

Light-Activated Ion Pumps and Channels for Temporally Precise Optical Control of Activity in Genetically Targeted Neurons¹

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Abstract

The ability to turn on and off specific cell types and neural pathways in the brain, in a temporally precise fashion, has begun to enable the ability to test the sufficiency and necessity of particular neural activity patterns, and particular neural circuits, in the generation of normal and abnormal neural computations and behaviors by the brain. Over the last 5 years, a number of naturally occurring light-activated ion pumps and light-activated ion channels have been shown, upon genetic expression in specific neuron classes, to enable the voltage (and internal ionic composition) of those neurons to be controlled by light in a temporally precise fashion, without the need for chemical cofactors. In this chapter, we review three major classes of such genetically encoded “optogenetic” microbial opsins—light-gated ion channels such as channelrhodopsins, light-driven chloride pumps such as halorhodopsins, and light-driven proton pumps such as archaerhodopsins—that are in widespread use for mediating optical activation and silencing of neurons in species from *Caenorhabditis elegans* to nonhuman primates. We discuss the properties of these molecules—including their membrane expression, conductances, photocycle properties, ion selectivity, and action spectra—as well as genetic strategies for delivering these genes to neurons in different species, and hardware for performing light delivery in a diversity of settings. In the future, these molecules not only will continue to enable cutting-edge science but may also support a new generation of optical prosthetics for treating brain disorders.

Key words: Channelrhodopsin, Optogenetics, Photosensitive proteins, Retinal, Halorhodopsin, Archaerhodopsin, Light-sensitive cation channel, Light-sensitive chloride pump, Light-sensitive proton pump, Photocontrol of behavior

1. Introduction

The ability to turn on and off specific cell types and neural pathways in the brain, in a temporally precise fashion, has begun to enable the ability to test the sufficiency and necessity of particular neural activity patterns, and particular neural circuits, in the generation of normal

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and abnormal neural computations and behaviors by the brain. Most electrophysiological and imaging experiments in neuroscience are correlative—comparing a neural signal observed in the brain to a behavior or pathology. In contrast, the power to manipulate specific cells and circuits is opening up the ability to understand their causal roles in brain functions. Over the last 5 years, a number of naturally occurring light-activated ion pumps and light-activated ion channels have been shown, upon genetic expression in specific neuron classes, to enable the voltage (and internal ionic composition) of those neurons to be controlled by light in a temporally precise fashion. These molecules are microbial (type I) opsins, seven-transmembrane proteins naturally found in archaea, algae, fungi, and other species, and which possess light-driven electrogenic activity or contain light-gated ion pores. These molecules, when heterologously expressed in neurons or other cells, translocate ions across cell membranes in response to pulses of light of irradiances that are easily achievable with common laboratory microscopes, LEDs, and lasers. These molecules have begun to find widespread use in neuroscience due to their ease of handling and use (each is a single gene, under 1 kb long, encoding for a monolithic protein), their lack of need for chemical supplementation in many species (they utilize the naturally occurring chromophore all-*trans* retinal, which appears to occur at sufficient quantities in the mammalian nervous system), and their high speed of operation (they can respond within tens of microseconds to milliseconds, upon delivery of light, and shut off rapidly upon cessation of light, as needed for neuroscience experiments).

Three major classes of such “optogenetic” microbial opsins have been described to date. The first class, channelrhodopsins, is exemplified by the light-gated inwardly rectifying nonspecific cation channel channelrhodopsin-2 (ChR2) from the green algae *Chlamydomonas reinhardtii* (1), which, when expressed in neurons, can mediate sizeable currents up to 1,000 pA in response to millisecond-timescale pulses of blue light (2–5), thus enabling reliable spike trains to be elicited in ChR2-expressing neurons by blue light pulse trains (Fig. 1b). Several additional channelrhodopsins useful to biologists and bioengineers have been discovered or engineered, with faster or slower kinetics, red-shifted activation, and cell-region specific targeting, and explored in detail below (6–10). The channelrhodopsins have been used to activate neurons in neural circuits in animals from worms to monkeys, and have proven powerful and easy to use. The second class of microbial opsins utilized for biological control to date, halorhodopsins, is exemplified by the light-driven inwardly directed chloride pump halorhodopsin, from the archaeal species *Natronomas pharaonis* (Halo/NpHR/pHR; (11)), which, when expressed in neurons, can mediate modest inhibitory currents on the order of 40–100 pA in response to yellow light illumination (12, 13), enabling moderate silencing of neural activity (Fig. 1c). Halorhodopsins have some intrinsic kinetic limitations,

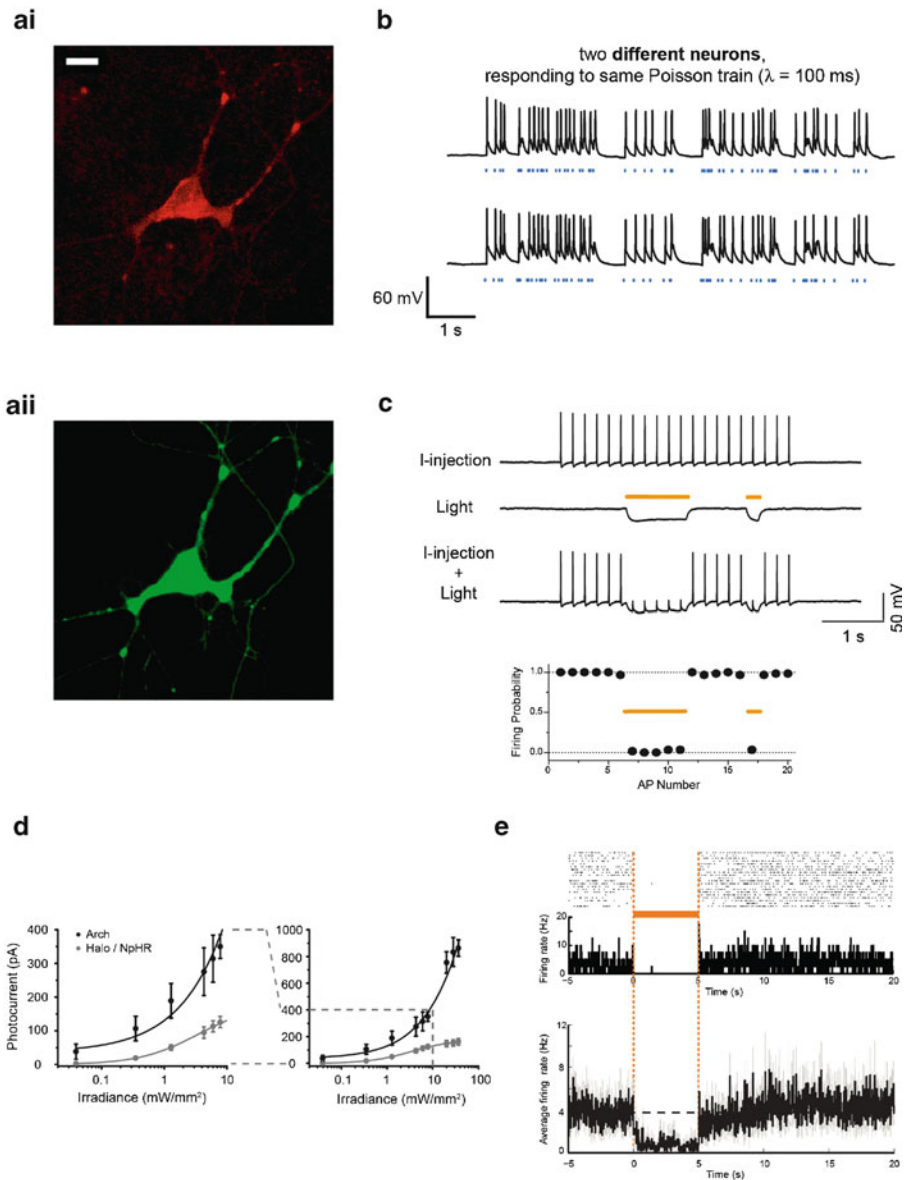


Fig. 1. Three classes of microbial opsins that enable optical neural activation and silencing tools. **(a)** Neuron expressing hChr2-mCherry **(ai)**; bar, 20 μm) and Halo-GFP **(a ii)**. **(b)** Poisson trains of spikes elicited by pulses of blue light (*blue dashes*), in two different neurons. **(c)** Light-driven spike blockade, demonstrated (*Top*) for a representative hippocampal neuron, and (*Bottom*) for a population of neurons ($N = 7$). *I-injection*, neuronal firing induced by pulsed somatic current injection (300 pA, 4 ms). *Light*, hyperpolarization induced by periods of yellow light (*yellow dashes*). *I-injection + Light*, yellow light drives Halo to block neuron spiking, leaving spikes elicited during periods of darkness intact. *Panels a–c* adapted from references Boyden et al. (3) and Han and Boyden, (12). **(d)** Photocurrents of Arch vs. Halo measured as a function of 575 ± 25 nm light irradiance (or effective light irradiance), in patch-clamped cultured neurons ($N = 4$ –16 neurons for each point), for low (i) and high (ii) light powers. The line is a single Hill fit to the data. **(e)** *Top*, Neural activity in a representative neuron before, during, and after 5 s of yellow light illumination, shown as a spike raster plot (*top*), and as a histogram of instantaneous firing rate averaged across trials (*bottom*; bin size, 20 ms). *Bottom*, population average of instantaneous firing rate before, during and after yellow light illumination (*black line*, mean; *gray lines*, mean \pm SE; $n = 13$ units). *Panels d–e* adapted from reference Chow et al. (15).

with some photocycles taking tens of minutes to complete (e.g., Fig. 4a, b; (12, 14)), and halorhodopsins also require improved trafficking for expression at high levels (15–17). A third class of microbial opsin, the bacteriorhodopsins, is exemplified by the light-driven outward proton pump archaerhodopsin-3 (Arch/aR-3), from the archaeal species *Haloarubrum sodomense*. Arch can mediate strong inhibitory currents of up to 900 pA in vitro (Fig. 1d), and in vivo is capable of mediating near-100% silencing of neurons in the awake behaving mouse or monkey brain in response to yellow-green light (Fig. 1e, (15, 18)). Protons are extremely effective as a charge carrier for mediating neural silencing, and proton pumps have greatly improved kinetics with respect to halorhodopsins (Fig. 4c, d), as well as a fast photocycle, and efficient trafficking to membranes. Furthermore, outward proton pumps, perhaps surprisingly, do not alter pH to a greater extent than do other opsins (such as ChR2) or than does normal neural activity. The broad and ecologically diverse class of outward proton pumps, which includes blue-green light drivable outward proton pumps such as the *Leptosphaeria maculans* opsin Mac (Fig. 5), enables, alongside the yellow-red drivable Halo, multicolor silencing of independent populations of neurons (15). Also, because the neural silencers Halo and Arch are activated by yellow or yellow-green light, and the neural depolarizer ChR2 is driven by blue light, expression of both a silencer and a depolarizer in the same cell (either by using two viruses, or by using the 2A linker to combine two opsins into a single open reading frame (12, 19)) enables bidirectional control of neural activity in a set of cells, useful for testing necessity and sufficiency of a given set of neurons in the same animal, or disruption of neural synchrony and coordination through “informational lesioning” (19).

In the sections below we describe the properties of these three opsin classes, as well as genetic (e.g., viral, transgenic) and hardware (e.g., lasers, LEDs) infrastructures for using these opsins to parse out the function of neural circuits in a wide variety of animal nervous systems. A theme of this field is that extremely rapid progress and adoption of these technologies has been driven by technology development curves in other fields such as gene therapy and optical imaging. Our hope is to convey a snapshot of this rapidly moving field as of 2011, summarizing the first half-decade of its existence, to teach both neuroengineers hoping to innovate by inventing new tools, as well as neuroscientists hoping to utilize these tools to answer new scientific questions. We will first survey the general properties of these opsins (Sect. 2), then go into the channelrhodopsins (Sect. 3), followed by the neural silencing pumps (halorhodopsins and bacteriorhodopsins, Sect. 4), the molecular strategies for delivering these genes to cells for appropriate expression in vitro and in vivo (Sect. 5), and the hardware for illumination of these opsin-expressing neurons in vitro and in vivo (Sect. 6).

2. Properties of “Optogenetic” Microbial (Type I) Opsins

The three classes of molecule described to date are from organisms such as unicellular algae, fungi, and archaea, whose native environments and membrane lipid composition are very different from those of mammalian neurons. Thus, the performance of these molecular tools in neurons can be difficult to predict based solely upon their properties in other species, and must be assessed empirically for assurance of efficacy and safety. Nevertheless, there are several molecular properties that contribute to efficacious, temporally precise optical control of neurons, which can be explored in a unified and logical fashion:

- *Initial protein expression levels.* The efficiency of ribosomal translation of a molecule is largely affected by codon optimization. It is recommended that genes be used that are codon-optimized for the target species.
- *Membrane insertion properties, protein folding, and interactions with local environment.* Increased membrane localization will result in more functional molecules and thus increased photocurrents. This property may also be inversely associated with the potential property of *toxicity*, since poorly trafficked or folded molecules may aggregate in the cytosol and endoplasmic reticulum. On the other hand, if a molecule has adverse intrinsic side effects, enhanced trafficking may exaggerate them. Furthermore, any given channel or pump will best operate under defined conditions (e.g., chloride conductance, pH, lipid environment, etc.), which may not exist in a given target cell type.
- *Innate conductance and permeability.* Channels translocate more ions per photocycle than pumps, since they open up a pore in the membrane. On the other hand, pumps can move ions against concentration gradients, unlike channels. Each opsin furthermore passes a precise set of ions in a specific cellular context, and not others.
- *Photocycle kinetics.* Both light-driven channels and pumps are described by a photocycle, the list of states that a molecule goes through after light exposure, including ion-translocating or ion pore-forming steps. The faster the photocycle, the more temporally precise the molecular function might be, and for a pump, the more ions will be translocated. (For a channel, a faster photocycle may result in the channel entering the ion pore-forming state more often, but may also reduce the time spent in the open state.) If a molecule enters an inactive

photocycle state for an enduring period of time, it may be effectively nonfunctional.

- *Photosensitivity.* Molecules may require different amounts of light to begin moving through their photocycle, based on the chromophore absorption efficiency. Furthermore, from an end-user standpoint, effective photosensitivity will appear to be a function of the overall photocycle; for example, a pump that has a slow photocycle may appear to be light insensitive (because incident photons may have no effect on the molecule during the photocycle), whereas a channel that inactivates extremely slowly may appear to be light sensitive (because each photon will result in large charge transfers).
- *Action spectrum.* Different molecules are driven by different colors of light. Multiple cell types can be orthogonally addressed with different colors of light, if they express opsins whose action spectra minimally overlap.
- *Ion selectivity.* Unlike traditional electrodes, microbial opsins can generate ion specific currents, since they will pass specific ions such as chloride (Cl^-) or calcium (Ca^{+2}). This opens up novel kinds of experimental capability, such as the ability to test the sufficiency of a given ion, in a given location, for a given biological function.

We will, in the following sections, frame current knowledge about cell-type specific optical control of neurons, in the context of decades of research in structure–function relationships of microbial (type I, or archaeal) opsins. In many ways, these molecules are similar in tertiary structure to mammalian (type II) rhodopsins (20), the pigments that confer photosensitivity to the rods and cones of the human retina. Both types are composed of seven transmembrane (7-TM) α -helices, linked by six loop segments, and their photosensitivity is enabled by a retinal bound to a specific lysine residue near the C-terminus, forming a Schiff base that undergoes a *trans–cis* or *cis–trans* isomerization upon illumination, that then induces conformational changes in the protein. However, they are evolutionarily unrelated, and their differences have important implications for their use in perturbing neuronal activity. Mammalian rhodopsins (21) are very sensitive photon detectors, optimized for sensitivity rather than speed. They utilize 11-*cis* retinal as the primary chromophore, which isomerizes to all-*trans* retinal upon absorbing a photon. The resultant structural change activates an associated G-protein, transducin, which then initiates a cascade of secondary messengers. The all-*trans* retinal dissociates from the opsin, is converted back to its 11-*cis* form, and then reassociates with the apoprotein to reconstitute a functional molecule—a process that typically takes hundreds of milliseconds, too slow to enable fast control of neurons in the central nervous system.

On the other hand, a microbial opsin utilizes all-*trans* retinal as its chromophore, which isomerizes to 13-*cis* retinal upon absorbing a photon. The chromophore does not undergo a quasi-irreversible dissociation event, but rather thermally relaxes to its active all-*trans* form in the dark (although this process can be facilitated by light). The *trans*–*cis* isomerization sets off several coupled structural rearrangements within the molecule that accommodate the passive conduction or active pumping of ions (22–24). Ultimately, this means that at the expense of light sensitivity, archaeal opsins can deflect the membrane potential of a cell on the scale hundreds of microseconds to a few milliseconds. However, it should be noted that genetically targetable, optical neural silencing has also been demonstrated using mammalian G-protein coupled receptors, which can couple to potassium channels (4), and genetically targetable optical neural activation has been demonstrated using melanosins and invertebrate-style rhodopsins, at the price of temporal precision (25, 26).

As an exemplar of a well-characterized microbial opsin reagent, with crystal structure and photocycle both well-described, Fig. 2 shows the crystal structure of the light-driven chloride-pump halorhodopsin (Fig. 2a; (27)), as well as schematized structural rearrangements that are hypothesized to occur as the molecule pumps a chloride ion across the membrane and into the cytoplasm (Fig. 2b; (24)). While it is convenient to consider the molecular tools discussed here as toggle switches for turning neurons on and off, it is critical for use of these opsins to realize that the translocation of ions by microbial opsins is not as simple as a two-state toggle switch. The structural rearrangements constitute an active advancement through a complex photocycle with various intermediate states beyond the initial phototransition (Fig. 2c). There exists a very rich literature on type I microbial opsins from an evolutionary and protein structure–function perspective. The canonical molecules include the proton-pumping bacteriorhodopsin (BR), the chloride-pumping halorhodopsin (HR/sHR/HsHR) from *Halo-bacterium salinarum* (*halobium*), and the halorhodopsin from *N. pharaonis* (Halo/NpHR/pHR). These were some of the first membrane proteins crystallized, and a myriad of structure–function studies have been performed on these molecules (11, 14, 20, 22–24, 27–45). These studies are not reviewed here, but it is important to point out their existence because much of what we know with respect to the photocycle and structure of opsins comes from these studies and from sequence homology of novel opsins to these canonical molecules. As we show later, for example, a deep understanding of the literature has enabled some researchers to derive powerful new variants of channelrhodopsin (5, 6, 8, 10), even though no crystal structure exists for this molecule.

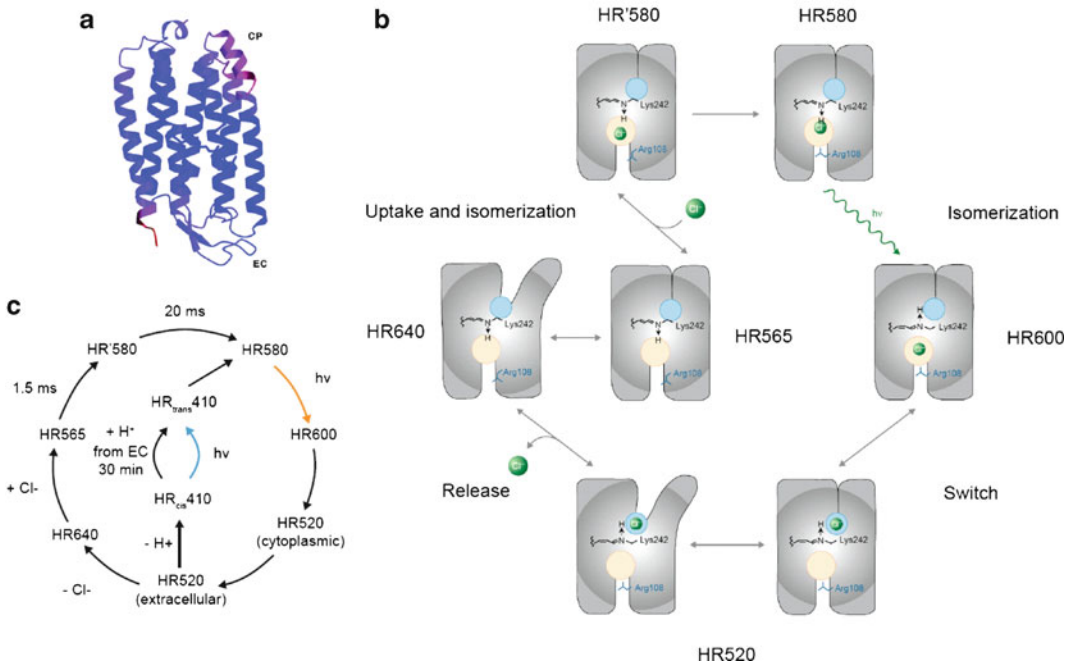


Fig. 2. Structure and function of halorhodopsin. (a) Crystal structure of halorhodopsin, which is composed of 7-transmembrane α -helices (7-TM) and a retinal chromophore that forms a Schiff base to a lysine near the C-terminus (from Kolbe et al. (27)). (b) Schematic of the halorhodopsin structural rearrangements and their relation to pumping activity at various points in the photocycle. Image modified from Essen (24). (c) The halorhodopsin photocycle at high-power continuous illumination on the timescale of a typical photocycle (i.e., conditions used for neural silencing, >few milliseconds). The HR410 intermediate is the origin of the long-lived inactivation in neural silencing (e.g., Bamberg et al. (14); Han and Boyden (12)).

3. Optical Neural Stimulation: Channel-rhodopsins

Channelrhodopsins are the primary photoreceptors in the eyespot of the unicellular algae that are responsible for phototactic and photophobic responses (46–48). Their name is derived from the fact that despite the sensory function, the 7-TM segment is in itself a light-activated ion channel. While the channel pore and properties remain poorly understood, it has recently been realized that channelrhodopsins are likely proton pumps like many other microbial opsins, but with a leaky step in the photocycle during which the opsin lets positive charge into cells (49). In *C. reinhardtii*, two separate channelrhodopsins were originally identified (48), one with fast kinetics but poor light sensitivity, channelrhodopsin-1 (ChR1) (50), and another with slower kinetics but improved sensitivity, channelrhodopsin-2 (ChR2) (1). Two more channelrhodopsins from *Volvox carteri* (VChR1, VChR2) have also been identified (7, 51), and as we discuss later, many more ChRs are expected to

exist. ChR1-style channelrhodopsins have red-shifted action spectra (peak $\lambda_{\text{ChR1}} = 500$ nm, $\lambda_{\text{VChR1}} = 535$ nm) relative to ChR2 (peak $\lambda_{\text{ChR2}} = 470$ nm), and thus in principle ChR1-style and ChR2-style opsins could be used together to drive separate sets of neurons with two different colors of light, if suitably spectrally separated opsin pairs could be found.

3.1. Conductance, Permeability, and Context

Channelrhodopsins (abbreviated as ChRs or chops) are light-activated, inwardly rectifying cation channels that are, at neutral pH, permeable to physiologically relevant cations such as H^+ , Na^+ , K^+ , and Ca^{2+} , with permeabilities (relative to sodium) of 1×10^6 , 1, 0.5, and 0.1 respectively (1, 6, 46, 50). It is of particular note that the proton conductance (G_{H^+}) is 10^6 -fold larger than the sodium conductance (G_{Na^+}), and thus near physiological pH, perhaps half the photocurrent is carried by protons (46); thus, ChRs may rapidly equilibrate the intracellular pH with its environment (10). Kinetic selectivity analysis has shown that the mechanism of ion selectivity is likely to be due to differential binding affinity of channelrhodopsin channel residues for different ions, not differential ion transport rates (46).

It was originally believed that ChR1 was a selective proton channel (50); however, it was later discovered that the poor photocurrents at mammalian pH were likely attributable to poor membrane localization (6), and the apparent lack of sodium currents in the original report were due to the low pH used to perform experiments in that study; the sodium conductance of ChR1 lessens at low pH (6, 46), unlike that of ChR2 (6). This highlights what is a recurrent theme throughout this chapter, that effective conductance in a heterologous system is determined not only by the innate kinetic and transport properties of the molecule but also by its trafficking and performance in the environment of the heterologous system.

The single ion channel conductance of ChR2 has been estimated at 50 fS (1), which corresponds to approximately 3×10^4 ions per second, or 300 ions per photocycle event, assuming a 10 ms turnover. This is considerably less than a typical voltage dependent sodium channel that may have a conductance on the order of ~ 10 pS. It has been estimated from electrophysiological data that 10^5 – 10^6 membrane embedded ChR2 molecules are required to cause reliable spiking in cultured rodent hippocampal neurons (52), with saturation blue light densities of several milliwatts per square millimeter both in vitro (10) and in vivo (53).

3.2. Basic View of Kinetics and Wavelength Selectivity

Figure 3a shows a typical photocurrent trace from a voltage-clamped neuron expressing ChR2 (top), and the spiking pattern that would result in current-clamp mode (bottom). There is a large transient peak with an opening time constant near 1 ms (1, 6, 10), although photocurrent onset can be measured at <200 μs (1, 3,

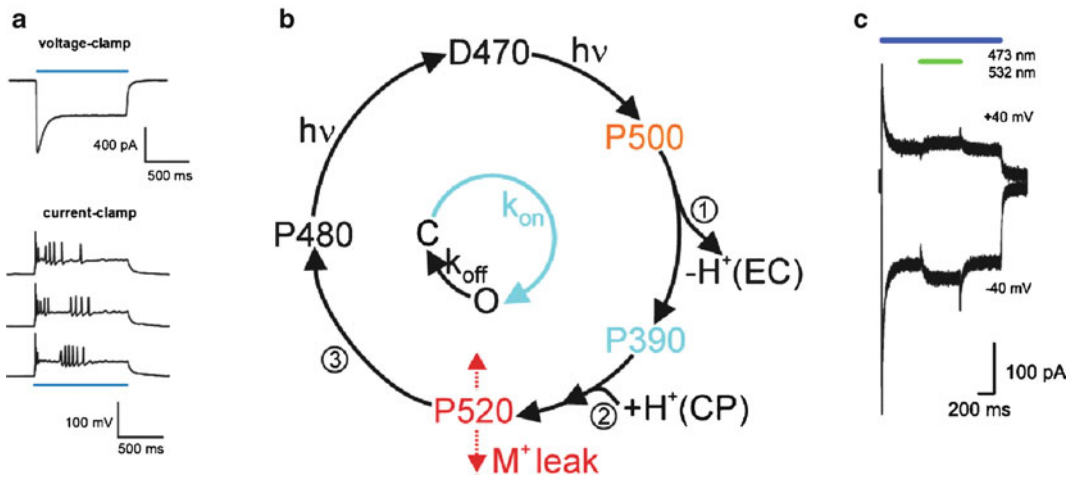


Fig. 3. Channelrhodopsin kinetic and photocycle properties, and impact on neural activity. (a) ChR2 currents elicited in a voltage-clamped hippocampal neuron expressing ChR2 and illuminated by blue light (*top*), and ChR2-driven spikes elicited in a current-clamped hippocampal neuron (three repetitions of the same blue light pulse in the same neuron) (*bottom*), under 1 s of blue light illumination. Adapted from Boyden et al. (3). (b) The photocycle of ChR2 determined by a combination of spectroscopy, site-directed mutagenesis, and electrophysiology, adapted from Feldbauer et al. (49). The *inner circle* summarizes the effective appearance of the photocycle, an approximation to the outer photocycle. (c) The interplay between photocycle, wavelength, and electrophysiological activity. ChR2 is excited with blue light for a brief period, and then a green light is turned on. The photocurrent initially diminishes because the channel is forced to close, but then increases because the green light also pumps the molecule into its most highly efficient state. Image modified from Bamann et al. (54).

54); this transient peak quickly decays to a stationary component that is typically <20–50% of the initial peak photocurrent (1, 3, 6, 10). Upon removing the light, ChR2 closes with a time constant of 10–20 ms (1, 6, 10). The transient photocurrent peak is highly dependent on the illumination intensity (51, 55) and history (1, 3, 10); the history dependence results from a desensitization of the transient component that takes ~5 s to recover from in the dark (3). The stationary component on the other hand, is less photosensitive and effectively history-independent (55). ChR2 absorbs maximally at 460 nm (1, 10), and the action spectra of both temporal components are nearly identical in ChR2.

The large and fast-onset peak enables ChR2-expressing neurons to spike with exquisite temporal precision on the millisecond timescale (Fig. 1b), the timescale of an action potential. However, the large inactivation (or alternatively, the small stationary component) and its slow recovery in the dark, as well as the slow closing rate of ~10–15 ms, ultimately limit the ability to drive reliable spike rates >25 Hz (3, 10) because (1) the stationary photocurrent may be too small to sufficiently depolarize a neuron to spike threshold, and (2) the channel cannot physically close quickly enough to enable de-inactivation of sodium channels. It should be noted, though, that many neurons, such as pyramidal cells, seldom fire

action potentials at this rate on the individual neuron level (vs. population synchrony or rhythmogenesis).

ChR1-style channelrhodopsins (VChR1, ChR1) (7, 50) on the other hand demonstrate dramatically faster kinetics than ChR2-style channelrhodopsins (VChR2, ChR2). The stationary photocurrents of ChR1s are >70% of the peak photocurrents, and the channels open and close approximately two- to threefold faster than does ChR2. Therefore, one would expect that given comparable expression, protein folding, membrane localization, and photosensitivity (i.e., factors contributing to effective conductance), ChR1s would be capable of driving spike rates with greater fidelity than ChR2s. However, poor membrane expression limits the performance of natural ChR1-style channelrhodopsins (7, 50). Chimeras composed of the first five helices of ChR1 and last two helices of ChR2 have been constructed (6, 10, 56), and these new variants exhibit the small inactivation and action spectrum of ChR1, but the overall effective conductance of ChR2. These structure–function studies are discussed in detail later in this chapter. A point mutant of this chimera dubbed “ChIEF” (based on its composition as a [Ch]annelrhodopsin chimera with an [I]190 V substitution with domains swapped between ChR1 helix-[E] and ChR2 helix-[F]), developed by Tsien and coworkers (10), appears to be a highly improved tool for stimulating neurons. Its large stationary photocurrent and very fast channel closing, the latter conferred by the I190 V mutation, contribute to far more reliable spiking (up to 100 Hz) than ChR2.

Based on the available characterization of the channelrhodopsins from *V. carteri* (7, 51), the general characteristics are similar to those of the analogous molecules in *C. reinhardtii*. VChR2 and ChR2 have nearly identical photocycles and action spectrum (51). VChR1 and ChR1 exhibit the similar reduced inactivation, and are both red-shifted from their respective VChR2/ChR2 counterparts. It has been proposed that VChR1 could be used for multicolor optical stimulation in conjunction with ChR2, which is blue-shifted by ~70 nm, but further improvements are likely required for reliable spiking because the VChR1 photocurrents are unfortunately approximately four- to fivefold smaller (7), and also there is significant spectral overlap between VChR1 and ChR2.

3.3. Detailed Models of Kinetics and Wavelength Selectivity

As previously mentioned, it is critical to realize that the translocation of ions by opsins is not as simple as the operation of an on/off switch, but rather these opsins traverse a complex photocycle with various intermediate states beyond the initial opening of the channel. Figure 3b shows the photocycle of ChRs based on photophysical studies performed primarily by laser flash spectroscopy, physiology, and site-directed mutagenesis (8, 49, 51, 54, 57). Importantly, the intermediates of the photocycles themselves can also undergo photoreactions, and thus they may be optically driven

or “short-circuited” (54) between photointermediates at much faster rates (Fig. 3c). The ChR2 photocycle initially begins in its closed dark-adapted state D470 (where the number in the state name corresponds to the peak light absorption, in nm, of the molecule in that state). The channel opens when D470 absorbs a photon, after which the molecule will become a green absorbing photoproduct or P-intermediate, P520, via thermal relaxation from shorter lived photoproducts. This initial cascade of events takes 0.2–1.5 ms, depending on the transmembrane potential. The open ChR2 can be closed by either optically pumping P520 → D470 with green light, or by decaying to P480 (via a yet to be determined intermediate), a process that takes ~6 s. The inactivation toward the stationary photocurrent may be due to molecules making the P520 → P480 transition, rather than the optically induced P520 → D470 transition that would allow the molecule to quickly open again. Assuming that ChR1 and ChR2 photocycles are topologically similar, e.g., the ChR2 D470 and P480 equate to the ChR1 peaks at 464 and 505 nm, this interpretation of the transient and stationary photocurrents is consistent with the finding that, for ChR1, the stationary photocurrent is red-shifted from the transient photocurrent (6).

The various wavelengths of absorption of rhodopsins and their intermediates throughout the photocycle arises from the different conformations of the chromophore and its local environment, which influences the chromophore charge distribution and the protonation of the retinylidene Schiff base. Figure 3c demonstrates this complex interplay in an experiment by Bamberg and coworkers (54). After blue light-excited ChR2 has reached its steady state, a green light costimulation is introduced. The photocurrent briefly diminishes because the open channel is forced to close, but the stationary photocurrent quickly improves because many molecules have been pumped back into their highly efficient, peak producing state. Thus, it is possible that slightly red-shifted or broadband illumination of ChR2 may strike a balance between optimally exciting the dark state (transient component) and repriming the dark state (driving the red-shifted intermediate photoproduct). As we discuss, optimal silencing with *N. pharaonis* halorhodopsin is analogously achieved by using both yellow light to hyperpolarize the neuron and blue light to drive the molecule out of its inactive state (12, 14).

3.4. Mutants and Variants

As previously mentioned, even though no ChR crystal structure exists at the time of this writing, useful structure–function studies have been performed based largely on sequence homology to *H. salinarum* bacteriorhodopsin. The E90Q mutation (57) has increased sodium selectivity (with respect to G_{H^+}) vs. wild-type ChR2, and the H134R mutant (5) demonstrates increased conductance by approximately twofold. Various mutations to C128 (8)

corresponding to bacteriorhodopsin T90, drastically slow down the rate of ChR2 closure from the open state, thus effectively creating a bistable open P520 state until illuminated with green light. By lengthening the time that ChR2 spends open on a per-photon basis, this mutation effectively decreases the amount of light needed to activate the channel, at the expense of temporal precision. In contrast, the E123T mutant, combined with the H134R mutant, speeds channel closure and increases the precision of neural action potential firing at the expense of photocurrent and light sensitivity (58), resulting in a reagent nicknamed ChETA.

Chimeras of ChR1 and ChR2 have been constructed by several researchers (6, 10, 56), one of which was that composed of ChR1 helices A–E and ChR2 helices F–G (called abcdeFG, ChEF, or ChR1/2_{5/2} by various investigators). These chimeras displayed the small inactivation of ChR1, but the large photocurrents of ChR2 on account of improved membrane localization and light sensitivity (based on quantitative confocal fluorescence microscopy, (6)). An I190 V substitution to ChEF led to the molecule, “ChIEF,” capable of driving more reliable fast spiking due to the much larger stationary current and faster channel closing kinetics after light offset (10). During these studies, it was also discovered that a single point mutation to wild-type ChR1, E87Q, eradicates its pH-dependent spectral shifts, and increases inactivation during illumination (56).

The fact that the poor effective conductance of ChR1 can be largely attributed to membrane localization rather than its photo-physical properties highlights the importance of considering and improving the trafficking of heterologously expressed molecules. In particular, ChRs are not localized to the outer membrane, but rather the eyespot, in *C. reinhardtii*, and the membrane composition of the organism is less than 20% phosphoglyceride (59), a primary lipid type in mammalian neurons. As we later discuss in the context of halorhodopsins, the use of signaling peptides can improve outer membrane localization and reduce aggregation in the cytosol, endoplasmic reticulum, and Golgi apparatus.

Along similar lines of using signal peptides to alter trafficking, the myosin binding domain (MBD) peptide promotes subcellular localization of opsins to neuronal dendrites (9). This subcellular localization strategy may prove to be helpful for enabling driving of electrical activity in specific neural compartments, or for high-resolution connectomic mapping in vivo. Two-photon excitation is a powerful laser excitation technique that enables submicron resolution in 3-D (60) relatively deep into the brain (~750 μm, or a significant fraction of the thickness of the mouse cortex), but its ability to induce action potentials in a neuron expressing ChR2 is limited by the interplay between molecule density and the extent of optical depolarization with respect to time (61, 62). The probability of inducing an action potential, at low powers

that are not destructive to tissue, is relatively low using a traditional raster scan because the fraction of molecules excited at any point in time is small, and most photons that do hit the membrane are wasted (since the open time of ChR2 is long relative to a femtosecond laser photon delivery rate). Thus, with most conventional two-photon laser scanning methods, the aggregate contributions of the serially excited molecules never sufficiently depolarize the whole neuron to spike threshold. However, Rickgauer and Tank have demonstrated that neurons expressing ChR2 can be reliably excited by two-photon microscopy by optimizing the scan pattern to deliver light optimally to the cell membrane, in a fashion that reaches the maximum surface area while minimizing wastage of photons on already-light-driven channelrhodopsin molecules (62).

3.5. Diversity

Unlike microbial rhodopsins from archaea, significantly less is known about the photoelectrogenic molecules of unicellular algae, the only organisms known to date to have naturally occurring light-activated channels. Photoelectric responses have been measured in several green flagellates, as well as phylogenetically distant cryptophytes (47, 63–65). Interestingly, the two-component phototaxis strategy employed by *C. reinhardtii*, in which the response is mediated by a fast (ChR1) and slow (ChR2) rhodopsin, appears to be general (47), which begs the question whether chimeras of their respective rhodopsins will also result in kinetic improvements and variants with interesting properties. Thus, as more phototaxis-mediating rhodopsins are isolated and sequenced, or as perhaps new depolarizing rhodopsin types are discovered, new molecular tools for controlling neurons will surely emerge.

4. Optical Neural Silencing: Halorhodopsins and Bacteriorhodopsins

Whereas traditional electrodes can stimulate neurons with temporal precision (albeit without cell type specificity), they are incapable of silencing neurons in order to assess their necessity for given neural computations, behaviors, and pathologies. Therefore, there is a large need for spatio-temporally precise methods for optical inhibition of neurons. Inwardly rectifying chloride pumps and outwardly rectifying proton pumps, halorhodopsins (HRs, hops) and bacteriorhodopsins (BRs, bops), respectively, are electrogenic pumps that when heterologously expressed are capable of sufficiently hyperpolarizing a neuron to silence its activity (Fig. 1c–e; (12, 15, 66)). They are thus far known to exist in every kingdom except for animals: archaea (22, 23, 67–69), bacteria (70–76), fungi (77, 78) and algae (79). In addition to their opposite electrophysiological effect, HRs and BRs differ primarily

from channelrhodopsins in that their physiological functions is chiefly due to their role as pumps as opposed to operating as passive channels, and thus can translocate ions against concentration gradients (but, typically only one ion per photocycle). Much is known about the photocycles and structure–function relationships of HRs and BRs because they have been crystallized (24, 27, 28, 80, 81) and heavily characterized via spectroscopy, mutagenesis, and physiology for decades.

This section focuses on two molecules in particular: *N. pharaonis* halorhodopsin (Halo/NpHR) and *H. sodomense* bacteriorhodopsin (Arch/AR-3), also known as an archaerhodopsin (that is, a bacteriorhodopsin from the *halorubrum* genus). Halorhodopsins were shown in 2007 to be capable of mediating modest optical neural hyperpolarizations, and since have been improved in trafficking to boost their currents (12, 13, 16); bacteriorhodopsins were shown in 2009 to be able to mediate very powerful and kinetically versatile silencing of multiple neural populations with different colors of light (18). We discuss in the following sections “Conductance, permeability, and context” and “Kinetics and wavelength selectivity” of halorhodopsins and bacteriorhodopsins for these two classes separately, followed by a joint discussion of the “Mutants and variants” and genomic “Diversity” in a unified section.

4.1. Halorhodopsins: Conductance, Permeability, and Context

N. pharaonis halorhodopsin (NpHR, Halo) is a highly selective, inwardly rectifying, chloride pump, which can also conduct larger monovalent anions (82). It has a reversal potential of approximately -400 mV (82), and its chloride-dependence of pumping activity (full- and half-saturating chloride concentrations: $[\text{Cl}^-]_{\text{saturation}} = 20$ mM, $[\text{Cl}^-]_{1/2} = 2.5$ mM) (83) is appropriate for operation in mammalian cells, in contrast to *H. salinarum* halorhodopsin, which is not capable of effective operation in mammalian neurons (15), presumably because of its large chloride dependency: $[\text{Cl}^-]_{\text{saturation}} = 5$ M and $[\text{Cl}^-]_{1/2} = 200$ mM (83, 84). In the absence of any signal peptide sequences to improve trafficking and membrane localization, Halo has been reported to generate 40–100 pA of hyperpolarizing current (12, 18, 66), with the differences in measured photocurrents between studies largely attributable to the power and wavelength of excitation used. This photocurrent is approximately 10–25-fold less than typical peak depolarizing currents generated by ChR2, highlighting one potential disadvantage inherent to a pump that translocates one ion per photocycle (e.g., Halo) vs. a channel that conducts 300 ions per photocycle (e.g., ChR2). To mediate these currents, there are an estimated ten million membrane-embedded Halo molecules per neuron (as assessed in hippocampal neuron culture). Because the expression levels are so high, Halo is known to form puncta or intracellular blebs, aggregating in the endoplasmic reticulum (ER) and Golgi apparatus (16, 17). These issues are somewhat addressed by attaching trafficking enhancement sequences to

the molecule, e.g., sequences from the Kir2.1 protein (eNpHR, eNpHR3.0), which increases the effective conductance several fold by increasing membrane expression (16, 85).

Recently, we have discovered that the crux-halorhodopsin (HR from the *haloarcula* genus) from *Haloarcula marismortui*, canonically known as cHR-5 (69, 86), produces similar photocurrents to Halo with more uniform expression; even when highly overexpressed under high copy number transfection conditions, no puncta or intracellular blebbing is observed (15). This molecule may better express than Halo in vivo, but it is unknown at this moment whether it will ultimately be more efficacious at altering mammalian behavior, given their statistically insignificant difference in photocurrent. However, the prolactin (Prl) ER-location sequence in conjunction with a signal sequence from a MHC class I antigen triples the Halo photocurrent (15, 18); we are now trying out multiple trafficking sequences in combination to see if they boost current further. However, it is important to note that if halorhodopsins have other side effects that are due to the protein's intrinsic properties—as an example, one paper quantitates the significantly altered neuronal capacitance that results from expressing halorhodopsin in neurons in vivo (87)—then boosting expression may only make such side effects worse.

4.2. Halorhodopsins: Kinetics and Wavelength Selectivity

N. pharaonis halorhodopsin is capable of silencing weakly firing neurons on the millisecond timescale with its ~100 pA-scale currents, with rapid onset and offset (12), but during long periods of illumination, all halorhodopsins that we have tested so far and that have current (from *N. pharaonis*, *H. sodomense*, *Haloarcula vallismortis*, *H. marismortui*, and *Salinibacter ruber*) inactivate by approximately 30% every 15 s of illumination at 1–10 mW/mm² yellow (593 nm) light (Fig. 4a, b; (12, 18)). This slow inactivation stands in contrast to ChR2, which responds to light with a large transient peak that decays within seconds, followed by a stable stationary photocurrent. For all of the halorhodopsins named above, recovery in the dark from light-induced inactivation is slow, with a time constant of tens of minutes, as has been described for some halorhodopsins earlier (12, 14, 39) (Fig. 4a, b). This long-lasting inactivation property may hinder the use of halorhodopsins for silencing for prolonged periods, e.g., during repeated behavioral trials. Importantly, for all halorhodopsins investigated, the inactive photoproduct can be driven back into its active pumping state with a short (e.g., subsecond duration) pulse of blue or UV light (12, 14); thus, optimal use of Halo for neural silencing requires both yellow and blue light to be delivered to the same set of neurons, which is possible (88) but can complicate optics setups.

The Halo photocycle is shown in schematic form in Fig. 2c. The time constants listed are the limiting ones, with all other transitions <100 μs. It should be noted that the names for

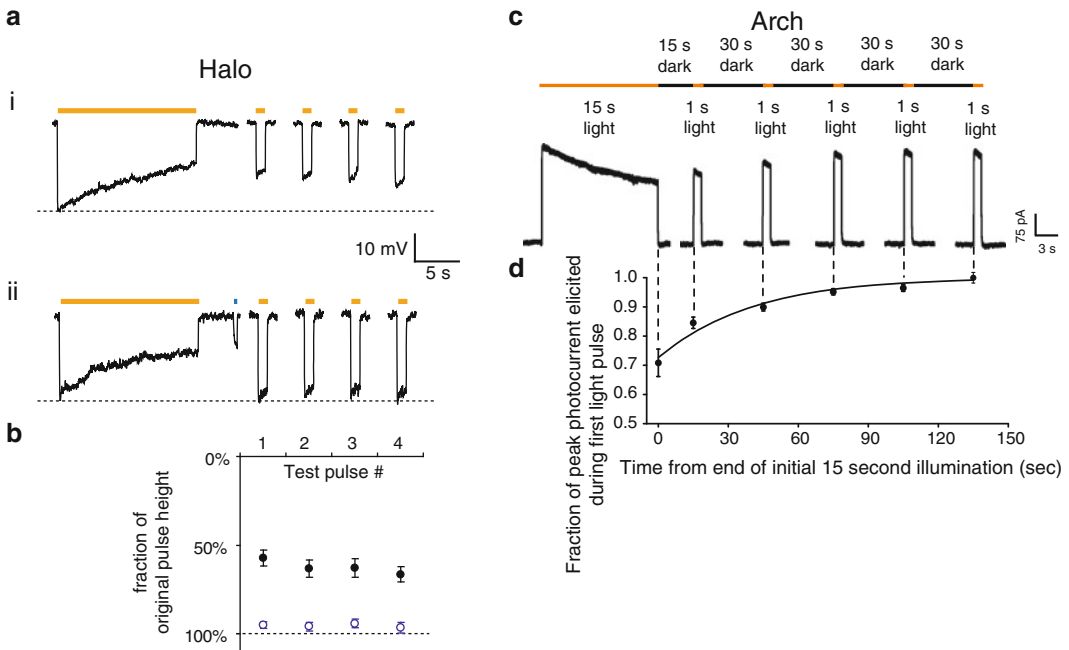


Fig. 4. Kinetic comparisons between halorhodopsins and archaerhodopsins. **(ai)** Time course of Halo-mediated hyperpolarizations in a representative current-clamped hippocampal neuron during 15 s of continuous yellow light, followed by four 1-s test pulses of yellow light (one every 30 s, starting 10 s after the end of the first 15-s period of yellow light). **(a ii)** Time course of Halo-mediated hyperpolarization for the same cell exhibited in **(ai)**, but when Halo function is facilitated by a 400-ms pulse of blue light in between the 15-s period of yellow light and the first 1-s test pulse. **(b)** Population data for blue-light facilitation of Halo recovery ($N = 8$ neurons). Plotted are the hyperpolarizations elicited by the four 1-s test pulses of yellow light, normalized to the peak hyperpolarization induced by the original 15-s yellow light pulse. *Dots* represent mean \pm S.E.M. *Black dots* represent experiments when no blue light pulse was delivered (as in Fig. 5ai.). *Open blue dots* represent experiments when 400 ms of blue light was delivered to facilitate recovery (as in Fig. 5a ii.). **(c)** Raw current trace of a neuron lentivirally infected with Arch, illuminated by a 15 s light pulse (575 ± 25 nm, irradiance 7.8 mW/mm²), followed by 1 s test pulses delivered starting 15, 45, 75, 105, and 135 s after the end of the 15 s light pulse. **(d)** Population data of averaged Arch photocurrents ($N = 11$ neurons) sampled at the times indicated by the *vertical dotted lines* that extend into Fig. 4c.

canonical spectroscopic states of *H. salinarum* halorhodopsin—the K, L, N, and O photointermediates—have not been used here because the order of the N- and O-states in *N. pharaonis* halorhodopsin is still somewhat debated (42, 89, 90). In the dominant photocycle, Halo absorbs a photon and then within tens of microseconds, quickly releases a chloride ion into the cytoplasm during the HR520 \rightarrow H640 transition, via short-lived intermediates that “switch” the chloride location within the molecule from the extracellular loading domain to the cytoplasmic release domain. The molecule then reisomerizes from the HR640 state and takes up a chloride ion from the extracellular side, a process that takes ~ 1.5 ms; it then forms the HR' state, which finally relaxes to the active ground state with a time constant of ~ 20 ms. However,

halorhodopsins can enter an alternate photocycle (middle trajectory within Fig. 2c), most notably under prolonged or bright illumination, as might occur during *in vivo* neural silencing. The 13-*cis* retinaldehyde Schiff base becomes deprotonated (releasing a proton into the cytoplasm and thus introducing a small depolarizing proton current) and forms a long-lived intermediate HR410 (14, 39). In the dark, the halorhodopsin will remain in this inactive state for a duration on the order of 30 min (39). This formation of HR410 is the origin of the long inactivation observed in neurons expressing halorhodopsin and the ability to recover the active state using the short blue light pulse (12, 14). In contrast, as we discuss in detail in the next section, archaerhodopsins (bacteriorhodopsins from the *Halorubrum* genus) spontaneously recover in the dark under physiological conditions.

4.3. Bacteriorhodopsins: Conductance, Permeability, and Context

Arch, canonically known as archaerhodopsin-3 (AR-3) from *H. sodomense*, is a yellow-green-light sensitive outwardly rectifying proton pump with nearly an order of magnitude increase in hyperpolarizing current over any characterized natural halorhodopsin (15, 18), attaining neuronal currents up to 900 pA *in vitro* in response to light powers easily achievable *in vitro* or *in vivo*. The efficacy of these proton pumps is surprising, given that protons occur, in mammalian tissue, at a millionfold-lower concentration than the ions carried by the optical control molecules described above. This high efficacy may be due to the fast photocycle of Arch (see also (91, 92)), but it may also be due to the ability of high-pKa residues in proton pumps to mediate proton uptake (91, 93).

Arch is a highly efficacious tool *in vivo*, with cortical neurons in the awake behaving mouse undergoing a median of 97.1% reductions in firing rate for periods of seconds to minutes (Fig. 1e) (18), and safely expresses for months in both mice and monkeys when virally delivered *in vivo*. Due to the larger currents, Arch enables very large (e.g., order of magnitude-scale) increases in addressable volume of tissue silenceable, over earlier reagents. We thoroughly investigated the safety of Arch function. To date, blebbing issues that have affected the usage of halorhodopsins have not been observed *in vitro* or *in vivo* for Arch, but membrane trafficking sequences may still prove effective at boosting expression beyond the natural state (the Prl sequence, which greatly magnifies Halo current, slightly increases Arch current in neurons). Furthermore, we have not observed changes in cell membrane capacitance or other passive neural properties, as has been reported with halorhodopsin expression (see above). From an end-user standpoint, illumination of Arch neurons was safe: spike rates measured *in vivo* were not significantly different before vs. after periods of optical neural silencing. Biophysically, pH changes in neurons expressing Arch and undergoing illumination were minimal, plateauing rapidly at alkalinizations of ~0.1–0.15 pH units; the fast stabilization of pH;

may reflect a self-limiting influence that rapidly limits proton concentration swings, and may contribute to the safe operation of Arch in neurons, as observed in mice and monkeys. Indeed, the changes in pH observed in cells expressing Arch and being illuminated are comparable in magnitude to those observed during illumination of ChR2-expressing cells (10) (due to the proton currents carried by ChR2 (1, 46)) and are also within the magnitudes of changes observed during normal neural activity (94–97). We have observed that other archaerhodopsins from other *Halorubrum* strains are also particularly powerful molecular reagents (work in progress). In contrast, the canonical *H. salinarum* bacteriorhodopsin, well known to poorly function in *Escherichia coli*, successfully produced modest photocurrents in mammalian neurons, which highlights the importance of not assuming all molecules will express and traffic the same in different organisms. (In contrast, *E. coli* does not support any detectable expression of ChR2, which expresses well in neurons; target species influences in modulation of opsin function should not be underestimated).

4.4. Bacteriorhodopsins: Kinetics and Wavelength Selectivity

Unlike all of the halorhodopsins we have screened to date (including not only the natural halorhodopsins described above, but also products of halorhodopsin site-directed mutagenesis aimed at improving kinetics), which after illumination remained inactivated for tens of minutes, Arch spontaneously recovers function in seconds in the dark (Fig. 4c, d), more like the light-gated cation channel channelrhodopsin-2 (ChR2) than like halorhodopsins. This feature is particularly useful for *in vivo* behavior work because it dramatically simplifies the necessary optical hardware; the need to use only one wavelength of light also increases the available bandwidth for multicolor silencing in multiple cell-types. This spontaneous recovery has also been observed by us with other archaerhodopsins, and thus may be a general feature of archaerhodopsins as a whole (work in progress).

Arch is maximally excited with green-yellow light ($\lambda = 561$ nm), a fairly common peak wavelength for proton pumps. Thus, it is backward compatible with halorhodopsin-driving equipment. Proton pumps naturally exist that are activated by many colors of light, in contrast to chloride pumps, which are primarily driven by yellow-orange light (even with significant mutagenesis of retinal-flanking residues, (15)). The light-driven proton pump from *L. maculans*, here abbreviated Mac, has an action spectrum strongly blue-shifted relative to that of the light-driven chloride pump Halo (Fig. 5a). We found that Mac-expressing neurons could undergo 4.1-fold larger hyperpolarizations with blue light than with red light, and Halo-expressing neurons could undergo 3.3-fold larger hyperpolarizations with red light than with blue light, when illuminated with appropriate powers and filters (Fig. 5b). Accordingly, we could demonstrate selective silencing of spike firing in Mac-expressing

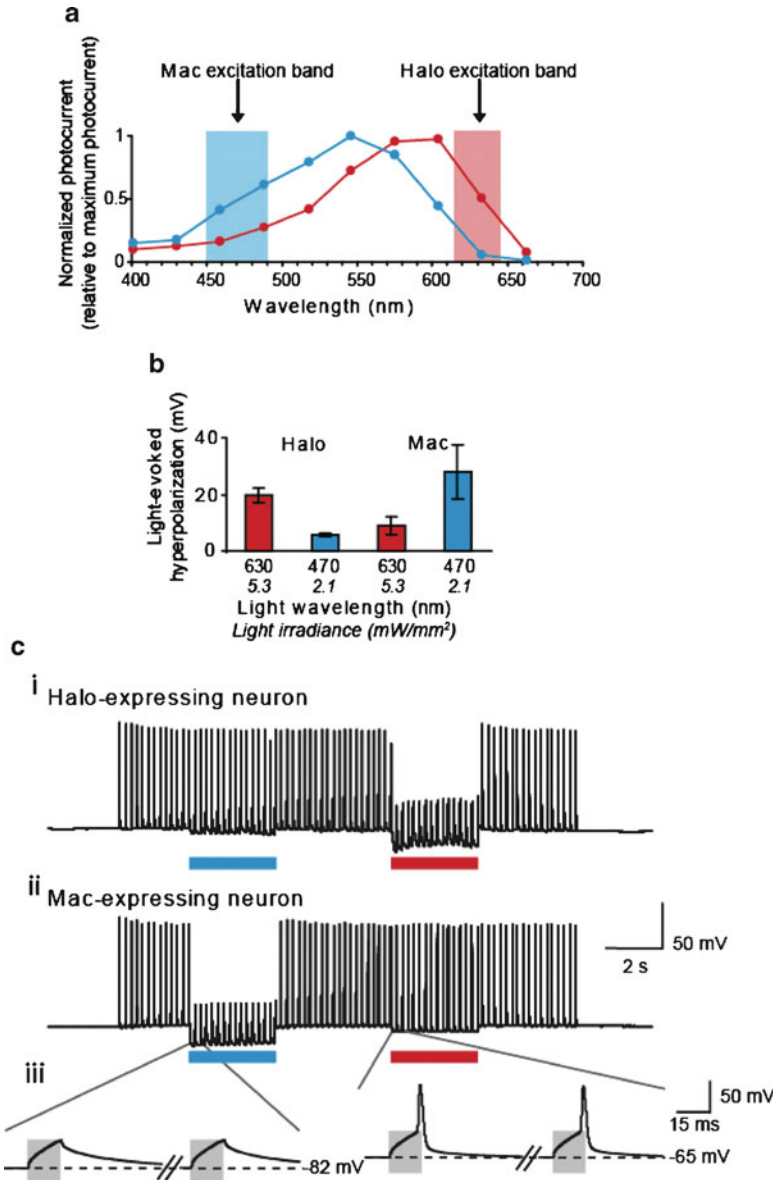


Fig. 5. Multicolor silencing of two neural populations, enabled by blue- and red-light drivable ion pumps of different classes. (a) Action spectra of Mac vs. Halo; *rectangles* indicate filter bandwidths used for multicolor silencing in vitro. Blue light power is via a 470 ± 20 nm filter at 5.3 mW/mm^2 , and red light power is via a 630 ± 15 nm filter at 2.1 mW/mm^2 . (b) Membrane hyperpolarizations elicited by blue vs. red light, in cells expressing Halo or Mac ($N = 5$ Mac-expressing neurons, $N = 6$ Halo-expressing neurons). (c) Action potentials evoked by current injection into patch clamped cultured neurons transfected with Halo (ci) were selectively silenced by the red light but not by the blue light, and vice versa in neurons expressing Mac (cii). *Gray boxes* in the *inset* (ciii) indicate periods of patch clamp current injection.

neurons in response to blue light, and selective silencing of spike firing in Halo-expressing neurons in response to red light (Fig. 5c). Thus, the spectral diversity of proton pumps points the way toward independent multicolor silencing of separate neural populations.

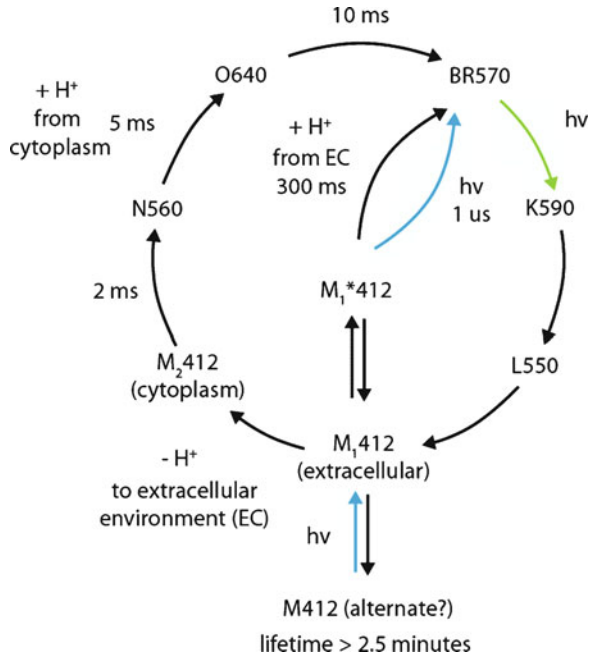


Fig. 6. The photocyclus of the *H. salinarum* bacteriorhodopsin. As in Figs. 2c and 3b, the photocyclus has been simplified to reflect the dominant photocyclus at large continuous illumination on the timescale of a typical photocyclus (i.e., conditions used for neural silencing, >few milliseconds). The M412 alternate intermediate is the origin of long-lived inactivation with bacteriorhodopsin. In contrast, Arch spontaneously quickly recovers from this state in the dark.

This result opens up novel kinds of experiment, in which, for example, two neuron classes, or two sets of neural projections from a single site, can be independently silenced during a behavioral task.

Figure 6 shows the photocyclus of the canonical *H. salinarum* bacteriorhodopsin, as representative of the photocyclus of the class of proton pumps (the photocyclus of archaerhodopsins are not as well characterized, and may well be different) (22, 93). The data that has led to the synthesis of the modern model of the bacteriorhodopsin photocyclus provide many of the insights that have led to solid investigations into this field as a whole. As in Figs. 2c and 3b, it has been simplified to represent the dominant photocyclus expected at large and continuous illumination on the timescale of a typical photocyclus (e.g., many milliseconds, as would be used for neural silencing). Upon absorbing a photon, bacteriorhodopsin forms its L550 intermediate (L = lumi) within microseconds, after quickly transferring a proton from the retinylidene Schiff base (at the Lys-216 position) to the Asp-85 proton acceptor. This transfer triggers the proton releasing group (PRG), containing Glu-204 and Glu-194, to release its own proton (98, 99), during the L → M transition. After a “switching” step during which the Schiff base reorients itself on the cytoplasmic side, it is then

reprotonated during the M → N transition via the Asp-96 donor residue, which in turn picks up a proton from the cytoplasm in the next transition (N → O). The chromophore re-isomerizes to the all-*trans* state during this transition as well. Finally, the bacteriorhodopsin relaxes back to its ground state as the proton release group is reloaded from the Asp-85 residue. Like halorhodopsin, bacteriorhodopsin can become trapped in a long-lasting light-unresponsive state (Fig. 6, “M412 alternate”) that requires blue light to reenter the normal photocycle; this could partly explain the lower currents observed with BR when compared to Arch.

4.5. Halorhodopsins and Bacteriorhodopsins: Mutants and Variants

For decades, researchers have been making mutants of bacteriorhodopsins and halorhodopsins for structure–function studies reviewed in many earlier publications (e.g., (22, 24, 93)). However, point mutations that improve these molecules as neural silencing tools have yet to be reported. Given that the conductance of a pump is limited by the fact that they move only one ion per photocycle, it would be highly desirable to mutate halorhodopsin into a channel, or channelrhodopsin into an anion channel. Tuning the action spectrum of both bacteriorhodopsins and halorhodopsins may be achieved by mutating the retinal-flanking residues (75, 76, 100, 101). Mutations that alter ion selectivity, such as the most well-known example of this is the bacteriorhodopsin D85T mutation that converts it into a chloride pump (102), could allow ion-specific currents to be mimicked. A Ca²⁺ selective pump, for example, would have powerful impact on enabling powerful studies of plasticity, synaptic transmission, and cellular signaling. Given that crystal structures for many bacteriorhodopsins and halorhodopsins exist, we anticipate that functionally oriented and applied site-directed mutagenesis will be a highly active field of research in neuroengineering. The fact that archaeorhodopsins on the whole represent a class of molecules that express particularly well in mammalian cells possibly indicates properties of their lipid-interacting residues, or perhaps “signal sequence”-like activity by their loop regions. The known crystal structures of these molecules (80, 81) may provide key insights into the trafficking of heterologously expressed molecules and their membrane insertion.

4.6. Diversity

Light-activated proton and chloride pumps are known to exist in far more organisms than do light-gated cation channels, and proton pumps are particularly prevalent, as all opsins described to date likely have at least some proton pumping capability (22, 23, 67–79, 103, 104). Even though many proton pumps maximally absorb blue-green to green wavelengths (which opens up, as shown in Fig. 5, the possibility, alongside yellow-light driven chloride pumps, for multiple-color silencing of distinct neural populations), light-activated hyperpolarizing currents carried by protons have been observed across the whole visible spectrum, from deep blue

(via a sensitizer) (105) to far red (106) (>650 nm), although the red-light sensitive current likely originates from a receptor that triggers an H⁺-ATPase, as opposed to direct light-mediated ion translocation.

The discovery or creation of a purely genetically encoded light-activated inhibitory channel would be highly desirable. In addition to seeking natural molecules and site-directed mutants, linking a non-light-gated ion-channel to a type I archaeal rhodopsin may be another promising approach to creating such a molecular tool (107). In this way, a light-activated shunt could be created, which would more closely mimic natural mechanisms of neural inhibition in the brain.

5. Molecular Targeting of Microbial Opsins to Different Cell Types

The number of papers using optical neural control in species ranging from *C. elegans* to mouse to nonhuman primate is increasing exponentially each year, and so we do not attempt to review the literature comprehensively. In each species, opsins have been used to test the necessity and sufficiency of neurons, cell types, muscles, neural pathways, brain regions, and other entities in behaviors, pathologies, and neural computations. Opsins have proven valuable in exploring neural dynamics in multiple mammalian brain structures as well. We focus on highlighting principles that govern how to best use these opsins in various settings, from a molecular biology standpoint (Sect. 5) and from a physical–optical standpoint (Sect. 6). From a molecular biology standpoint, these opsins can be delivered to neurons in almost any conventional way that genes are delivered into cells or into organisms. Transgenic mice have been made with ChR2, for example (108), mice and monkeys have been injected with lentiviruses, adeno-associated viruses (AAV), and other viruses encoding for light-gated proteins (2, 52, 109–111), and rodents and chicks have been electroporated in utero with plasmids encoding for light-gated proteins (4, 53, 112). In each case, different parameters of the technique can be selected so as to enable specific cell types, pathways, or regions to be selectively labeled or to selectively express the opsin. Transgenic *C. elegans* have been made expressing ChR2 using conventional methods (5), as have transgenic *Drosophila* (113) and zebrafish (114). For these latter species, supplementation with all-*trans*-retinal may be beneficial, whereas mammalian brains seem to operate microbial opsins without need for supplementation. (It is possible that in the future, genetically engineering retinal-lacking organisms, such as invertebrates, to produce retinal within their nervous systems may be of use, e.g., by expressing within them enzymes that can produce retinal from vitamin precursors (115)).

For mammalian nervous systems, a large variety of possible strategies exist for conveying opsin genes into specific cell types. For example, transgenic mice can be made through BAC transgenic, knock-in, or other methods, but such strategies are not common for other species yet. For viral delivery, cell type specific promoters can be inserted upstream of the opsin to target various excitatory, inhibitory, and modulatory neurons (e.g., (19, 116–119)); the size of the promoter is limited by the virus type (AAV viruses hold typically 4–4.5 kb total, whereas lentiviruses hold typically 8–10 kb maximum). The surface or coat proteins that a virus bears can also modulate which cell types will take up the virus; for example, lentiviruses may favor excitatory neurons of the cortex, whereas certain AAV serotypes may favor inhibitory cells (120). Lentiviruses can be pseudotyped—fabricated with a coat protein of desired targeting capacity, e.g., with rabies glycoprotein that leads to lentiviruses that travel retrogradely (121)—whereas AAVs can be engineered with biotinylation sites that enable, upon streptavidin conjugation, targeting of potentially arbitrary substrates (122). Retroviruses, which preferentially label dividing cells, have been used to deliver ChR2 to newborn neurons of the dentate gyrus of the hippocampus (123). Other viruses such as rabies virus and pseudorabies virus can be used, with unique tracing capabilities including the ability to go retrogradely across multiple synapses (124, 125). Viral particles typically have to be injected directly into the brain, often through stereotactic targeting to a specific brain area, since the potent blood–brain barrier typically precludes systemic delivery of large viral particles (although see (126)). We have recently described a parallel injector array that can deliver viruses into complex three-dimensional configurations (127). Finally, in utero electroporation of ChR2-GFP into embryonic mice at embryonic day ~15.5 has been found to selectively label pyramidal cells in layers 2/3 (53, 112).

Of potential interest is the possibility of a new generation of neural prosthetics, which can accomplish the synthetic neurobiology mission of repairing the nervous system by enabling optical input of information to sculpt neural dynamics and overcome pathology. To deliver these genes in a safe, efficacious, and enduring way, viruses such as AAV may be valuable; AAV has been used in over 600 human patients in gene therapy clinical trials without a single serious adverse event (128) and has been successfully used with opsin delivery. The ability to optically control specific targets within the brain may enable more potent and side-effect free therapies than possible with existing electrical and magnetic neuromodulation therapies, or with drugs which often are nonspecific and have side effects. Already several groups have prototyped blindness therapies that may enable new approaches to a

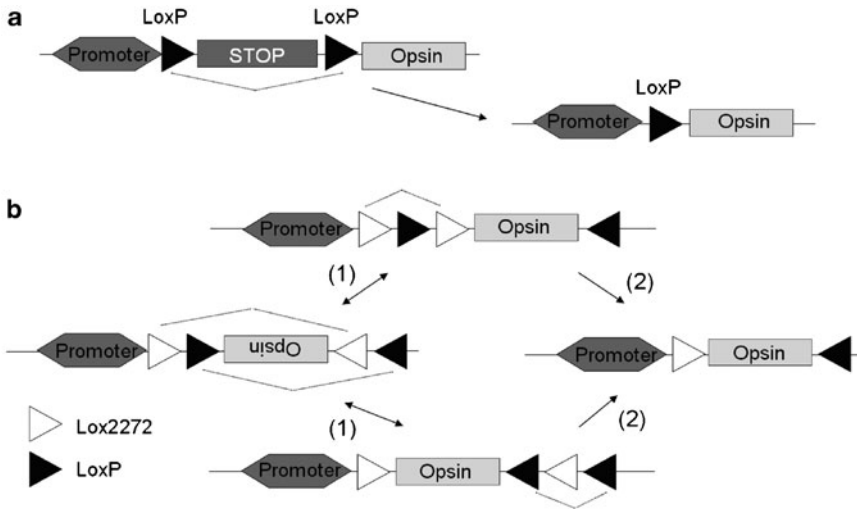


Fig. 7. Transgenic mouse expressing Cre within specific cells, coupled to lox-containing AAV viruses, enable cell-type specific opsin expression. (a) A pair of loxP recombination sequences mediate the removal of the transcriptional and translational stop cassette containing multiple polyadenylation signals (STOP), in the presence of Cre (provided in transgenic mice within specific cell types), to initiate gene expression. (b) Two pairs of loxP-type recombination sequences (FLEX) for stable inversion proceeds in two steps: (1) inversion followed by (2) excision. loxP and lox2272 are orthogonal recombination sites. (Adapted from Kuhlman and Huang (111) and Atasoy et al. (110)).

currently intractable set of disorders, those in which photoreceptors degenerate within the retina (129, 130). To that end, the recent assessment of brain and immune function in nonhuman primates after ChR2 expression and activation, which showed a lack of harmful effects in a preliminary study (109), may pave the way toward new ideas for neural prosthetics for humans.

One strategy that has become widely popular, is to inject the brain of a mouse expressing Cre recombinase in a specific cell type, with a virus that either bears an opsin preceded by a lox-flanked stop cassette, or a virus that bears an opsin reversed and flanked by pairs of lox sites in a specific configuration (Fig. 7; (110, 111)). Given the very large number of Cre transgenic mice in existence, and that are being generated, this strategy is likely to be very useful, at least for mice. Transgenic mice can be made that express Cre in extremely cell specific ways (e.g., through 3' UTR knockins at the ends of cell type-specific genes), and then viruses can be rapidly made as new opsins are created, thus enabling cell type-specific expression without requiring the difficult process of trimming cell-specific promoters to fit within the small viral payload, or the difficult process of making transgenic mice for each new opsin tool that is developed (given the rapid pace of innovation, as shown in Sects. 1–4).

6. Hardware for Optical Neural Control

For *in vitro* use, xenon lamps (e.g., Sutter DG-4, Till Photonics Polychrome) equipped with fast-moving mirrors or monochromators can be used for flexible delivery of fast (millisecond-timescale), bright light pulses to biological samples on microscopes. Fluorescence filters can be used to deliver light of the appropriate wavelength (e.g., GFP excitation filter for Chr2, rhodamine or Texas Red excitation filter for Arch, Texas Red excitation filter for Halo, and GFP or YFP excitation filter for Mac). Recently, many companies such as Thorlabs have begun to sell LEDs or LED arrays compatible with microscope fluorescence illuminators, which sell for a small fraction of the price of a full lamp setup. Or fiber-coupled LEDs can simply be placed nearby to the sample (131). Confocal and two-photon microscopes, or more generally scanning laser methodologies, can be used to drive opsins, as have been described in a variety of papers (112, 132, 133). Recently, digital micromirror displays (DMDs) have come forth as potentially useful for photostimulating in complex patterns, comprising millions of individual pixels that can be toggled either on or off (134, 135).

In vivo, the brain scatters light starting within a few hundred microns of an optical source, and absorbs light starting within a few millimeters of distance. Thus, most efforts for *in vivo* neuromodulation focus on delivering light to a volume of tissue, ranging from a very small volume containing a few hundred cells to a large volume (say, a few cubic millimeters) containing many thousands of cells. One widespread method is to use a laser coupled to an optical fiber (Fig. 8a shows a versatile setup that couples multiple colors of laser light into a single fiber; simpler commercially available single-color laser-coupled fibers with TTL control are also available), and to insert the fiber into a cannula implanted in the brain (Fig. 8b shows a hand-built one for mice; commercial versions from companies such as Plastics One can also be built) or directly into the brain (Fig. 8c shows a setup for monkey), or to couple the fiber to an implanted fiber via a ferrule. An optical commutator (e.g., from Doric Lenses) can be placed between the fiber that inserts into the brain and the fiber that connects to the laser, to allow free rotation (66). Arrays of custom-targetable optical fibers, each coupled to a miniaturized light source (e.g., a raw die LED) and targeted to a unique target, will open up the ability to drive activity in complex 3-D patterns, enabling the perturbation of complexly shaped structures as well as the ability to perturb targets in a patterned fashion (136, 137).

Aside from the key advantage of being able to manipulate a specific cell type, another key advantage of optical stimulation is the lack of electrical artifact as compared to conventional electrical

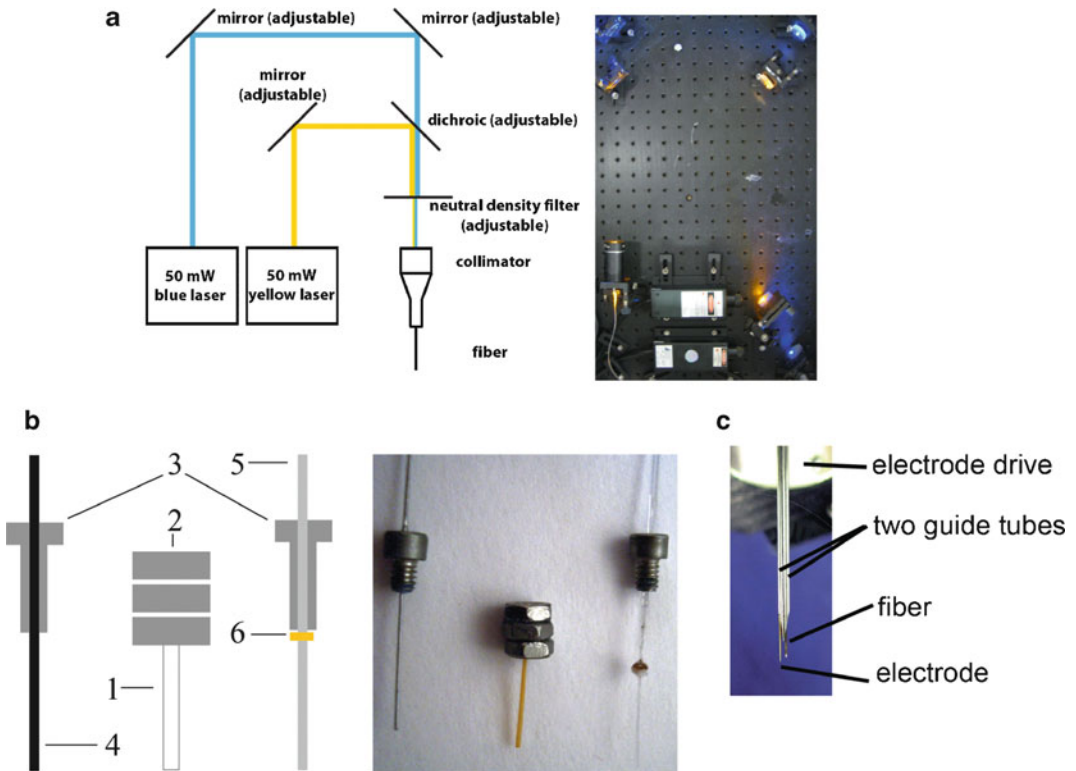


Fig. 8. (a) Schematic design (*left*) and picture (*right*) of an optics assembly used to couple blue and yellow laser light into a single optical fiber for in vivo neural modulation. A pictured assembly, lacking a neutral density filter, shows the hardware laid out on a standard optics breadboard. (b) Schematic design (*left*) and picture (*right*) of a system for targeting and securing optical fibers within the brain. A polyimide cannula (1, 250 μm ID), designed to terminate at the locus of optical modulation, is epoxied to a stack of hex nuts (2, sized 2–56) which will be secured to the skull with dental cement. Vented screws (3, sized 2–56), which have holes in their centers, screw into the nuts while leaving a path open to the cannula. A dummy wire (4, 230 μm stainless steel wire) may be epoxied to the screw to seal the craniotomy when the optics are not in use. An optical fiber (5, 230 μm OD silica fiber) is allowed free rotation without vertical displacement by a plastic washer (6, homemade) which is epoxied to the fiber and sandwiched in between the vented screw above and the cannula below. (c) Apparatus for optical activation and electrical recording. Photograph, showing optical fiber (200 μm diameter) and electrode (200 μm shank diameter) in guide tubes. Adapted from Han et al. (19).

stimulation methods. However, despite the lack of electrical artifact, light does produce a voltage deflection when the electrode tip was illuminated (19, 138). For example, Fig. 9 shows traces recorded on a tungsten electrode in saline being illuminated by a pulsed laser beam, as adapted from (19). This voltage deflection slowly evolved over many tens of milliseconds, and accordingly was only recorded when the electrode voltage filtered at 0.7–170 Hz to examine local field potentials, (Fig. 9, top traces of each panel). This voltage deflection was not recorded when the electrode voltage was filtered at 250–8,000 Hz to detect spike signals; (Fig. 9, bottom traces of each panel). When light illuminated parts of the electrode other than the tip, no artifact was recorded; only illumination of the

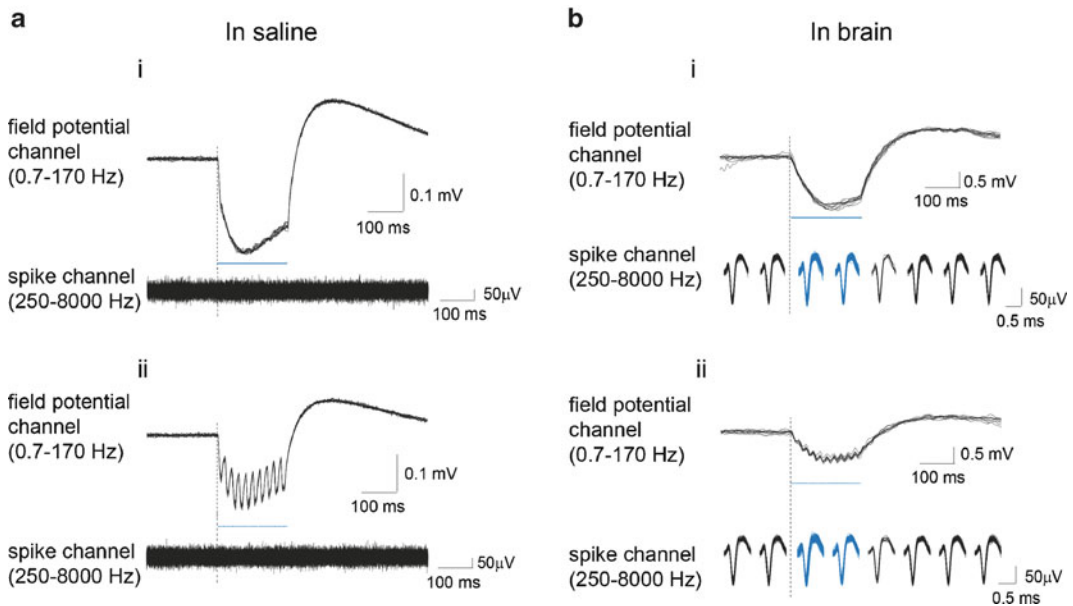


Fig. 9. Voltage deflections observed on tungsten electrodes immersed in saline (a) or brain (b), upon tip exposure to 200 ms blue light pulses (bi) or trains of 10 ms blue light pulses delivered at 50 Hz (bii). Light pulses are indicated by *blue dashes*. Electrode data was hardware filtered using two data acquisition channels operating in parallel, yielding a low-frequency component (“field potential channel”) and a high-frequency component (“spike channel”). For the “spike channel” traces taken in brain (b), spikes were grouped into 100 ms bins, and then the binned spikes were displayed beneath corresponding parts of the simultaneously acquired “field potential channel” signal (59 and 53 repeated light exposures for bi and bii respectively). (Shown are the spikes in eight such bins—the two bins before light onset, and the four bins after light cessation.) For all other signals shown, ten overlaid traces are plotted. From Han et al. (19).

tip–saline interface resulted in the voltage transient. This phenomenon is consistent with a classical photoelectrochemical finding, the Becquerel effect, in which illumination of an electrode placed in saline can produce a voltage on the electrode (139, 140). Consistent with the generality of the Becquerel effect as a property of electrode–electrolyte interfaces, this artifact is observed on various electrode materials, such as stainless steel, platinum–iridium, silver/silver chloride, gold, nichrome, or copper. Similar slowly evolving voltage deflections were observed when tungsten electrodes were used to record neural activity in the brain, during optical stimulation. Because the optical artifact was slowly evolving over many tens of milliseconds, spike waveforms were detected without corruption by the artifact. However, local field potentials and field oscillations, which reflect coherent neural dynamics in the range of Hertz to tens of Hertz, may be difficult to isolate from this Becquerel artifact using the electrodes here tested. Notably, we have not seen the artifact with pulled glass micropipettes (such as previously used in (3) and (12), or in the mouse recordings with pulled glass pipettes in (109)). Thus, for recordings of local field potentials and other slow signals of importance for neuroscience, hollow glass electrodes may prove useful.

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