

# Optogenetics: Turning the Microscope on Its Head

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Look outside your window. You will likely see green plants, perhaps some yellow, pink, or white flowers, maybe a bird with blue, brown, or red in its feathers and eyes. The world is full of living color, and life has evolved a dizzying variety of chromophores for signaling and photoreceptors for sensing the dynamically changing photic environment.

Scientists are now identifying these chromophores, tweaking them, and then reinserting the genes responsible for them under control of cell-type-specific promoters into species separated by up to two billion years of evolution (1) (Fig. 1). This molecular mix-and-match has led to mice whose neurons are multicolored like an electronics ribbon cable, fish in which brain activity-induced changes in calcium concentration cause active brain regions to light up, and recently, molecular tools by which one can use light to turn on or off the expression of nearly any gene in the genome.

Equally important has been a radical change in how scientists use the microscope. Since the time of Leeuwenhoek, microscopes conveyed light from a sample, greatly magnified, to a viewer. Now microscopes are also used to illuminate a sample with light in precisely sculpted patterns of space, time, color, and polarization. The light tickles molecular actuators, leading to activation of cellular processes in patterns of space and time determined at the whim of the experimenter.

This review describes how scientists are identifying, modifying, and applying optically active proteins, the instrumentation being developed for precisely targeted illumination, and open challenges that a bright student might solve in the next few years.

## The fluorescent protein palette

The term optogenetics was coined in 2006 to describe genetic targeting of optically responsive proteins to particular cells, combined with spatially or temporally precise optical actuation of these proteins (2). The field actually started more than a decade before, with the discovery that the gene for green fluorescent protein (GFP) could be transferred from the jellyfish *Aequorea Victoria* to the worm *Caenorhabditis elegans* (3), lighting up that worm's neurons.

Triggered by this discovery, scientists adopted a twofold approach to finding fluorescent proteins (FPs) with more colors and better optical properties. Some tweaked the protein scaffold, looking for mutations that increased brightness, photostability, or folding speed or changed the color. Others swam around coral reefs with fluorescence spectrometers, identifying fluorescent creatures and cloning out genes for new FP scaffolds. Both approaches have been spectacularly successful (4). The GFP-derived palette ranges from far blue (emission peaked at 424 nm) to yellow-green (emission peaked at 530 nm).

One of the motivations for these explorations was to develop red-shifted FPs because of the relatively greater transparency and lower background fluorescence of tissue in the near infrared range compared with visible wavelengths (5). One source of red-shifted FPs is the bacterium, *Rhodospseudomonas palustris*, found, among other places, in swine waste lagoons, which produces bacteriophytochrome-based FPs that require a biliverdin chromophore to fluoresce. These proteins enable one to peer deep into the body of a mouse, watching, for instance, a tumor grow under the skin (6).

Perhaps the most dramatic application of the FP palette is in the so-called “Brainbow” mouse (7) (Fig. 2 A). Through a clever combination of random genetic rearrangements, each neuron in this mouse produces a distinct set of fluorescent markers derived from a coral, a jellyfish, and a sea anemone. The beautiful multi-hued labeling permits scientists to track the delicate axons and dendrites of individual cells, which otherwise would appear as an impenetrable monochrome tangle.

## Blinking, highlighting, and binding

Rainbow-colored mice are visually appealing and scientifically useful, but the capabilities of FPs go far beyond simply tagging structures. Many of these proteins fluoresce to different degrees and in different colors depending on the local environment around the chromophore. This property has found a dizzying array of applications.

### Blinking

At the single-molecule level, many FPs spontaneously blink on and off. Some colors of illumination favor the dark state and others the bright state, and proteins can be coaxed in and out of the fluorescent state under optical control. In photoactivation light microscopy, individual FP molecules are turned on sparsely, localized with subdiffraction precision,

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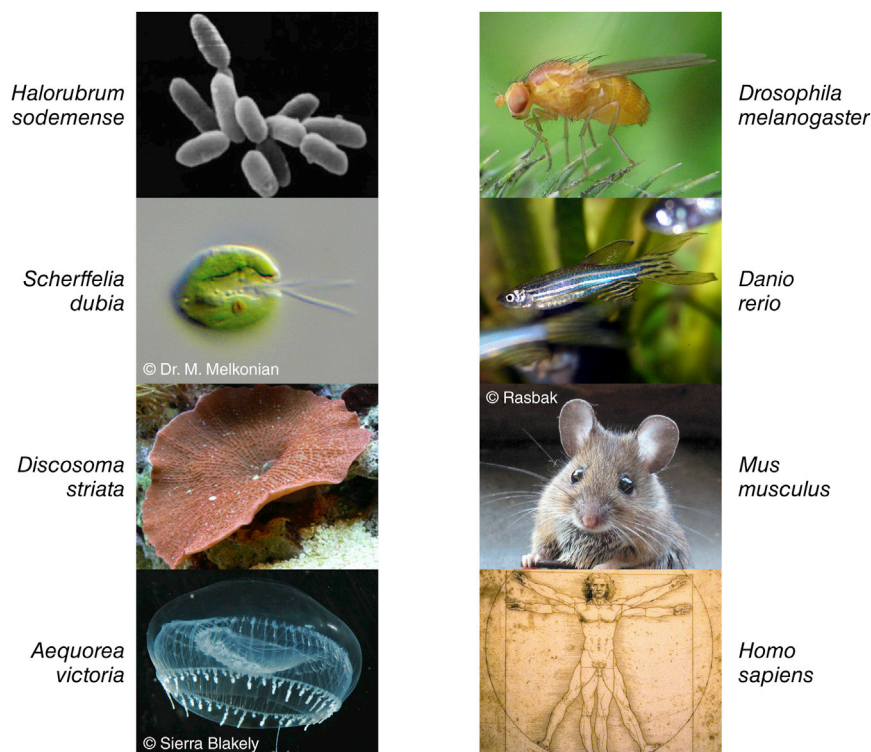


FIGURE 1 Optogenetic mix-and-match. (*Left*) Organisms whose genes have yielded new optogenetic tools. (*Right*) Organisms into which scientists have transferred these genes. To see this figure in color, go online.

and then turned off. Iterating this process hundreds of times builds up a pointillist image of the sample, with resolution far below the diffraction limit (8). This advance was recognized in the 2014 Nobel Prize in Chemistry.

### Highlighting

Photoactivatable and photoswitchable FPs have served as optical highlighters for tracking the flow of matter in a cell. One can tag a cellular structure with a flash of light and then follow the motion of that structure through the cell. This enables one to probe how mitochondria move through neurons and track the assembly and disassembly of microtubules.

### Binding

Many nonfluorescent proteins change shape when they bind a ligand or a partner. The chromophore in most FPs must pack snugly among surrounding amino acids to fluoresce. Crack open the protein barrel or expose the chromophore to water, and the fluorescence goes away. This combination of features has been exploited by constructing circularly permuted FPs in which the two ends of the amino acid chain are linked, and a new break is introduced near the chromophore. A slight tug on the new ends of the chain can reversibly disrupt the fluorescence and, by fusing nonfluorescent sensor domains to circularly permuted FPs, one can make fluorescent sensors that report ATP, calcium, membrane voltage, and ligand binding to G protein-coupled receptors.

The most dramatic applications of fluorescent sensor proteins come from the GCaMP family of  $\text{Ca}^{2+}$  indicators (Fig. 2 B). The concentration of this ion blips upward every time a neuron fires. Expression of GCaMP-based reporters in the brains of worms, flies, fish, and mice has led to spectacular movies of the coordinated activation patterns of thousands of neurons.

Within the last year, scientists have started to engineer more complex combinations of functions into GFP-based optogenetic tools. For instance, the calcium-modulated photoactivatable ratiometric integrator (CaMPARI) protein starts life as a fluorescent calcium indicator, and, in the simultaneous presence of neural activity and violet illumination, converts from green to red (9). This behavior lets one record a photochemical imprint of the calcium level in a large volume of tissue at a defined moment in time. One can then image the tissue at leisure, with high resolution in space, to map this snapshot of activity.

Many new types of sensors are still needed. A fluorescent reporter for glutamate has been described (10), but reporters for many other neurotransmitters (gamma-aminobutyric acid, dopamine, serotonin, and acetylcholine) are still in development. It also is challenging to sense physical forces. Fluorescent reporters for membrane voltage (11) and cytoskeletal tension (12) have been developed, but we lack voltage indicators that perform well enough to be used in vivo or that can be targeted to intracellular membranes (mitochondria, vesicles, and endoplasmic reticulum). We also lack fluorescent reporters for many of the subtle, but

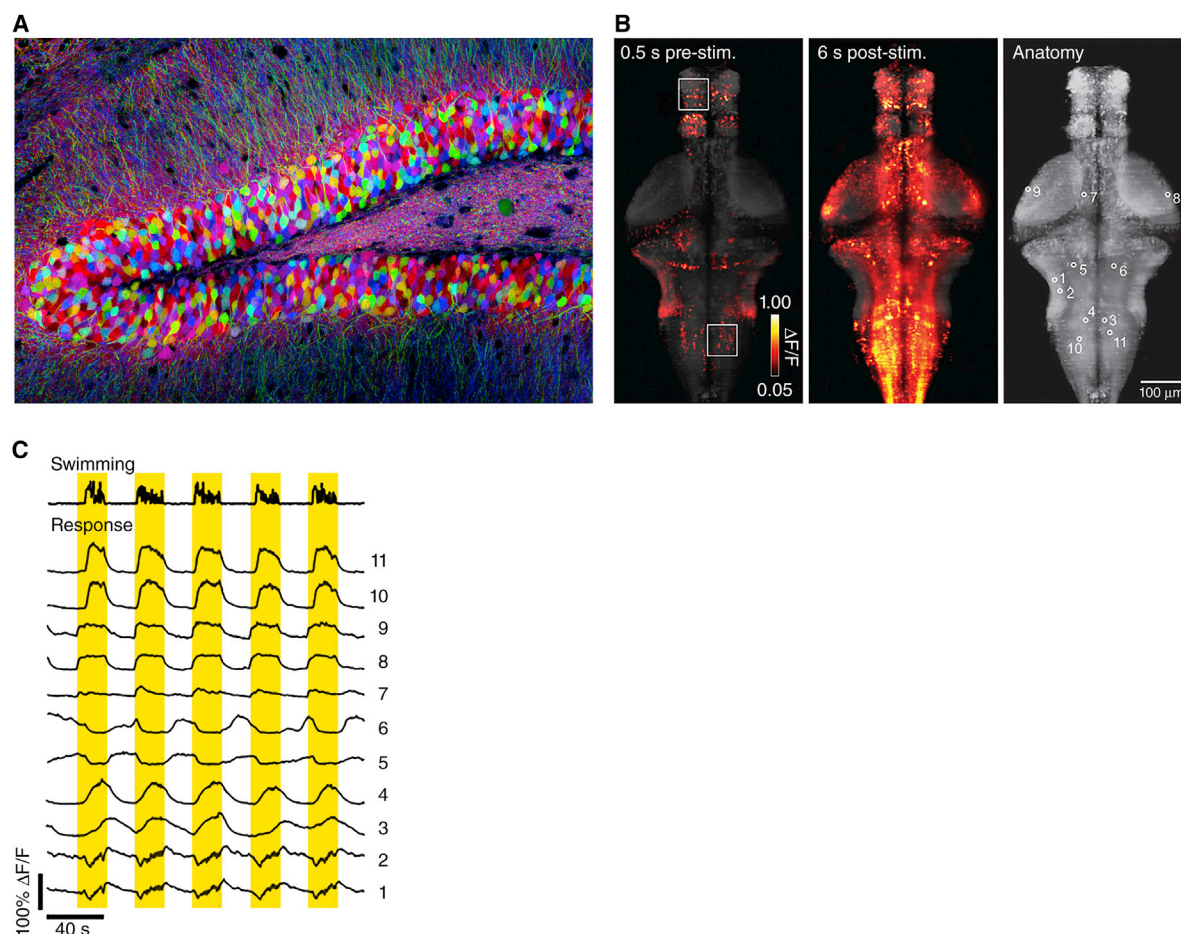


FIGURE 2 Fluorescent protein-based sensors light up the brain. (A) Brainbow mouse hippocampus. Random genetic recombination events turn on a different subset of fluorescent proteins in each neuron, giving each one a unique hue. (B) Imaging neural activity in the brain of a zebrafish via the calcium indicator GCaMP6s. The fish was immobilized over an image of a drifting grating. When the grating started to move (*stim*), the fish tried to swim to maintain its position within the visual field. The central image shows the brain regions that activated when the fish swam. (C) Activity of neurons indicated in the right panel of (B) during swimming. (A is from Jeff Lichtman; B and C are from (23).) To see this figure in color, go online.

likely important, physical forces in biology: plasma membrane tension, osmotic pressure, or stresses between cells and their neighbors or the surrounding extracellular matrix.

In the applications described above, light interacts with the FPs—eliciting fluorescence, changing the brightness, or changing the color—but the light does not fundamentally change the underlying biological process (at least not intentionally; phototoxicity is a constant concern for these experiments). The true power of optogenetics emerged when scientists started to use light to perturb the underlying biology in a precise way.

### Microbial rhodopsins bring light to the membrane

Most living things sense and respond to changes in light. A diverse set of transducers has evolved to couple light into biochemical signals. Here, we focus on the microbial rhodopsins as a paradigmatic example. The first microbial rhodopsin was discovered in the early 1970s in a halophilic archaeon, *Halobacterium salinarum*, in the salt marshes of

San Francisco Bay. The protein has seven transmembrane  $\alpha$  helices and a retinal chromophore covalently bound in its core. Upon illumination, the retinal undergoes a *trans-to-cis* isomerization, which induces a series of shape changes in the protein that lead to pumping of a proton from inside the cell to the outside. The protons return back into the cell through the ATP synthase, powering the metabolism of the host.

More than 5000 types of microbial rhodopsins have been identified by metagenomic sequencing. They are found in archaea, prokaryotes, and eukaryotes. Most are uncharacterized, and these proteins mediate a huge variety of interactions between sunlight and biochemistry. Some act as light-driven proton pumps (e.g., bacteriorhodopsin, proteorhodopsins, and archaerhodopsins) and others act as light-driven chloride pumps (e.g., halorhodopsin), light-activated signaling molecules (sensory rhodopsins), or light-gated cation channels (channelrhodopsins).

The discovery that channelrhodopsin 2, derived from the green alga *Chlamydomonas reinhardtii*, functioned as a



light-gated cation channel triggered a race to apply this protein to control neural firing with light. A first-person historical account has been written by one of the chief protagonists, Ed Boyden (13), and a thorough review of the early literature has also been written by Karl Deisseroth, another key protagonist, and his colleagues (14). Early demonstrations in cultured neurons were quickly followed by demonstrations in mouse brain slice, chick spinal cord, worms, flies, and zebrafish. Control of rodent behavior started with simple whisker movements, but then quickly expanded to control of locomotion, sleep, feeding, aggression, memory, and social interactions (Fig. 3 A).

Recent work on pup rearing demonstrates the sophistication and precision that optogenetic stimulation has reached (15). In male or female mice showing parenting behavior, a subpopulation of neurons became active in the medial pre-optic area. These cells were genetically targeted with a Cre-dependent channelrhodopsin construct and, in virgin male mice that normally show aggression toward pups, optogenetic actuation reversibly switched the animals into a grooming mode. These and many other optogenetic experiments demonstrate that seemingly complex rodent behaviors can be elicited by precise actuation of relatively small numbers of neurons in genetically defined circuits.

### Converting microbial rhodopsins into reporters

Efforts to engineer better optogenetic neural modulators relied heavily on mechanistic insights obtained from decades of detailed biophysical studies of the photocycle of bacteriorhodopsin and its homologs. A standard technique in this arena was transient absorption spectroscopy, which

triggers the photocycle with a flash of light and subsequently records absorption spectra as a function of time. Motion of the proton through the protein core was accompanied by shifts in the absorption spectrum.

My lab discovered that microbial rhodopsin proton pumps are weakly fluorescent and that this fluorescence varies depending on the location of a proton in the core of the protein. Changes in membrane voltage could reposition the proton, thus changing the fluorescence. We realized that this phenomenon might provide a novel route toward one of the longest-standing challenges in neuroscience: to develop a fast and sensitive optical reporter of membrane voltage.

The initial proteorhodopsin-based voltage indicator (called PROPS) functioned only in bacteria and led to the discovery that *Escherichia coli* generate spontaneous electrical spikes (16). Neither the underlying mechanism nor the biological function of this spiking is well understood. Of the millions of species of bacteria in the world, we know almost nothing about the electrophysiology of any of them.

PROPS did not work in mammalian cells because the protein did not traffic to the plasma membrane. After an unsuccessful year-long effort to engineer membrane trafficking into PROPS, we switched to Archaeorhodopsin 3 (Arch), a protein derived from a Dead Sea microorganism, *Halorubrum sodomense*, which was discovered in the early 1980s by an Israeli microbiologist. Arch immediately showed voltage-sensitive fluorescence in mammalian cells (17). Further protein engineering eliminated the photocurrent and improved the sensitivity and speed of the protein, leading to the QuasAr family of voltage indicators (18).

By good fortune, the fluorescence of microbial rhodopsins is excited by red light and emits in the near infrared.

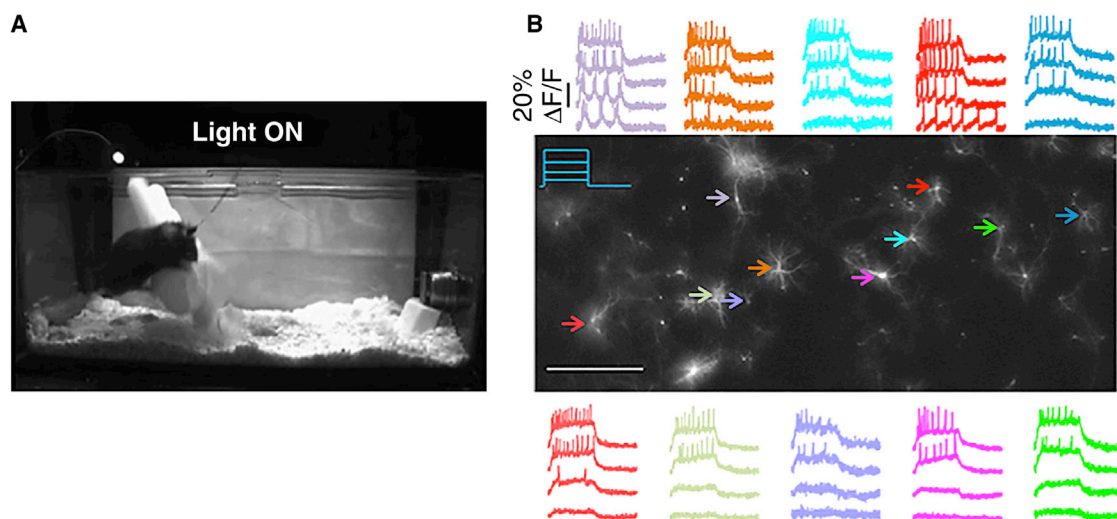


FIGURE 3 Optogenetic control and readout of neural activity. (A) Optogenetic control of aggression. In this still from a movie, the mouse is expressing Channelrhodopsin 2 in its ventromedial hypothalamus, ventrolateral subdivision (VMHv1). Illumination of this region through an optical fiber causes the animal to attack an inflated rubber glove, which it would otherwise ignore. (B) All-optical electrophysiology. These rat hippocampal neurons express the Optopatch constructs, comprising a blue light-activated channelrhodopsin variant (CheRiff) and a red light-activated voltage indicator (QuasAr2). Illumination with 500 ms pulses of blue light triggers intensity dependent neural activity. Scale bar, 0.5 mm. (A is from (24); B is from (25).) To see this figure in color, go online.

This feature leaves the rest of the visible spectrum open for other applications: combination with other GFP-based reporters or pairing with optogenetic actuators. We developed a system for all-optical electrophysiology based on a combination of the QuasAr voltage indicators with a new optogenetic actuator derived from a freshwater alga from a pond in the south of England. With this Optopatch construct, one could stimulate a neuron to fire with a flash of blue light and record the response with red excitation and near infrared fluorescence (Fig. 3 B). Optopatch has enabled high-throughput functional phenotyping of neurons in culture. It is now being applied to the study of human induced pluripotent stem cell-derived neurons with mutations associated with ALS, epilepsy, and schizophrenia. A key challenge with the microbial rhodopsins is to increase the brightness of their fluorescence so they can be used in tissue and in vivo.

### Optogenetic control inside the cell

In recent years, the toolchest of optogenetic actuators has grown dramatically. For nearly any cellular process, someone is working to bring it under optical control. See the article by Zhou et al. (19) for an excellent recent review. Animal rhodopsins have been engineered to control

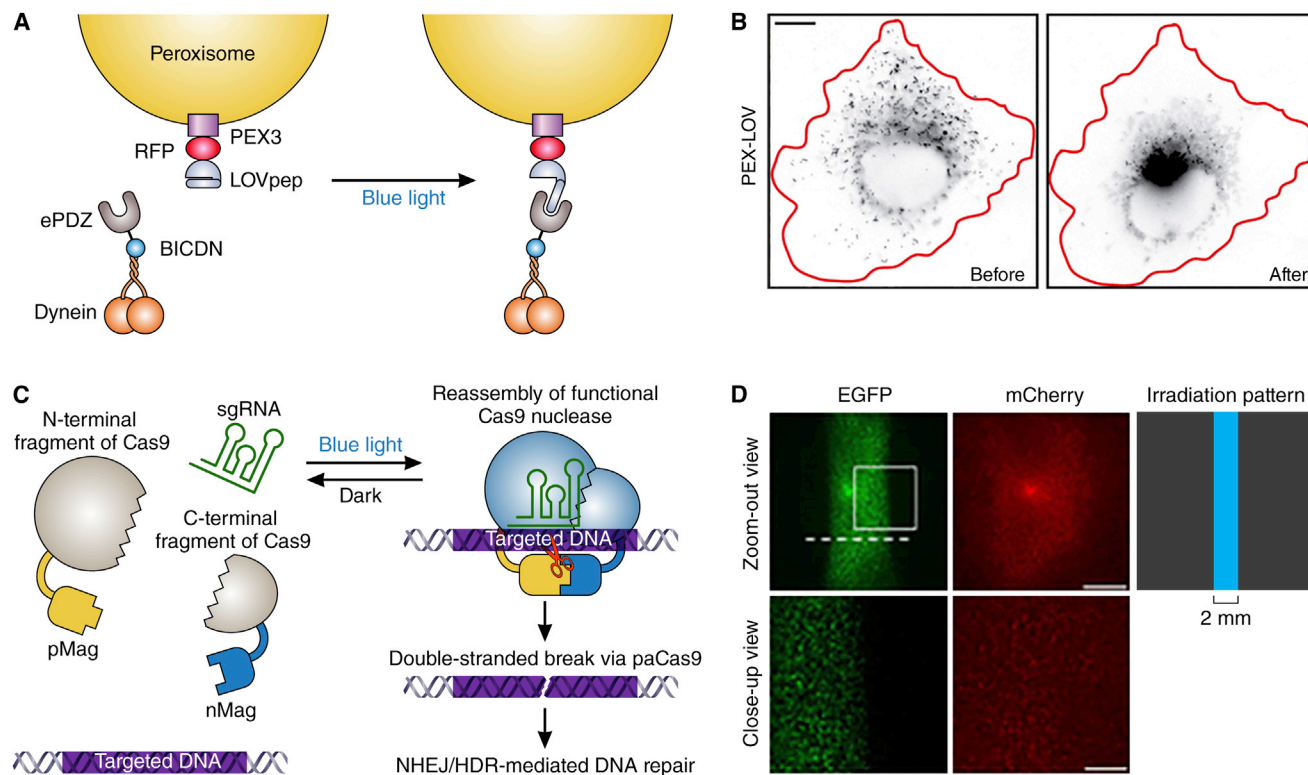
signaling by G proteins, opioid pathways, and serotonin pathways. For control in the cytoplasm, proteins have been developed to regulate enzyme activity and trigger signaling cascades as well as optical control of organelle trafficking (20) (Fig. 4, A and B).

Developments around optical control over the processes of DNA editing and transcription have been particularly exciting. Transcription activator-like effectors and, more recently, the CRISPR/Cas9 system enable targeting of proteins to arbitrary DNA sequences. Once there, depending on the effector domain, the protein can cut the DNA or turn transcription on or off. Light-activated variants have been made that enable optical control of the activity of nearly any gene in the genome (21,22) (Fig. 4, C and D).

### The microscope as a two-way tool

Much activity in the optogenetics world has focused on molecular transducers. Innovations in the targeted delivery of light are equally important. The concept of the microscope as a passive observation tool is being replaced by the idea that light provides precise handles for tugging and pushing on molecular machines.

Advances in video projector technology have been a key driver of the instrumentation. Digital micromirror devices



**FIGURE 4** Optogenetic control of intracellular processes. (A) Control of trafficking. Light-induced association between the LOVpep and ePDZ domains tethers the peroxisome to the motor protein dynein. (B) Upon illumination, the dynein drags the peroxisomes toward the nucleus. (C) Control of gene editing. Light-induced association of the pMag and nMag domains brings together the two pieces of a split Cas9 nuclease, restoring its ability to cut DNA. The Cas9 cuts at the location determined by the sequence of the guide sgRNA. (D) In cells expressing a genetic construct where DNA cleavage led to expression of eGFP, eGFP fluorescence was seen only in the illuminated region. (A and B are from (26); C and D are from (27).) To see this figure in color, go online.

(DMDs) comprise an array of typically  $\sim 10^6$  microfabricated mirrors, each of which can be electrostatically deflected between two orientations. One orientation reflects light to the sample, the other to a beam dump. If one places a DMD in the image plane of a microscope, then each micromirror maps to a single spot on the sample. Thus, one can have  $\sim 10^6$  points of light, each individually controllable at up to  $\sim 10$  kHz. The optical path between a DMD and the sample is identical to the path between the sample and the camera, only the arrows on the light rays are reversed.

Another important advance is the development of liquid crystal spatial light modulators (SLMs), which modulate the phase, rather than the amplitude, of the light. This capability can be used to focus or diffract light into user-specified patterns. The SLM has the advantage over the DMD that it can achieve higher illumination intensity at specified points; the SLM redirects light from regions that should be dark to regions that should be bright, whereas the DMD simply blocks light from reaching the dark regions. However, the SLM is not as fast as the DMD, and it is more complex to control because there is not a simple relationship between the pattern on its pixels and the pattern on the sample.

### Future opportunities

For almost any cellular function or biochemical process, one can imagine using optogenetics to gain control. For instance, one would like to use light to tag RNA molecules for subsequent pulldown and sequencing, to tag protein molecules for subsequent analysis by mass spectrometry, or to control the cell-cell interactions in a developing embryo or a healing wound. These capabilities have not yet been developed, but they are readily envisioned.

To achieve maximum flexibility, one would need robust two-photon-activated optogenetic constructs. Then one could turn on or off any endogenous or exogenous gene in intact tissue on the basis of an arbitrary measurement. A challenge here is the low efficiency of two-photon photochemistry. The use of two-photon excitation to trigger autocatalytic amplification cascades may provide a route.

There are many instrumentation challenges. We need better ways to localize optical excitation at greater depths and with greater spatial precision in highly scattering tissues or to image fluorescence emission in three dimensions in scattering tissues. Structured illumination or optical coherence techniques may help bypass optical scattering, and the integration of imaging with computation represents one of the forefronts of optogenetics.

With the right combinations of genes and optical hardware, one can imagine exciting new directions in biology. If one could control cell-cell interactions optically, one could perhaps optically sculpt tissues with novel or unusual shapes and functions. Optogenetic stimuli might mimic the spatially patterned gradients of morphogen signaling that guide embryonic development, but with the much greater

flexibility of light compared to diffusion, one might coax cells to grow into multicellular structures that could not arise by natural means.

Optogenetics will likely find applications in humans. Companies are currently working to develop channelrhodopsins for vision restoration. Light-controlled proteases may one day provide an ultra-precise surgical tool or an optically triggered viral infection could enable spatially targeted gene therapy. Tattoos with reporter proteins (or the genes encoding them) could provide simple diagnostics, allowing the facile transdermal readout of physiological state.

### CONCLUSIONS

Optogenetics as a field cuts cleanly across traditional disciplinary boundaries. Ecology and genetics provide a source of proteins; advanced spectroscopy and structural biology elucidate molecular mechanisms; molecular biology and biochemistry are used to engineer proteins; sophisticated instrumentation delivers light. An understanding of cell biology or neuroscience is needed to develop reasonable biological questions, and rigorous computation is essential to process the torrents of data that often result. The development of optically instrumented life forms promises to continue for the decades ahead.

Optogenetics also illustrates the difficulty in predicting where basic science will lead. The Optopatch constructs combine genes from an archaeon from the Dead Sea, an alga from England, an FP from a coral, an FP from a jellyfish, and a peptide from a pig virus. The discoverers of these individual genes likely never suspected that they would be combined one day and used in human neurons to study a cell-based model of neurodegeneration in amyotrophic lateral sclerosis.

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