avoid excessive mechanical force on cells, through a ~31 gauge needle), and then add serum-containing media (to halt the trypsinization) before plating the final mixture on glass coverslips. For neuron culture, mouse or rat hippocampal or cortical neurons should be cultured from P0 pups or E18 embryos at moderate densities, using standard protocols (Boyden, Zhang et al. 2005; Han and Boyden 2007; Chow, Han et al. 2010). Multiple experimenters in our laboratory have independently found that the best recordings from opsin-expressing neurons are often from ones in areas of sparse neuron density, often at the edge of the area occupied by cells. The preferred method for HEK and neuron culture transfection is calcium phosphate precipitation of DNA, e.g. using commercially available kits. The calcium phosphate precipitation-based process can be harsh on neurons; accordingly, precautions should be taken, if needed, to limit neuronal excitotoxicity, for example adding AP5, a NMDA receptor antagonist, to the medium. The best transfection rates in neurons, in our hands, are achieved when neurons are transfected 3–4 days *in vitro*, with rapidly diminishing efficiency beyond then (although the genes encoding for quickly and highly expressed proteins, like Arch, can be delivered at 5 days *in vitro*).

Viral vectors, such as lentiviral vectors, can also be useful for assessing opsin function, because they can result in a high yield of opsin-expressing cells in a cultured cell environment, and they can also be used to insure a precise gene dosage into a cell of interest. For neurons, they also present lower toxicity, at a higher cellular yield, than achieved commonly with calcium phosphate transfection. Many lentiviral preparation protocols, involving the transfection of opsin-containing and helper plasmids into carefully cultivated

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and healthy HEK cells, exist that work well with opsins, e.g. (Boyden, Zhang et al. 2005; Han, Qian et al. 2009; Chow, Han et al. 2010). One key consideration is that recombination can be a major issue when preparing lentiviral vectors (and other viral vectors), due to the presence of repetitive sequences within the genomic vector of the virus, i.e. the payload-encoding plasmid. In theory any *E. coli* with loss of function mutation in rec gene(s) should be suitable for growing up such plasmids. However in our experience working with lentiviral plasmids, Stbl3 (recA13-) *E. coli* have a much lower rate of recombination compared to other rec- cells such as XL1-Blue (recA1-). XL10-Gold *E. coli* may work as well, with AAV plasmids. It is recommended to try out different types of rec- cells to find the optimal one for a particular viral vector, as recombination events can cause loss of vectors, and require time-consuming plasmid reconstruction. It is also important to check if any special considerations are needed for utilizing these specialized viral plasmid-optimized competent cell lines. For example Stbl3 is endA+, and thus the endA endonuclease will need to be removed with appropriate washing when purifying the DNA, to prevent DNA degradation. To check for recombination, viral plasmids should regularly be verified in both sequence and topology, using DNA sequencing and restriction digestion respectively. Both methods are recommended because sequencing short regions, such as the cloned insert, will only inform you whether the sequence is locally correct, but recombination can also occur between unpredictable locations, so that the cloned sequence is largely locally correct but different in global topology. Therefore it is highly recommended to perform multiple restriction digests to verify that the global sequence topology has not deviated from the designed plasmid. When cloning payloads into viral vectors, it is important to use only parent vectors that have also been tested for recombination, and it is important to perform both sequencing and restriction digests periodically as a viral plasmid stock is generated and propagated, ideally minimizing the number of generations that a stock is propagated to the minimum possible.

IV. Physiological Assays

Once a molecule is chosen, and expressed in a target cell type for characterization, it must be physiologically characterized by an observation method (e.g., patch clamp, dye imaging) – in the case of opsins, using illumination. Below we discuss illumination hardware, solutions in which to perform experiments, strategies for selecting cells to be analyzed, and methods for cellular readout.

The millisecond-scale resolution of optogenetic tools enables the remote control of cellular physiology with unprecedented resolution, but also requires illumination sources with increased temporal resolution than what is achievable with conventional fluorescence illuminators. A commonly used programmable excitation source is the Sutter DG-4, which uses a galvanometer mirror to direct light from the lamp into one of four filter slots within the lamp, to determine the excitation wavelength (*i.e.* no excitation filter should be placed within the actual fluorescence cube in the microscope, if the excitation light is filtered within the DG4 itself); a second mirror is used to shutter and/or adjust the intensity of light by modulating how much is directed to the light collection optics for delivery from the DG4 into the microscope. The output of the DG4 can be fed into an illuminator port of practically any microscope used for fluorescence imaging or electrophysiology. Laser based systems are also useful; since the action spectra of microbial rhodopsins are quite broad, typically with 100–150 nm bandwidths (full-width at half-maximum), sub-optimal excitation at a given wavelength of illumination can easily be compensated for by increased illumination power. For example, a 532 nm solid-state green laser is an order of magnitude cheaper than a 593 nm solid-state yellow laser, but will still excite the yellow light-sensitive *N. pharaonis* halorhodopsin quite effectively, just by increasing the delivered power slightly over the amount that would be required if a 593 nm laser were used. Action spectra (but not true

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absorbance spectra, which requires flash photolysis) can be measured during electrophysiological recording, by scanning through the visual spectrum with a Till Photonics Polychrome V or analogous color-programmable light source, coupled to a microscope through a standard fiber optic cable. In this particular illuminator, broadband light from a xenon lamp is passed through a programmable monochromator, so as to emit light with narrowband (10 nm bandwidths) properties, centered at various wavelengths. Light-emitting diodes (LEDs) have become increasingly popular due to their cheapness and fast switching times; a recent report (Albeanu, Soucy et al. 2008) offers excellent instructions for constructing a high-power and fast illuminator with two LEDs co-aligned for dual-spectral excitation, and commercial systems from Thorlabs and other vendors are also available. Most LEDs can be switched on and off with very fast (e.g., nanosecond) resolution, so the temporal resolution of various LED systems is largely limited by the drivers or power sources. LEDs are particularly useful for ultraviolet, orange, red, and infrared wavelengths, since many lamps are only weakly irradiant in these spectral bands.

Solutions used in electrophysiological characterization of mammalian cells (e.g., during patch clamp or imaging) are in some ways more complicated than typical solutions used in molecular biology. We highly suggest preparing electrophysiology solutions from scratch, instead of purchasing pre-made solutions. These solutions must be osmotically balanced to prevent cell death, ideally within 1–5 mOsm, and pH balanced, ideally within 0.1 pH units; extreme precaution must be taken to avoid contamination of reagents (as even a small change in a low-concentration ion, like calcium, can greatly change the health or electrical properties of a cell under electrophysiological study). For example, our laboratory (and many other electrophysiology laboratories) avoid insertion of spatulas into stock containers to dispense solids for preparing electrophysiological solutions; chemicals are instead poured from stock containers whenever possible. Solutions should be sterile filtered immediately following preparation, to maximize cell health and available recording time, as use of sterile aliquots of solutions can improve cell health. Our bath solution of choice for *in vitro* experiments using both HEK cells and neurons is Tyrode's solution, a HEPES buffer-based saline solution (Boyden, Zhang et al. 2005; Han and Boyden 2007; Chow, Han et al. 2010). Artificial cerebrospinal fluid (ACSF), which is bicarbonate-buffered, may improve cell health in certain circumstances over that obtained from use of Tyrode's Solution, but requires fluid manifolds to perfuse CO_2-saturated solutions in order to maintain physiological pH levels, and the added inconvenience is often not justified. For the use of dyes that indicate the levels of ions such as H+ or Ca+2 (e.g., SNARF, fura-2, Oregon Green BAPTA), the manufacturers' instructions provide a good starting point for deriving protocols for the loading and imaging of the dyes, although some optimization of loading conditions and imaging conditions may be required for given cell types and given conditions of joint photostimulation and imaging (Lin, Lin et al. 2009; Chow, Han et al. 2010; Prigge, Rosler et al. 2010).

Even within a cell type, different cells will vary in their levels of opsin expression, appearance, and sustained photocurrents, potentially to great degrees. Excessive overexpression of an opsin can lead to poor cell health, so simply picking the very brightest cells to record electrophysiologically may yield unrepresentative data, and accurate characterization of the performance of an opsin ideally is performed with unbiased selection of cells. Beginning experimenters may have slightly different, even unconscious, biases in cell choice strategy, e.g. choosing the biggest cells in the field of view. To address some of these problems, we often normalize observed photocurrents by cell capacitance, thus obtaining the photocurrent density, which helps compensate for the varying size of the cells being recorded. We often have multiple experimenters in our lab validate key results when photocurrent magnitude is the question, to additionally address this issue (for example, there

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were no statistically significant differences in photocurrents measured between the two co-first author experimenters in (Chow, Han et al. 2010)).

Expression of opsins in a cell can increase over time, as the process of protein expression and trafficking can be slow. For full characterization of an opsin, it is recommended to assess opsin function at various times after transfection, e.g. between a few days and a few weeks, to understand the timecourse of expression and trafficking. Importantly, different opsins, and opsins expressed using different gene delivery mechanisms, will present with different timecourses of functional expression. In neurons, we have noted a trend for many microbial rhodopsins from archaea to express and traffic to the membrane more quickly than do those from fungus and plants, although specific opsins within these families can violate this trend. Typically, the photocurrents measured in neurons from archaeal rhodopsins (both bacteriorhodopsins and halorhodopsins) using the protocols in Section IV do not change after 5–6 days post-transfection or 10 days post-viral infection; photocurrents of channelrhodopsin-2 take a few extra days to plateau, compared to the archaeal opsins. These are the times that it takes for currents to saturate; fluorescence levels may saturate much earlier, perhaps because although opsins are rapidly expressed at the level of protein, it may take some time for them to traffic, and assemble within the membrane in functional form (e.g., some may potentially require multimerization within the membrane to attain full function). It is possible that adding trafficking sequences, or inducing mutations, can result in slowed down or sped up functional protein expression, versus the wild type form of the opsin.

Similar trends in expression and membrane localization rates as a function of kingdom of origin area also observed in transfected HEK cells, with faithful expression of rhodopsins from archaea requiring two days and ones from fungi and plants requiring up to three days (although again, individual opsins may violate these rules of thumb). These multi-day expression times may present difficulties because HEK cells will divide a few times during this period, and this counteracts the goal of performing reliable recordings on isolated cells (or on cells with minimal shared membrane with other cells, as described in Section III). The addition of sodium butyrate (Dunlop, Bowlby et al. 2008) or lowering the cell culture incubator temperature (32°C instead of 37°C) can extend the time between cell divisions and allow for more time for membrane expression (Wang, Sugiyama et al. 2009).

V. Conclusion

The process of assessing the physiological function of a heterologously expressed protein in a target cell is complex. Such explorations are critical for understanding the potential uses of a given synthetic physiology tool, for evaluating potential side effects or toxicity of a candidate tool, or for screening for novel or optimized tools. As a closing example, it was originally believed that channelrhodopsin-1 (ChR1) was a light-gated proton channel, but multiple reports since then have demonstrated that it is indeed a nonspecific cation channel like ChR2 when evaluated at neutral pH and expressed at sufficiently high levels (Nagel, Ollig et al. 2002; Berthold, Tsunoda et al. 2008; Wang, Sugiyama et al. 2009). Thus, considerations of the cellular environment in which a protein is evaluated, e.g. pH and expression level, are key for understanding the physiological power of a given molecular tool. In summary, assessing the function of a given physiological driver is complex because of the many variables that can modulate the expression and performance of physiological drivers, and the quantitative, high-speed nature of the signal being driven. The creation of new model systems that can replicate key features of targeted physiological systems, in a fashion that could support high-throughput tool assessment or tool optimization, may greatly enhance the ability to generate novel and impactful synthetic physiology tools.

Acknowledgments

ESB acknowledges funding by the NIH Director's New Innovator Award (DP2OD002002) as well as NIH Grants 1R01DA029639, 1RC1MH088182, 1RC2DE020919, 1R01NS067199 and 1R43NS070453; the NSF CAREER award as well as NSF Grants EFRI 0835878, DMS 0848804, and DMS 1042134; Benesse Foundation, Jerry and Marge Burnett, Department of Defense CDMRP Post-Traumatic Stress Disorder Program, Google, Harvard/MIT Joint Grants Program in Basic Neuroscience, Human Frontiers Science Program, MIT Alumni Class Funds, MIT Intelligence Initiative, MIT McGovern Institute and the McGovern Institute Neurotechnology Award Program, MIT Media Lab, MIT Mind-Machine Project, MIT Neurotechnology Fund, NARSAD, Paul Allen Distinguished Investigator Award, Alfred P. Sloan Foundation, SFN Research Award for Innovation in Neuroscience, and the Wallace H. Coulter Foundation. The reported methods were developed with additional contributions from Xue Han (currently Assistant Professor, Boston University), Xiaofeng Qian, and Aimei Yang of the Synthetic Neurobiology research group at MIT, and with the helpful discussion of Dr. John Y. Lin (UCSD). Thanks also to Xue Han for critically reading the manuscript.

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