ACCOUNTS of chemical research

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Painting with Rainbows: Patterning Light in Space, Time, and Wavelength for Multiphoton Optogenetic Sensing and Control

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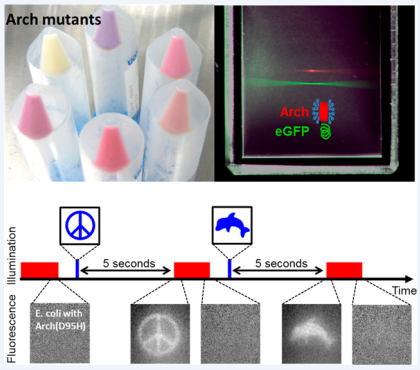
CONSPECTUS: Photons are a fascinating reagent, flowing and reacting quite differently compared to more massive and less ephemeral particles of matter. The optogenetic palette comprises an ever growing set of light-responsive proteins, which open the possibility of using light to perturb and to measure biological processes with great precision in space and time. Yet there are limits on what light can achieve. Diffraction limits the smallest features, and scattering in tissue limits the largest. Photobleaching, diffusion of photogenerated products, and optical crosstalk between overlapping absorption spectra further muddy the optogenetic picture, particularly when one wants to use multiple optogenetic tools simultaneously.



But these obstacles are surmountable. Most light-responsive proteins and small molecules undergo more than one light-driven transition, often with different action spectra and kinetics. By overlapping multiple laser beams, carefully patterned in space, time, and wavelength, one can steer molecules into fluorescent or nonfluorescent, active or inactive conformations. By doing so, one can often circumvent the limitations of simple one-photon excitation and achieve new imaging and stimulation capabilities. These include subdiffraction spatial resolution, optical sectioning, robustness to light scattering, and multiplexing of more channels than can be achieved with simple one-photon excitation.

The microbial rhodopsins are a particularly rich substrate for this type of multiphoton optical control. The natural diversity of these proteins presents a huge range of starting materials. The spectroscopy and photocycles of microbial rhodopsins are relatively well understood, providing states with absorption maxima across the visible spectrum, which can be accessed on experimentally convenient time scales. A long history of mutational studies in microbial rhodopsins allows semirational protein engineering. Mutants of Archaerhodopsin 3 (Arch) come in all the colors of the rainbow. In a solution of purified Arch-eGFP, a focused green laser excites eGFP fluorescence throughout the laser path, while a focused red laser excites fluorescence of Arch only near the focus, indicative of multiphoton fluorescence. This nonlinearity occurs at a laser intensity $\sim 10^{10}$-fold lower than in conventional two-photon microscopy! The mutant Arch(D95H) shows photoswitchable optical bistability. In a lawn of *E. coli* expressing this mutant, illumination with patterned blue light converts the molecule into a state that is fluorescent. Illumination with red light excites this fluorescence, and gradually resets the molecules back to the non-fluorescent state. This review describes the new types of molecular logic that can be implemented with multi-photon control of microbial rhodopsins, from whole-brain activity mapping to measurements of absolute membrane voltage.

Part of our goal in this Account is to describe recent work in nonlinear optogenetics, but we also present a variety of interesting things one could do if only the right optogenetic molecules were available. This latter component is intended to inspire future spectroscopic, protein discovery, and protein engineering work.

INTRODUCTION

Optogenetic technologies are revolutionizing biology. Much effort has focused on engineering protein reporters and actuators, molecular tools that interconvert between optical signals and local biochemical conditions. But equally important is engineering of the light. By stimulating molecules with carefully tuned and timed flashes of light, one can choreograph molecular motions to achieve novel and exciting forms of biochemical logic. Indeed, one can think of light as a remarkably versatile reagent. Here we describe emerging concepts and instrumentation for implementing new forms of optogenetic sensing and control. We focus on microbial rhodopsins, though the principles and techniques generalize to other optogenetic and photochemical tools.

Many, perhaps most, light-sensitive proteins undergo more than one light-driven transition: light potentiates or spectrally shifts the fluorescence in photoactivatable and photoswitchable fluorescent proteins;[1−4] light of different wavelengths drives dimerization and dissociation in the Phy-PIF,[5] Cry-CIB,[6] and Dronpa[7] optogenetic tools; and light drives multiple transitions among the seven or more states in the photocycles of microbial

Received: August 13, 2016
Published: October 27, 2016

DOI: 10.1021/acs.accounts.6b00415
Acc. Chem. Res. 2016, 49, 2518−2526

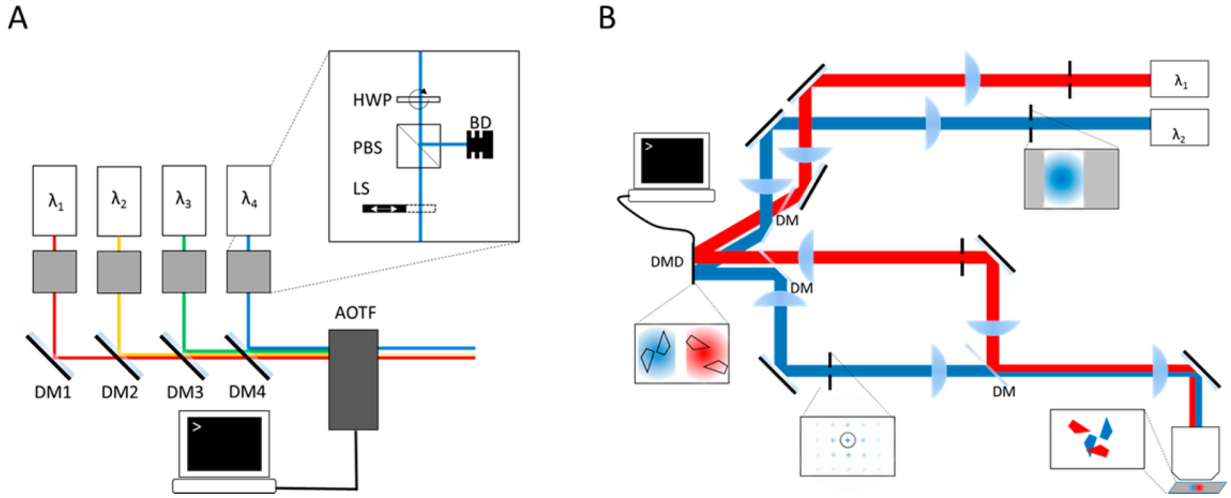


Figure 1. Optical systems for patterning light. (A) Spectral and temporal control via an acousto-optical tunable filter (AOTF), used to modulate the intensities of multiple laser lines simultaneously. Not shown are beam expansion and steering optics for each laser line. The combination of half-wave plate (HWP), polarizing beam splitter (PBS), and beam dump (BD) is used to modulate the intensity of each laser. A laser shutter (LS) provides binary beam blanking. (B) Spatial patterning of two wavelengths using a digital micromirror device (DMD). Two laser beams are combined by a dichroic mirror (DM) and projected side-by-side onto adjacent halves of the DMD. The two wavelengths are then combined to overlap in the sample plane. An aperture is used to select the appropriate diffraction orders from the DMD.

rhodopsins.[8,9] Multimodal interactions with light also occur in small molecules: light of different colors reversibly activates and inactivates azobenzene-based reagents,[10,11] and dark states of cyanine dyes can be revived by exposure to violet light.[12] By sculpting light in space, structuring light in time, and combining multiple colors and polarizations, one can achieve unprecedented levels of control.

This insight draws inspiration from nature, where multiphoton interactions also play an important role. Sensory Rhodopsin I (SRI) mediates positive phototaxis under blue light, and negative phototaxis under violet light,[13] via opposing reactions of the retinal. Similar single-component color sensors are found in the phytochromes, whose bilin chromophore switches between red and far-red responsive forms[14] and in the diverse cyanobacteriochromes whose linear tetrapyrrole chromophores mediate a variety of bidirectional switching behaviors.[15]

SPECTRAL PAINTBRUSHES

To achieve precise optogenetic control, one needs tools to pattern light in wavelength, space, and time. Commercially available equipment has not yet achieved sufficient flexibility, but fortunately custom apparatus can be constructed with modest budget and expertise. Figure 1 shows motifs that we find particularly useful. To achieve spectral control, multiple lasers are combined using dichroic mirrors and then selected among using an acousto-optic tunable filter (AOTF, Figure 1A). One can typically modulate up to 8 spectral lines independently with ∼5 μs time resolution. The AOTF only provides extinction ratios of $\sim 10^2-10^3$, so it is important to incorporate a neutral density filter and shutter in front of each laser to prevent bleedthrough of undesired wavelengths.

To achieve spatial control, it is common to use either a digital micromirror device (DMD) or a liquid crystal spatial light modulator (SLM). SLMs can redirect light from one sample region to another, enabling high-intensity local stimulation. DMDs are purely subtractive (they block light in unilluminated regions) but have higher time resolution (up to ∼20 kHz), have a simple 1:1 relation between pixels and points in the sample, and work across the visible spectrum. Thus, for most applications, we use DMD-based optical patterning. Figure 1B shows a system for using a single DMD to pattern two wavelengths simultaneously. While there are, of course, infinite variations on the optical layouts of Figure 1, we have found these motifs particularly useful in studying multiphoton control of microbial rhodopsins and anticipate them to be equally useful in photopharmacology and photouncaging of small molecules.

MICROBIAL RHODOPSIN PHOTOCYCLES ENABLE MULTIPHOTON CONTROL

Microbial rhodopsins are a large and diverse family of transmembrane proteins that transduce sunlight into a huge variety of biochemical cues. Their common motif is a retinal chromophore bound via a Schiff base to one of seven transmembrane α-helices. Microbial rhodopsins provide particularly rich possibilities for optical control because the absorption spectrum of the retinal is exquisitely sensitive to the local chemical and electrostatic environment.[16] This fact has been used for decades to probe the photocycles of microbial rhodopsins: by initiating the photocycle with a brief flash of light and then recording absorption spectra as a function of time, one can follow the molecular motions of the photocycle.[17]

Several years ago, we hypothesized that these conformation-dependent spectral shifts might provide a route to optical sensing of membrane voltage. Our logic was simple: in proton-pumping rhodopsins, directional motion of a proton across the membrane was accompanied by dramatic shifts in absorption spectrum. We hypothesized that changes in membrane potential might create electrostatic forces, which would reposition the proton and thereby induce similar spectral shifts.

At the time, it was widely believed that fluorescence of microbial rhodopsins was exceedingly weak[18] and thus unlikely to yield useful signals. While attempting to measure a Raman spectrum of green-absorbing proteorhodopsin (GPR) in *Escherichia coli*, we serendipitously discovered that this protein emitted near-infrared fluorescence under red light excitation and that the fluorescence appeared sensitive to membrane

DOI: 10.1021/acs.accounts.6b00415
Acc. Chem. Res. 2016, 49, 2518−2526

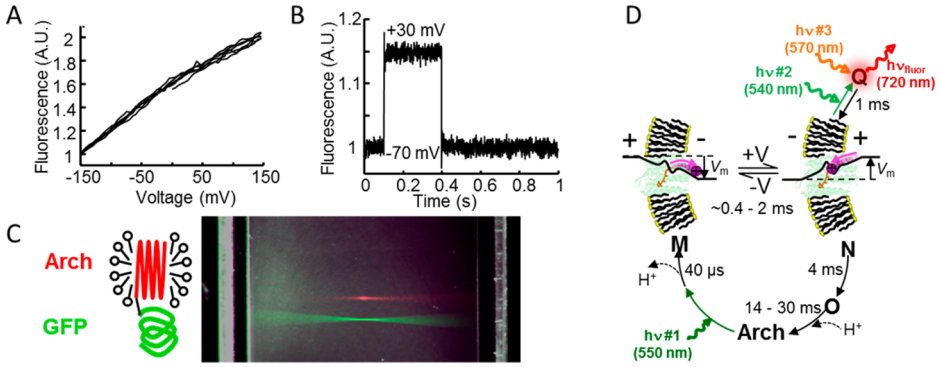


Figure 2. Voltage-sensitive fluorescence in Arch arises from a three-photon process. (A) Fluorescence of wild-type Archaerhodopsin 3 expressed in a HEK293 cell as a function of membrane voltage. Fluorescence was excited at 640 nm, and emission was collected from 660−760 nm. (B) Response of wild-type Arch fluorescence to a step in membrane potential from −70 to +30 mV. The response time of the indicator is 0.6 ms. (C) Nonlinear fluorescence of Arch. Two laser beams were focused inside a cuvette containing an Arch−eGFP fusion. The top beam (594 nm) excited fluorescence from Arch. The identically shaped bottom beam (473 nm) excited fluorescence from eGFP. Arch fluorescence was localized to the focus, whereas eGFP fluorescence occurred throughout the beam. Image is a pseudocolored composite of three exposures taken under different camera settings. (D) Model for voltage-sensitive fluorescence of Arch. A first photon initiates the photocycle. Membrane voltage controls the equilibrium between M and N states. In the N state, a second photon excites a transition to Q, where a third photon excites near-infrared fluorescence. Panels A and B reproduced with permission from ref 20. Copyright 2012 Nature Publishing Group. Panels C and D adapted with permission from ref 30. Copyright 2014 National Academy of Sciences.

potential. The original proteorhodopsin-based voltage indicator (PROPS) functioned only in *E. coli*,[19] wherein it revealed an unanticipated electrical spiking behavior. We later found that Archaerhodopsin 3 (Arch), from the Dead Sea microorganism *Halorubrum sodomense*, trafficked well in mammalian cells and showed rapid voltage-sensitive fluorescence[20] (Figure 2). Subsequently we[21,22] and others have developed a palette of rhodopsin-based voltage indicators derived from Arch,[23,24] macularia rhodopsin (Mac),[25] and acetabularia rhodopsin (Ace).[26] Indeed, voltage-sensitive fluorescence seems to be a common feature of the proton-pumping microbial rhodopsins. To increase the brightness of these reporters, we and others have fused conventional fluorescent proteins to the rhodopsin.[22,25] Voltage-dependent shifts in the absorption spectrum of the rhodopsin led to voltage-dependent quenching of the fluorescent reporter. Recent reviews have covered genetically encoded voltage indicators[27] and the physics of bioelectricity.[28]

Rhodopsin photocycles open the prospect of multiphoton control. Our first foray into multiphoton optogenetics came through a puzzling observation. Under laser illumination in the microscope, Arch fluorescence was relatively easy to observe, far easier than anticipated from the reported low fluorescence quantum yield of 10^{-4} to 10^{-3}.[29] We speculated that the fluorescence might come not from the ground state but from a photocycle intermediate. Indeed in a purified solution of Arch−eGFP, a focused blue laser elicited eGFP fluorescence throughout the beam path, while an identically focused orange laser elicited Arch fluorescence predominantly at the laser focus, suggesting a multiphoton process. Yet the orange laser intensity was ~10^{10}-fold lower than the peak intensity typically used in two-photon fluorescence microscopy![30] The low intensity needed to elicit nonlinear fluorescence in Arch suggested a sequential rather than concerted multiphoton mechanism.

Through a series of careful spectroscopic and electro-optical measurements, we deduced that the fluorescence of wild-type Arch arose through a sequential three-photon process; that the fluorescent state was likely an off-photocycle intermediate (called the "Q state") and that voltage likely controlled protonation of the Schiff base in the M and N photocycle intermediates (Figure 2 D).[30] The complex photocycle of Arch was initially an inconvenience, but we soon realized that the ability to guide the molecule optically enabled new kinds of measurement and control.

TWO-STATE OPTOGENETICS

We start with a simple example of two-state control with a channelrhodopsin. In the photocycle of native channelrhodopsin 2, the channel is opened by blue light (λ_{max} = 470 nm), and closed by green light (λ_{max} = 520 nm). However, the channel also closes spontaneously with a time constant of ~10 ms, so usually light is used only for activation. Berndt and co-workers found that the mutation C128S made the open state metastable, with an open lifetime in the dark of at least 30 min.[31] In these "step-function opsins", blue light rapidly opened the channel and green light closed it. While the photocycle contains several other states, for most purposes, the photocycle can be summarized as the two-state system shown in Figure 3A. The steady-state probability of the channel being open depends on the illumination intensities driving the forward and reverse reactions I_f and I_b:

$$P_{open} = \frac{I_f k_f}{I_f k_f + I_b k_b} \quad (1)$$

where k_f and k_b represent the corresponding wavelength-dependent action coefficients (equal to $\Gamma(\lambda) \times QY(\lambda)$, where Γ is the absorption coefficient of the initial state and QY is the quantum yield for the transition.) The forward and reverse transitions might have overlapping action spectra, in which case one must consider the possibility of crosstalk between the activation and inactivation wavelengths.

This seemingly simple two-state equilibrium enables many novel approaches to optogenetic control. The open probability depends in a nonlinear way on the ratio I_f/I_b. This ratio can create patterns in space and time that are impossible to create with a single beam. Here we give some examples.

Stoplight Rhodopsins

One often wishes to observe how evoked neural activity modulates physiological variables such as Ca^{2+} concentration or

DOI: 10.1021/acs.accounts.6b00415
Acc. Chem. Res. 2016, 49, 2518−2526

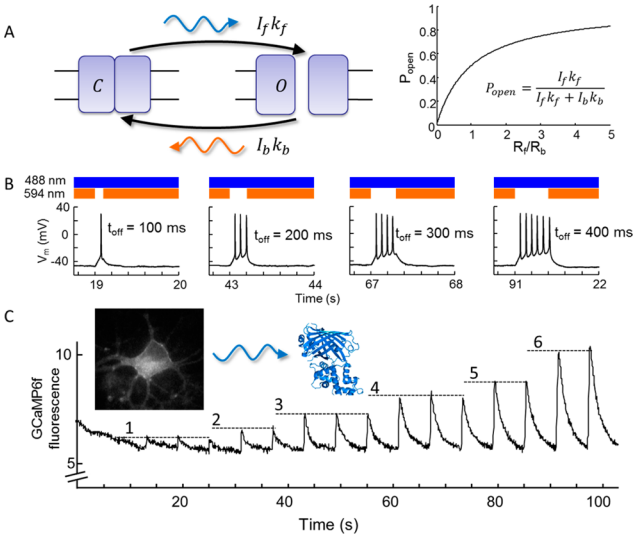


Figure 3. Stoplight technique enables simultaneous optogenetic control and imaging of GFP-based reporters. (A) Step-function opsins have two stable states. They are opened by blue light and closed by orange light; (right) open probability as a function of the ratio of the opening and closing rates. (B) Optogenetic control of neural firing via negative regulation with modulated orange light. (C) Fluorescence imaging of a GCaMP6f Ca^{2+} reporter excited by blue light, while different numbers of action potentials are triggered by modulating the orange light. Adapted with permission from ref 35. Copyright 2014 Elsevier.

ATP production. While a variety of approaches have been developed,[21,32−34] the task is challenging because of spectral crosstalk. Reporters based on GFP are by far the most diverse in terms of sensing modality, but all channelrhodopsin-based actuators, even the most red-shifted ones, retain at least 20% activation at the blue wavelengths used for GFP excitation.[35]

Under constant blue illumination, exposure to orange light negatively regulates the open-state population of step-function opsins. Neurons fire when the orange light turns off. This roundabout approach enables one to trigger neurons to fire by modulating orange light and to simultaneously monitor the physiological response with any GFP-based reporter under continuous blue light.

We developed a step-function channelrhodopsin derived from *Scherfellia dubia* rhodopsin (the same protein from which the ultrasensitive CheRiff actuator was derived).[35] Upon introducing the mutations sdChR(C138S,E154A), the protein showed step-function behavior and excellent expression in neurons. In a cultured rat hippocampal neuron coexpressing sdChR(C138S,E154A) and the Ca^{2+} indicator GCaMP6f, illumination with continuous blue light excited fluorescence of the Ca^{2+} reporter. Temporally modulated orange light induced spiking when turned off (Figure 3B). Fluorescence of GCaMP6f clearly reported the number of optically evoked spikes (Figure 3C). This strategy provides a versatile means to probe the effect of neural firing on any parameter that can be probed with a GFP-based reporter, though the requirement for intense orange light most likely restricts application to cultured neurons or to optically thin samples.

Spatial Localization below the Diffraction Limit

A further challenge in optogenetics is to localize optical stimuli to subdiffraction areas, for instance, to optically stimulate single dendritic spines, single synaptic vesicles, or other subdiffraction cellular compartments. Two-state optogenetics provides a path toward this capability too.

For reversibly switchable fluorophores, Hell and co-workers showed that one could achieve subdiffraction imaging by activating with a diffraction-limited spot and inactivating with a donut-shaped surround, a technique they called reversible saturable optical linear fluorescence transition (RESOLFT) microscopy.[36] This technique has enabled far-field optical imaging well below the diffraction limit.

The RESOLFT principle can also be applied to activation of photoreversible optogenetic tools. Figure 4A illustrates one

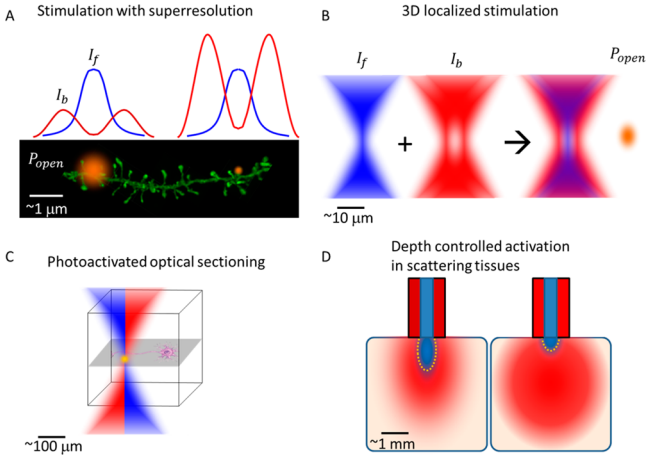


Figure 4. Possible applications of two-photon optogenetics. (A) Optogenetic stimulation with subdiffraction localization. A donut-shaped inactivating beam can suppress activation everywhere but at the center of the donut. (B) An inactivation beam with a localized minimum in intensity (formed, for example, by rapidly scanning a focused beam in a circle) can restrict activation in three dimensions. (C) When photoactivation at one wavelength potentiates fluorescence excitation at a second wavelength, then crossing of two beams can lead to localized fluorescence excitation. (D) In scattering media (e.g., tissue) activating and inactivating beams can compete to restrict the depth of activation.

prospective application, for subdiffraction excitation of channelrhodopsin variants. By activating with a Gaussian spot and inactivating with a donut surround, the photocurrent can be localized to a subdiffraction spot. In the limit where $I_b k_b \gg I_f k_f$, the spatial extent (fwhm) of the photocurrent, Δx_{open}, is given by a simple generalization of the well-known RESOLFT resolution formula:

$$\Delta x_{open} = \Delta x_b \sqrt{\frac{I_f k_f}{I_b k_b}}$$

where Δx_b is the fwhm of the hole in the inactivating beam. With this technique, one could easily produce optogenetic stimulation with spatial resolution ~10-fold below the diffraction limit.

The key challenge for practical implementation of this concept is the low unit conductance of most channelrhodopsin variants. As the activated zone shrinks in volume, the total photocurrent shrinks proportionally. Development of cation channelrhodopsins with higher unit conductance is thus an important research goal. The recent discovery of anion channelrhodopsins with exceptionally large unit conductance[37] bolsters optimism that this goal is achievable. The principles

DOI: 10.1021/acs.accounts.6b00415
Acc. Chem. Res. 2016, 49, 2518−2526

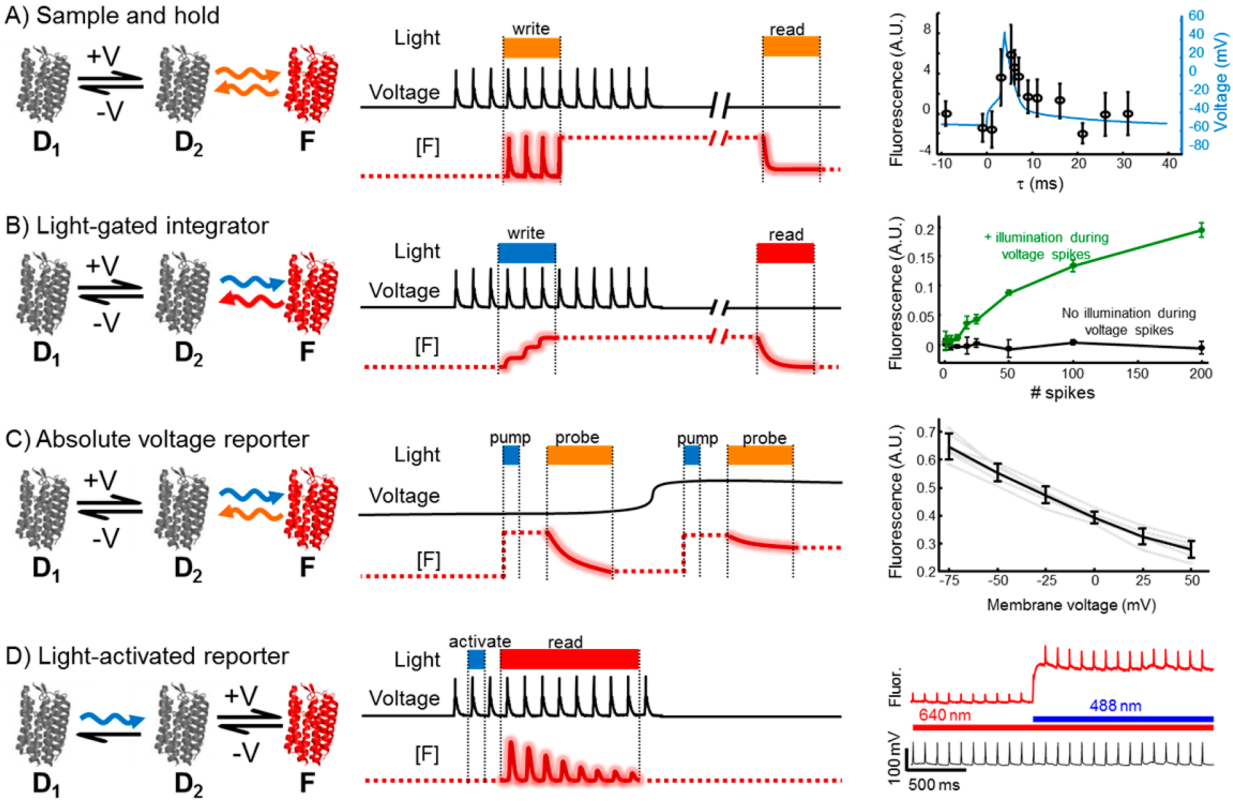


Figure 5. Three-state optogenetics enables new approaches to optogenetic sensing. (A) Sample-and-hold. The population of the F state tracks the voltage while the orange light is on and retains memory of the last voltage when the light is off; (right) example recording from a neuron, where flashes of light at the indicated times relative to an action potential led to fluorescence 1 s later. (B) Light-gated integrator. Blue light gradually builds population in F, in a voltage-dependent way; red light reads this population and resets the integrator; (right) example recording from a HEK cell where the population of a fluorescent state was proportional to the number of voltage spikes induced during a write pulse. (C) Absolute voltage reporter. A blue pump pulse initializes the population in the F state; the fractional change in fluorescence during the orange probe pulse indicates the absolute voltage; (right) example recordings from six HEK293 cells showing the small cell-to-cell variability in fluorescence signal when measured in this way. (D) Light-activated reporter. Illumination with blue light produces a population of molecules that show voltage-sensitive fluorescence; this population gradually decays back to the dark state; (right) example recording from a neuron showing blue light sensitization of voltage-sensitive fluorescence. Panels A and B adapted with permission from ref 46. Copyright 2014 American Chemical Society. Panel C adapted with permission from ref 53. Copyright 2014 Elsevier.

discussed here can also be applied to achieve subdiffraction activation of other photoreversible optogenetic actuators.

Spatial Localization above the Diffraction Limit

Factors other than diffraction can also limit the spatial resolution of optical excitation. Figure 4 shows some examples. In three dimensions, out-of-plane activation can be a problem, both for fluorescence imaging and for optogenetic actuation. Two-photon excitation provides a route to optical sectioning, but two-photon light sources are bulky and expensive, not all molecules show good two-photon cross sections, and the point-scanning requirements of two-photon excitation are inconvenient for high-speed stimulation or imaging. Indeed, despite recent progress, two-photon optogenetic stimulation[38] and two-photon voltage imaging[39] remain challenging to implement robustly.

It is commonly believed that one-photon optogenetic stimulation in tissue cannot achieve single-cell resolution due to spurious activation of channelrhodopsin molecules above and below the focal plane. Figure 4B shows a possible remedy. By forming a three-dimensional zone of inactivation light around a focused beam of activation light, one can localize the activation to a small region. Provided that the inactivation beam is brighter than the activated beam, scattered inactivating photons will counteract the effects of scattered activating photons, keeping the activation localized even in the presence of light scattering.

In the emerging field of photopharmacology, small molecules are rendered light sensitive by fusing with a light-responsive moiety, often an azobenzene derivative.[40] However, diffusion of activated molecules broadens the activation zone to a size larger than the laser focus. Even with highly localized two-photon activation, the steady-state concentration of the photoproduct decays as $1/r$ from the source. However, if one surrounds the focused activating beam (either one-photon or two-photon) by a shell of inactivating light, as in Figure 4B, then one can create a tunable bolus of photoproduct, contained within the bounds of the inactivating wall. For a spherical source of radius R in a medium where the product is photoreacting at a constant rate k_bI_b (assuming uniform profile of inactivating light), the steady-state concentration profile follows

$$C(r) = \frac{C_0 R}{r} e^{-r\sqrt{k_b I_b / D}}$$

where D is the diffusion coefficient of the molecule and C_0 is the concentration at the surface of the source. Real optical systems will not conform to this idealized spherical geometry, but the presence of a light-driven reverse reaction will always

DOI: 10.1021/acs.accounts.6b00415
Acc. Chem. Res. 2016, 49, 2518−2526

impart a sharper-than-diffusion dropoff in concentration outside the source region. This idea has recently been contemplated in the context of photoswitchable drugs.[41]

Two-state optogenetics may also provide benefit for imaging in optically scattering samples (Figure 4C). Many small-molecule and protein-based fluorescent reporters show reversible photoactivation, where light of one wavelength converts the molecule into a state that fluoresces under excitation at a second wavelength.[2,42,43] By intersecting an activation and a probe beam in a sample, one can achieve two-photon-like localization of fluorescence (signal proportional to the product of the two beam profiles), while using inexpensive continuous-wave lasers. The key to enabling fast scanning in such a geometry is to have a rapid rate of inactivation of the fluorophore, either spontaneous or driven by the imaging beam; otherwise the distribution of activated fluorophore will spread beyond the borders of the activation beam, degrading the spatial resolution. We suggest that development of rapidly inactivating photoactivatable fluorophores is a worthy goal for future protein engineering efforts.

The ability of an activation beam to modulate the fluorescence of an imaging beam also opens the possibility for lock-in imaging, where one records the component of the fluorescence at the modulation frequency of the activation beam.[44] Lock-in imaging can be used to reject background signals from molecules that do not show photoactivation behavior and has been used to image spiropyran-based organic photoswitchable dyes and the genetically encoded Dronpa fluorescent protein.[44] Recently, photoswitching was used in a beautiful demonstration of molecularly specific photoacoustic imaging with a photoswitchable bacterial phytochrome.[45]

Finally, two-state optogenetics may find application on length scales long compared to the photon scattering length, l_s, typically 30−100 μm depending on the wavelength and tissue. After scattering, photons continue to ricochet through a tissue, possibly causing spurious activation of optogenetic or photopharmacological reagents. By surrounding an activation beam with an inactivation beam, one can constrain the activation zone within the clouds of scattered photons (Figure 4D). Modulation of the intensity of the inactivation beam will modulate the depth over which activation dominates. This strategy may prove particularly useful in, for example, clinical applications of photopharmacology, where one wishes the drug action to remain topical, or in deep-brain optogenetic stimulation where one wishes to circumscribe the stimulated region.

THREE-STATE OPTOGENETICS

The Arch photocycle in Figure 2D shows a voltage-dependent equilibrium between the M and N states, connected by a light-driven reaction to a fluorescent Q state. This three-state motif governs the behavior of many Arch mutants. The main differences between mutants are in the voltage sensitivity, and in the spectra and kinetics of the transitions into and out of the Q state. A host of new capabilities emerges from this simple topology. Here we focus on microbial rhodopsin-based voltage sensors, though some similar principles have been applied to fluorescent Ca^{2+} sensors. Applications of this three-state system are shown in Figure 5.

Light-Gated Integrator and Light-Gated Sample-and-Hold

A common challenge in fluorescent sensing experiments is to probe the dynamics of an analyte over a large volume, for example, a piece of brain. This challenge is particularly severe when the dynamics are rapid. In a mouse brain with 75 million neurons, it is not possible, even conceptually, to image from a substantial portion of these cells simultaneously with the 1 ms time resolution needed to record their electrical activity. Three-state optogenetics provides a means to record within each neuron a photochemical imprint of the activity state—firing or not firing—across a wide brain region at a moment in time. This imprint is stored in a metastable fluorescent product, whose state can be probed at a late time and then optically reset for a subsequent measurement. We call this concept "flash memory".[46]

In the topology of Figure 5, voltage controls an equilibrium between two dark states. Light then converts one of these states into a fluorescent product. In the Arch(D95H) mutant, the transitions into and out of the fluorescent Q-state are both light driven, in contrast to the spontaneous relaxation from Q to N that occurs in the wild-type protein (Figure 2D). In Arch(D95H), transitions into and out of Q had distinct but overlapping action spectra. Illumination with orange light drove the transition in both directions, establishing a rapid equilibrium between the dark state, D_2, and the fluorescent state F. Extinction of the orange illumination decoupled state F from the voltage-sensitive manifold, storing a record of the voltage at the moment the light turned off. Fluorescence of the F population was then probed at a later time, using low-speed microscopy. We call this mode of operation "sample-and-hold" in analogy to the electrical circuit. A key merit of the sample-and-hold approach is that the photons used in the write pulse need not travel ballistically through the sample. While deep-tissue imaging is challenging due to light scattering, simply filling a tissue volume with light is comparatively easy.

The same photocycle topology can be used to store an integrated record of neural activity. Illumination with light of a blue wavelength drove the transition from the D_2 to the F state, but not the reverse. Thus, population transferred to F at a voltage-dependent rate. The total population in F represented the integrated activity during the illumination period, up to the point where the whole population was in F. Illumination with red or orange light probed the fluorescent population in F and reset the sensor. We call this mode of operation "light-gated integrator". Thus, by tuning the wavelength and intensity of the illumination wavelengths, one can tune the function implemented by these three-state fluorescent voltage indicators.

A similar concept has been applied in the CaMPARI light-gated Ca^{2+} integrator.[47] Here, violet light drives an irreversible photochemical transition in an mEOS fluorescent protein; but the transition can only occur when Ca^{2+} is bound to a sensing arm of the protein. The total quantity of fluorescent product depends on the integrated Ca^{2+} activity during the illumination epoch. The concept of separating the encoding and readout of neural activity could also be applied to many other fluorescent protein-based reporters.

Absolute Voltage Reporters

Another challenge in fluorescent sensing is to determine the absolute quantity of an analyte. For all their charms, fluorescent reporters are typically bad for quantitative measurements due to uncontrolled variation in expression level, illumination intensity, detection efficiency, and photobleaching. Dual-wavelength ratiometric reporters help somewhat but suffer from differential photobleaching of the two components, wavelength-dependent scattering, and challenges of accurately

DOI: 10.1021/acs.accounts.6b00415
Acc. Chem. Res. 2016, 49, 2518−2526

calibrating wavelength-dependent excitation and detection efficiencies.

Figure 5C shows how a three-state reporter can be used as an absolute sensor, in this case of voltage. A pump pulse (blue) drives the whole population into the fluorescent state, F. A probe pulse (orange) then establishes an equilibrium between F and D_2. The probe pulse must be at a wavelength that drives both the forward and reverse optical transitions, establishing a fixed steady-state ratio of D_2 to F. Voltage determines the steady-state ratio of D_1 to D_2 and thereby the steady-state population of F. In the example shown, the probe pulse also excited fluorescence of F, though one could use a different wavelength for that purpose if desired. The ratio of fluorescence at the beginning of the probe pulse to steady-state fluorescence reports the voltage. This quantity is insensitive to photobleaching or to variations in illumination or collection efficiency and thus provides an accurate measure of absolute voltage.

If one thinks of the electronically excited state of a fluorescent reporter as distinct from the ground state, then every fluorescent sensor is truly a three-state system. In some reporters, the relaxation dynamics of the electronic excited state depend on the membrane voltage or analyte concentration. Measurements of these dynamics via fluorescence lifetime imaging microscopy (FLIM) provide absolute accuracy,[48] just as in the much slower absolute voltage measurement scheme of Figure 5C. The OGB1 dye shows a calcium-dependent change in fluorescence lifetime.[49] We explored the voltage dependence of fluorescence lifetime in several genetically encoded voltage indicators and found that ASAP1 and a FRET-rhodopsin sensor showed this effect and thus could, in principle, be used as absolute voltage reporters.[50]

Light-Activated Reporters and Primed Photoconversion

To probe analytes in particular cells or subcellular regions, it is advantageous to localize a sensor to the targeted region. At the cellular and subcellular levels, this can sometimes be done by genetic or biochemical means; but sometimes one wishes to select the probed region dynamically. Optically activated fluorescent sensors provide a route to this goal. Several three-state sensors have been developed in which illumination at one wavelength potentiates fluorescence sensing at a second wavelength.

The photocycle topology of an Arch-based sensor with this property is shown in Figure 5D. Whereas Figure 3C showed photoactivated optical sectioning for structural imaging, Figure 5D shows an approach for functional imaging at the crossing of two beams. We have observed an Arch mutant that has this property, and Berlin and co-workers reported a family of light-activated Ca^{2+} sensors (termed "PA-GCaMPs").[51] In the PA-GCaMPs, illumination with violet light irreversibly potentiated the fluorescence, enabling targeted measurements of Ca^{2+} dynamics in single cells or cellular subcompartments. A related technique, called "primed photoconversion" uses two sequential single-photon absorption events to photoactivate a fluorophore.[3] Due to the requirement for two photons, this technique can achieve optical sectioning. Yet because the photons are used sequentially, rather than simultaneously, the laser intensity requirements are modest. The photoactivated fluorescent reporters can then be probed by subsequent one-photon or two-photon fluorescence imaging techniques. We expect that further development of photoactivated reporters will enable new approaches to functional imaging in deep tissue.

CONCLUSION

While physical reagents diffuse slowly and often stop at membrane barriers, light as a biochemical reagent follows a different set of rules. One can achieve chemical specificity by tuning wavelength and spatial and temporal specificity through electro-optical patterning. By combining multiple optically driven transitions, one can achieve levels of spatial control even beyond the capabilities of linear optics. With the advent of sophisticated acousto-optical, micromirror, and liquid crystal light modulators, one can now use light to poke and prod cellular machines with unprecedented finesse, using either protein-based or small molecule tools.[52] Key to realizing this potential, however, are detailed spectroscopic studies of the basic photoconversion mechanisms. Through concerted efforts in spectroscopy, protein engineering, chemical synthesis, and optical instrumentation, we anticipate an ever-growing palette for the optical paintbrush.

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ACKNOWLEDGMENTS

This work is supported by NIH Grants 1-R01-EB012498 and 1-DP2-OD007428, ONR Grant N00014-11-1-0549, the Gordon and Betty Moore Foundation, and the Howard Hughes Medical Institute.

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