

CHAPTER 1

A comprehensive concept of optogenetics

Guillaume P. Dugué^{†,*}, Walther Akemann[‡] and Thomas Knöpfel[‡]

[†] *Champalimaud Neuroscience Programme, Instituto Gulbenkian de Ciência, Oeiras, Portugal*

[‡] *RIKEN Brain Science Institute, Hirosawa, Wako City, Saitama, Japan*

Abstract: Fundamental questions that neuroscientists have previously approached with classical biochemical and electrophysiological techniques can now be addressed using optogenetics. The term optogenetics reflects the key program of this emerging field, namely, combining optical and genetic techniques. With the already impressively successful application of light-driven actuator proteins such as microbial opsins to interact with intact neural circuits, optogenetics rose to a key technology over the past few years. While spearheaded by tools to control membrane voltage, the more general concept of optogenetics includes the use of a variety of genetically encoded probes for physiological parameters ranging from membrane voltage and calcium concentration to metabolism. Here, we provide a comprehensive overview of the state of the art in this rapidly growing discipline and attempt to sketch some of its future prospects and challenges.

Keywords: optogenetics; optical imaging; optical control; introduction; fluorescent proteins; opsins; database; wiki.

A historical perspective on optogenetics

The term “optogenetics” was coined a few years after neurons had first been engineered to express opsins and other light-driven actuator proteins, and photoevoked firing had been obtained in cell cultures (Banghart et al., 2004; Boyden et al., 2005; Li et al., 2005; Zemelman et al., 2002, 2003) and behaving flies (Lima and Miesenböck, 2005). The

term initially served as a common denomination for approaches combining “genetic targeting of specific neurons or proteins with optical technology for imaging or control of the targets within intact, living neural circuits” (Deisseroth et al., 2006). A later definition of optogenetics as “the branch of biotechnology which combines genetic engineering with optics to observe and control the function of genetically targeted groups of cells with light, often in the intact animal” (Miesenböck, 2009) continued to gather under the same name two complementary approaches with intertwined histories: one consists in monitoring neuronal activity using

*Corresponding author.

Tel.: +351 210480116; Fax: +351 210480298

E-mail: guillaume.dugue@neuro.fchampalimaud.org

genetically encoded fluorescent reporters (sensors), while the other aims at controlling neuronal activity using genetically addressable light-activated tools (actuators). But with the lightning success of actuators in and outside neuroscience, a more restrictive definition of optogenetics started to gain increasing acceptance. In the *Nature Methods* issue of January 2011 featuring optogenetics as Method of the Year 2010, optogenetics was introduced as “the combination of genetic and optical methods to achieve gain or loss of function of well-defined events in specific cells of living tissue” (Deisseroth, 2011). In this section, we trace back the conceptual roots and history of the field and try to paint a comprehensive and balanced picture of what optogenetics encompasses today.

Early ideas

Scientific intuitions and representations of natural phenomena are often formed and conveyed in a visual form. The fact that neuronal activity relies on primarily invisible electrochemical phenomena makes its representation particularly uneasy. Yet an appealing popular depiction of brain function is an intricate mesh of neuronal processes traveled by evanescent bursts of light symbolizing electrical activity. But as this representation was being adopted by popular media for the sake of simplifying the communication of scientific contents, the idea of optically visualizing neuronal activity was evolving from a mere visionary fantasy to an existing technology. Charles S. Sherrington was probably the first to inspire this notion in an oft-quoted passage from his book *Man on His Nature* in which he imagined neuronal activity as points of light (Sherrington, 1940, pp. 176–178). Sherrington used this metaphor to describe the different stages of a sleep-to-wake transition, upon which the brain gradually becomes “an enchanted loom where millions of flashing shuttles weave a dissolving pattern.” Beyond Sherrington’s vision, it progressively became clear that light would be an interesting

tool not only to interrogate but also to manipulate neuronal activity, an idea which was publicized by Francis H. Crick. In a 1979 article entitled “Thinking about the brain,” Crick pointed out the need for “a method by which all neurons of just one type could be inactivated, leaving the others more or less unaltered” (Crick, 1979). Some 20 years later in a paper reviewing the current and future benefits of molecular biology in neuroscience, Crick explicitly envisioned that light might be used to control and monitor the activity of genetically defined neuronal populations (Crick, 1999): “One of the next requirements is to be able to turn the firing of one or more types of neuron on and off in the alert animal in a rapid manner. The ideal signal would be light, probably at an infrared wavelength to allow the light to penetrate far enough. This seems rather far-fetched but it is conceivable that molecular biologists could engineer a particular cell type to be sensitive to light in this way. [...] Most modern theories of brain action stress the firing (in one way or another) of not single neurons but groups of neurons. [...] One way-out suggestion is to engineer these neurons so that when one of them fires it would emit a flash of light of a particular wavelength. The experimenter could then follow the firing of that group of neurons alone.” Quite remarkably, Crick’s ideas would essentially come true within the next decade.

From organic to genetically encoded reporters of neuronal activity

The chemical approaches to ions and voltage sensing

The first ideas on how to implement an optical measure of neuronal activity emerged in the late 1960s from the study of changes in light scattering, birefringence, and fluorescence associated with action potentials (Cohen et al., 1968). Larger optical signals were obtained in the 1970s by introducing voltage-sensitive organic molecules

into neuronal membranes. This opened the field of voltage-sensitive dye imaging which is still intensively explored today (Peterka et al., 2011). Another step toward the realization of Sherrington's vision was taken during the next decade by Roger Y. Tsien who synthesized organic molecules which change their fluorescence with variations in the concentration of intracellular calcium and could therefore be used as reporters of neuronal activity (Göbel and Helmchen, 2007). These calcium-sensitive dyes have opened an avenue for noninvasive imaging of neuronal activity, a field which has exploded during the 1990s with modern imaging techniques such as two-photon microscopy (Denk et al., 1990; Göbel and Helmchen, 2007). Besides voltage and calcium sensing, a variety of other fluorescent indicators were developed to detect variations in sodium, chloride, zinc, and pH but have been much less intensively used in neurobiological research (Johnson and Spence, 2010).

The genetic approaches to sensing chemicals and voltage

Despite tremendously helpful, organic dyes carried intrinsic limitations which restricted the scope of their applicability. First, these dyes usually stain all cell types indiscriminately and therefore do not offer cell-type-specific activity readout. Second, they have to be added externally and useful staining typically lasts for less than a day, prohibiting chronic experiments such as the study of lasting neuronal plasticity. Third, the dyes themselves or the conditions to deliver them into neurons can present some toxicity. These reasons led to orienting efforts toward substituting these dyes for proteinaceous fluorescent indicators which would enable chronic staining of genetically defined neuronal populations. This new generation of fluorescent reporters was almost exclusively engineered based on the green fluorescent protein (GFP) cloned from the jellyfish *Aequorea victoria*

(Prasher et al., 1992). It is interesting to note that the work on *A. victoria*'s bioluminescence also led to the cloning of the calcium-sensitive luminescent protein aequorin almost 10 years before (Inouye et al., 1985; Prasher et al., 1985). Quite remarkably, aequorin had provided the first report on the use of an optical protein calcium sensor even earlier, when Ridgway and Ashley (1967) optically recorded calcium transients after microinjecting it into single muscle fibers of the barnacle.

To generate fully genetically encoded fluorescent reporters, the classical approaches consisted in fusing one or more fluorescent proteins (FPs) with various protein moieties offering sensitivity to signals such as transmembrane potential, ions (calcium, pH, chloride, or zinc), neurotransmitters (glutamate), or second messengers molecules like cyclic nucleotides (Chudakov et al., 2010; Tian and Looger, 2008). For the sensing process to be converted into a measurable change in fluorescence output of the FPs, these probes usually rely on two possible design strategies (Fig. 1). In the first one, the photophysical properties of a single FP are modulated by conformational changes imposed by the sensor. In most cases, two portions of the FP are interchanged and reconnected by short spacers (circularly permuted) so that its fluorescence becomes more sensitive to small structural rearrangements at its extremities. In the second one, conformational changes are used to modify the distance or orientation of two FP variants with spectral properties allowing Förster resonance energy transfer (FRET). While the work on certain reporters is still in its early phase, others like voltage-sensitive fluorescent proteins (VSFPs) and genetically encoded calcium indicators (GECIs) have already gone through multiple improvement steps and show promising results. These tools are reviewed in detail in Chapters 4 and 5, respectively. An overview of available optical reporters for probing the activity of genetically defined neurons is given in Fig. 1. A general overview of optogenetic reporters is provided in Chapter 12.

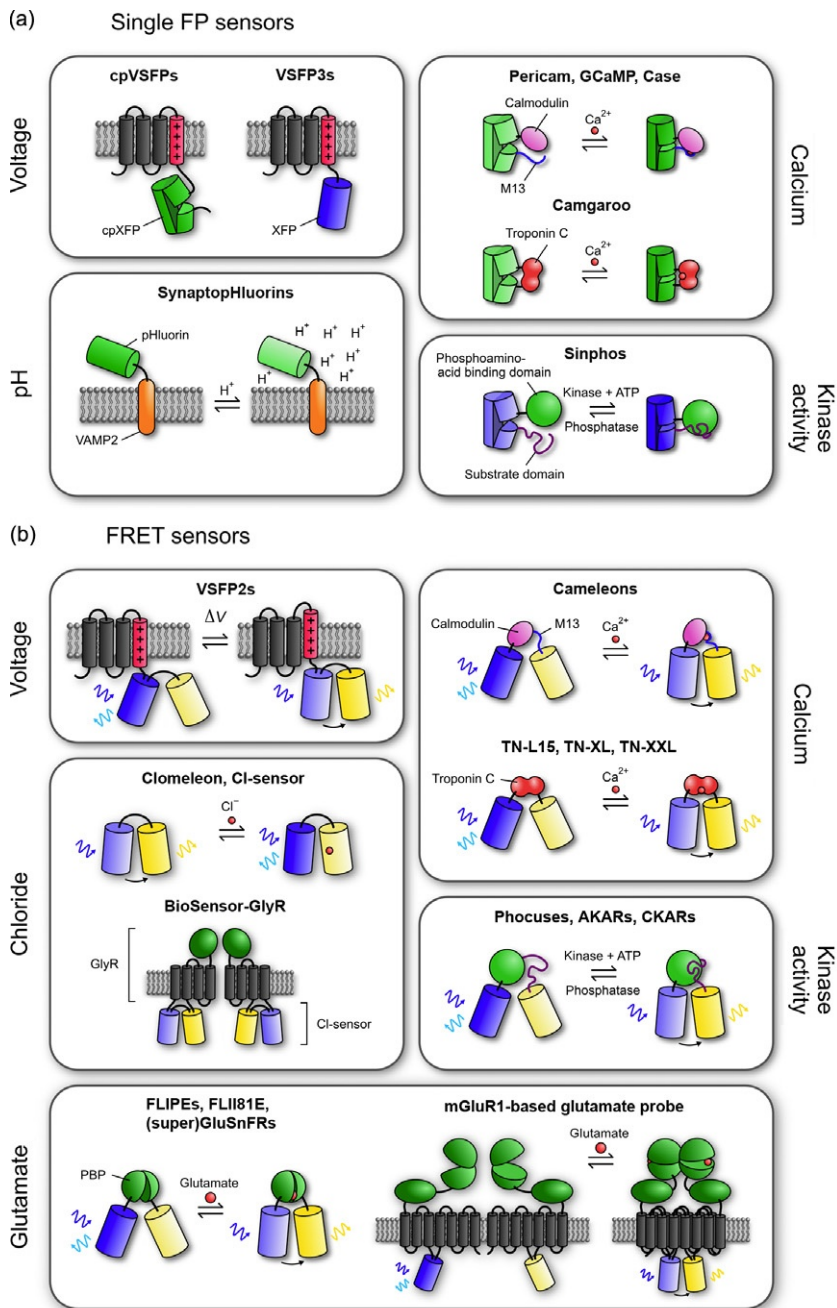


Fig. 1. Optogenetic tools for monitoring cellular signals. (a) Sensors based on single fluorescent proteins (FPs). These sensors usually incorporate intact GFP variants (XFPs) or their circularly permuted version (cpXFPs). Single FP-based voltage-sensitive fluorescent proteins (VSFPs, voltage probes) are derived from a combination of a membrane-integrated voltage sensor domain (gray and purple transmembrane domains) and cpXFPs (cpVSFPs) or XFPs (VSFP3s). Single FP calcium indicators include

Tools for controlling genetically defined neurons

Optogenetic approaches employing microbial rhodopsins for exciting and inhibiting neurons are covered in Chapters 2 and 3. We are providing here a historical overview of how modern strategies for controlling neuronal activity with light emerged over the past 10 years.

Photochemical approaches

The idea of using light-sensitive molecular tools for the optical control of neuronal activity (actuators) had been latently present in the literature well before Crick articulated their theoretical utility. In particular, photochemistry had already provided insights into how to convert a ligand from an inert state into a high-affinity form, a field which would become popular in neuroscience with the use of caged neurotransmitters (Nerbonne, 1996). Using this technique, synthetic photoconvertible ligands can be used to optically modulate neuronal activity through the activation of specific receptor proteins (Fig. 2a). To restrict the action of the ligand to genetically designated neurons, the receptor itself has to be targeted to these neurons (Zemelman et al., 2003). This method was used by Lima and Miesenböck (2005) to elicit specific behaviors in fruit flies using light as a trigger, providing the first example of an optically “remote-controlled” animal.

The photoactivation process can be made more efficient by linking the ligand to the protein through a covalent bond and obtaining a “photoswitched tethered ligand” (PTL, Fig. 2a), a technique used successfully to control nicotinic receptors (Bartels et al., 1971), ionotropic glutamate receptors (Volgraf et al., 2006), potassium channels (Banghart et al., 2004; Chambers et al., 2006; Fortin et al., 2011), and recently a chimeric potassium-selective glutamate receptor called HyLighter (Janovjak et al., 2010). One major drawback of photochemical approaches is the necessity of either delivering the ligand or conjugating the PTL to the target protein, which limits their use to easily accessible preparations like cultured neurons, brain slices, or small organisms such as fruit flies (Lima and Miesenböck, 2005) or zebrafish larvae (Janovjak et al., 2010). Photochemical approaches to control neuronal firing have been reviewed by Gorostiza and Isacoff (2007, 2008) and Miesenböck (2011).

Genetic approaches based on animal opsins

Fully genetically encoded light-gated actuators which do not require the addition of an exogenous cofactor appeared as a more viable solution for controlling neuronal activity *in vivo*. Not surprisingly, the hunt for candidates first concentrated on the phototransduction machineries underlying animal vision. The light-sensitive elements in these systems are membrane-embedded photopigments

scaffolds based on GFP and the calmodulin-M13 complex (such as Pericam, GCaMPs, and Case) and troponin-based scaffolds (Camgaroo). SynaptopHluorins are indicators of vesicle release and recycling, consisting of a pH-sensitive form of GFP (pHluorin) fused to the luminal side of a vesicle-associated membrane protein (VAMP). Sinphos are detectors of protein phosphorylation (kinase activity) made of a fusion between a cpXFP, a phosphorylatable substrate peptide, and a phosphoamino acid binding domain. (b) FRET sensors. These sensors are traditionally based on a FRET pair of FPs such as CFP and YFP. VSFP2s are FRET-based voltage sensors. FRET calcium indicators include cameleons (based on the calmodulin-M13 complex) and the TN sensor family (based on troponin). Chloride sensors (Clomeleon and Cl-sensors) take advantage of the fact that chloride can efficiently quench YFP fluorescence, thus reducing the FRET signal of a CFP-YFP pair. In BioSensor-GlyR, Cl-sensor proteins are grafted to the subunits of a glycine receptor (GlyR) in order to sense chloride ions flowing through the receptor. FRET kinase activity sensors (Phocuses and XKARs) have been developed using the same rationale as for Sinphos (see a). Finally, glutamate can be detected using FRET sensors based on bacterial periplasmic binding proteins (PBPs) or on a metabotropic glutamate receptor (mGluR1). Membranes are represented with the cytoplasmic side toward the bottom.

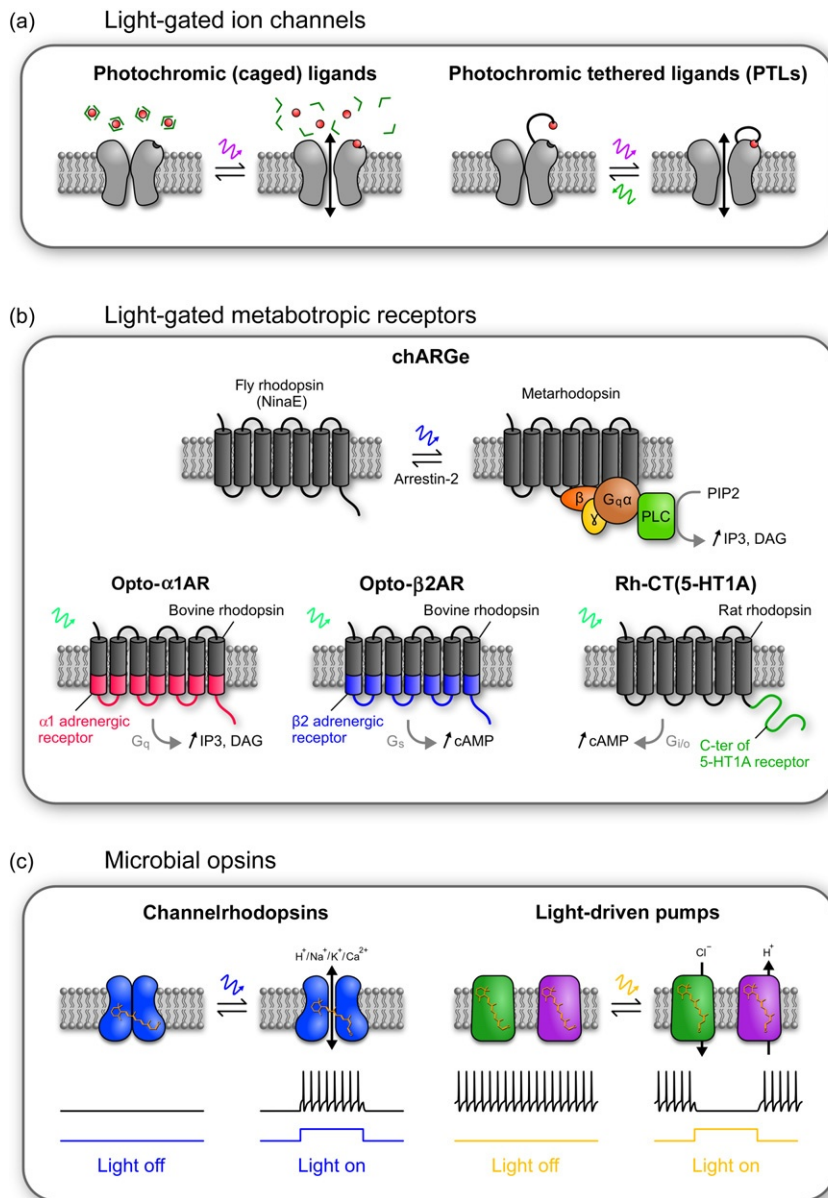


Fig. 2. Optogenetic tools for controlling neuronal activity. (a) Artificial gating of ion channels by light can be accomplished using photochromically caged ligands or tethered ligands with a photochromic activation switch. (b) Endogenous conductances can be modulated through light-dependent activation of intracellular second messenger cascades using animal opsins (type II opsins). This was achieved in mammalian neurons by reconstituting a minimal fly phototransduction machinery through the heterologous expression of three proteins (NinaE, arrestin-2, and $G_{q\alpha}$, a system called chARGE) or by expressing single vertebrate rhodopsin genes (not shown). Alternatively, the intracellular loops or C-terminal domain of vertebrate rhodopsins can be exchanged with the intracellular domains of specific metabotropic receptors to gain optical control over specific signaling cascades (Opto- α 1AR,

called rhodopsins, each rhodopsin molecule consisting of a protein called opsin (belonging to the family of G-protein-coupled receptors or GPCRs) covalently bound to a chromophore (a vitamin A-related compound called retinal or one of its derivatives). Upon illumination, the bound retinal molecule undergoes isomerization, which induces conformational changes in the opsin backbone and activates a G-protein signaling pathway.

This path was pioneered by Har Gobind Khorana in the late 80s, who observed light-dependent ionic currents in *Xenopus* oocytes transfected with a bovine rhodopsin gene (Khorana et al., 1988). The next significant step was taken in the early 2000s by the team of Gero Miesenböck who managed to reconstitute a minimal fly phototransduction cascade in mammalian neurons by coexpressing NinaE, a blue-sensitive rhodopsin and two of its natural partners: the α q G-protein subunit and arrestin-2, a protein required for deactivation of rhodopsin. Upon illumination, the excited rhodopsin activates an endogenous phospholipase C through the action of the G-protein, which, in turn, activates nonspecific cation channels through the production of second messengers (Fig. 2b). The system called “chARGe” was used to optically elicit action potential firing in cultured hippocampal neurons (Zemelman et al., 2002) but was fastidious to implement and carried intrinsic limitations like slow and variable activation and deactivation kinetics (a few hundred milliseconds to several tens of seconds). Following a similar rationale, subsequent studies showed that heterologous expression of single mammalian opsins in neurons was enough to modulate endogenous conductances through specific G-protein cascades, but with comparable slow kinetics (Gutierrez

et al., 2011; Li et al., 2005; Masseck et al., 2011; Melyan et al., 2005).

The slow kinetics observed in these approaches is inherent to the metabotropic nature of vertebrate and invertebrate opsin signaling, which challenges their relevance as strategies for temporally precise control of neuronal firing. However, in a different perspective, animal opsins were used successfully to gain optical control over specific intracellular transduction pathways. Building up on the work of Kim et al. (2005), the team of Karl Deisseroth engineered chimeric receptors by replacing the intracellular loops of the bovine rhodopsin with those of specific adrenergic receptors (Airan et al., 2009), taking advantage of common structure–function relationships among GPCRs. Using these tools, they were able to optically activate the intracellular pathways normally recruited by these receptors (the cAMP and IP3 pathways; Fig. 2b). Following a similar approach, the team of Stefan Herlitze produced a light-activated receptor which recruits the signaling cascade of a specific serotonin receptor (Oh et al., 2010). These emerging tools might be gathered under the name “opto-XRs” proposed by Airan et al. (2009), where X specifies the particular pathways which is being optically hijacked (e.g., opto- α 1AR for α 1 adrenergic receptors).

The revolution of microbial opsins

Ideal light-gated actuators would be single proteins rather than effector proteins activated by multicomponent signaling cascades. Unexpectedly, such tools were to be found in branches of biology which could have hardly been more distant from neuroscience: the study of phototropism

Opto- β 2AR, and Rh-CT(5-HT1A)). (c) Naturally occurring microbial opsins (type I opsins) and their synthetic variants provide a large panel of single-protein actuators which can be used to control neuronal firing with millisecond precision. Channelrhodopsins display an intrinsic light-gated cationic conductance and can be used to depolarize neurons. Microbial light-driven pumps can produce hyperpolarizing currents by translocating chloride ions into the cell (halorhodopsins) or protons outside (bacteriorhodopsins, proteorhodopsins, and archeorhodopsins). Membranes are represented with the cytoplasmic side toward the bottom.

in unicellular algae and of light-driven ion transport in halophilic archaeobacteria.

Indeed, animals are not the only realm that possesses retinal opsins. Unicellular photosynthetic organisms like green algae or euglenids also express rhodopsins in a photoreceptive organelle called the “eyespot apparatus” used to initiate phototropic reactions (swimming toward or away from light). In the green algae *Chlamydomonas*, the eyespot contains atypical rhodopsins displaying intrinsic light-gated ion conductance, called channelrhodopsins (Nagel et al., 2005). In 2002–2003, two channelrhodopsins were cloned from the species *Chlamydomonas reinhardtii*. The first one (ChR1) is selectively permeable to protons (Nagel et al., 2002), while the second one (ChR2) is also permeable to other cations (Nagel et al., 2003) and can thus mediate depolarizing currents irrespective of the extracellular pH. Due to their channel-like structure, these proteins provided extremely rapid responses to light when tested in *Xenopus* oocytes, with photocurrents occurring within tens of microseconds upon illumination with blue light (450–500nm). A couple years later, the teams of Karl Deisseroth and Stefan Herlitze expressed ChR2 in cultured hippocampal neurons and showed that ChR2-mediated photocurrents were rapid and large enough to fire these cells with millisecond precision (Boyden et al., 2005; Li et al., 2005). New channelrhodopsins cloned from two other species of green algae were recently used successfully in mammalian neurons: VChR1 from *Volvox carteri* (Zhang et al., 2008) and MChR1 from *Mesostigma viride* (Govorunova et al., 2011). Initially, there was considerable doubt whether this approach would be successful, as acknowledged in retrospect by Deisseroth himself (Deisseroth, 2010), because no one could tell whether the protein would fold and integrate correctly into the cytoplasmic membrane of mammalian cells and if endogenous retinal would be available at sufficient quantities. But subsequent experiments showed that within reasonable expression levels, ChR2 and its variants could be

used safely to control the activity of genetically defined neuronal populations in animal models ranging from flies to monkeys (Fenno et al., 2011). In only 5 years, channelrhodopsins emerged as a technical revolution at almost all levels of neurobiological research.

But channelrhodopsins were not the only players in this revolution. Other microbial rhodopsins behaving as light-driven ion pumps were long known to exist in halophilic archaeobacteria (Mukohata et al., 1999) and were discovered recently in bacteria (Beja et al., 2000) and some eukaryotes (Waschuk et al., 2005). Proteins like bacteriorhodopsins, proteorhodopsins, and archaerhodopsins extrude protons from the cytoplasm, building up a proton gradient used for the production of ATP. Others like halorhodopsins are used by certain halobacteria to maintain their osmotic balance by transporting chloride into their cytoplasm (Muller and Oren, 2003). Both types thus generate a hyperpolarizing photocurrent which can be used to silence neuronal activity. Interestingly, these pumps operate at different peak sensitivity wavelengths compared to ChR2, opening the possibility of coexpressing them with ChR2 to achieve bidirectional control of the same cell. Zhang et al. (2007) provided the proof of principle that this is indeed possible by coexpressing ChR2 and the halorhodopsin from *Natronomonas pharaonis* (NpHR) in acute brain slices and in *Caenorhabditis elegans*. But translating these light-driven pumps into usable tools was not as straightforward as for ChR2. A common problem with these proteins was impaired subcellular localization which decreased their tolerability when expressed at high levels. In particular, the proteins would accumulate at successive steps along the secretory pathway to the cell surface. A number of candidates isolated from various species were tested and modified by adding a series of trafficking signal peptides to improve their membrane localization (Gradinaru et al., 2010). Two families of pumps emerged as promising light-activated silencers: the series of proteins derived from NpHR (the latest being eNpHR3.0 described in Gradinaru et al., 2010) and the archaerhodopsins Arch from

Halorubrum sodomense (Chow et al., 2010) and ArchT from *Halorubrum* genus (Han et al., 2011). The diversity and biophysical properties of microbial opsins and their use in neuroscience were reviewed extensively (Boyden, 2011; Fenno et al., 2011; Hegemann and Moglich, 2011; Lin, 2011; Yizhar et al., 2011a,b,c). State-of-the-art methodologies to deploy these tools in mammalian cells were reviewed in detail by Chow et al. (2011). An overview of available tools for controlling genetically defined neurons is given in Fig. 2.

Emerging optogenetic approaches outside neuroscience

The origins of optogenetics are deeply rooted in neuroscience. As described above, the first efforts to engineer genetically encoded optical sensors were aiming at monitoring neuronal activity. Similarly, the first light-actuated control systems were designed to modulate neuronal firing. But today's developments in the field are addressing a much broader scope of unmet needs in the study of biological systems. Recent progress in bioengineering has provided a new panel of optogenetic readout and control strategies to study a variety of molecular and cellular processes (Miesenböck, 2011).

An expanding toolkit for sensing and monitoring cellular activities

Optical reporters have long been used outside neuroscience to monitor various biomolecules and enzymatic activities (Souslova and Chudakov, 2007). The range of substances and processes which can be optically tracked is quickly expanding. A new family of indicators has been designed from bacterial periplasmic binding proteins (PBPs) to sense metabolites such as carbohydrates and amino acids. These indicators use the Venus fly-trap-like conformational change of PBPs upon

binding to their substrate in order to generate a FRET signal (Deuschle et al., 2005). Other indicators were engineered to detect second messenger molecules like H₂O₂ (Markvicheva et al., 2011), enzymatic activities (kinase, protease, GTPase), and several cellular processes (cell cycle, actin dynamics). These recent developments are reviewed extensively in Lalonde et al. (2005), Okumoto et al. (2008), Frommer et al. (2009), and Okumoto (2010). Chapter 12 of this issue reviews the wide range of factors for which optogenetic reporters are now available.

An emerging repertoire of light-gated effectors to control cell physiology

A new repertoire of light-activated tools for manipulating identified biochemical events is emerging. These new tools include rhodopsin-based chimeric GPCRs like the opto-XRs which can trigger specific intracellular signaling cascades upon illumination (Airan et al., 2009; Kim et al., 2005; Oh et al., 2010). Following a similar logic, Ye et al. (2011) recently managed to functionally link the signal transduction pathway of a vertebrate rhodopsin to a specific gene transcription control mechanism in order to achieve light-induced transgene expression. Other research lines are exploiting and improving non-membrane-associated photoreceptor protein domains to build photoswitchable cytoplasmic effectors (Losi and Gärtner, 2011; Moglich and Moffat, 2010; Strickland et al., 2010). Such domains can be found in numerous species of bacteria, protists, fungi, and plants where they serve a great variety of functions. Recently, naturally occurring photo-activated adenylyl cyclases containing a BLUF domain have been used to control cAMP levels in various models (Nagahama et al., 2007; Schroder-Lang et al., 2007; Stierl et al., 2011) and reengineered to function as guanylyl cyclases (Ryu et al., 2010). Other studies have taken advantage of LOV domains to confer photosensitivity to DNA-binding proteins, enzymes, and

small GTPases (Lee et al., 2008; Moglich et al., 2009; Strickland et al., 2008; Wu et al., 2009) and more recently to activate endogenous calcium channels (Pham et al., 2011). One last exciting development consists in exploiting reversible light-dependent protein binding mechanisms found in plants (reviewed in Kami et al., 2010). These mechanisms involve identified partners such as phytochromes (Phy) and phytochrome-interacting factors which can be fused to proteins of interest to gain photocontrol over their association. The resulting “photoactivated dimerizers” can be used to investigate biological processes with exquisite spatiotemporal resolution or to create new molecular pathways. This strategy has already been used to achieve light-gated protein translocation, protein splicing, gene transcription, and DNA recombination (Kennedy et al., 2010; Levskaia et al., 2009; Shimizu-Sato et al., 2002; Toettcher et al., 2011; Tyszkiewicz and Muir, 2008; Yazawa et al., 2009) and is reviewed extensively in Chapter 6 of this issue.

Photosensitizers: using light to destroy proteins and cells

Interacting with cells and proteins in a rapid and reversible way is one key program of optogenetics. But light can also be used to produce targeted lesions, an approach which can be relevant for perturbing neural circuits and designing models of neurodegenerative disorders. One way of making these lesions specific is to target light-sensitive molecules called photosensitizers (PSs) to particular proteins or cells. When irradiated with light, PSs generate reactive oxygen species (ROS) which very rapidly react with any nearby biomolecule and can eventually kill cells through apoptosis or necrosis. The technique, called chromophore-assisted light inactivation (CALI), has been used extensively for the treatment of precancerous lesions and superficial tumors. Most available PSs are organic molecules which have to be introduced exogenously into living systems and offer very poor

selectivity for particular cell types or proteins. One solution to this issue is to rely on peptide-like (peptoids) PSs which are resistant to proteolysis and can be designed to bind specifically to virtually any given protein (Lee et al., 2010). But a more definitive solution for protein- and cell-type-specific CALI was the design of the first genetically encoded PS (Bulina et al., 2006a). The protein called KillerRed was isolated by screening a collection of GFP homologs for phototoxic effects on *Escherichia coli* cells. Although its ROS-production capacity is still inferior to chemical PSs, KillerRed has been used successfully in zebrafish embryos to induce cell death (Teh et al., 2010) and in cell cultures to achieve target protein inactivation (Bulina et al., 2006b) and reversible blockage of cell division (Serebrovskaya et al., 2011).

A comprehensive definition of optogenetics

As pointed out before (Miesenböck, 2009), the term “optogenetics” is a bit of a misnomer as it does not involve any interaction between light and the genome. But coming up with this label was definitely a smart move judging from how quickly it was adopted by the research community. The term is now firmly established both in the scientific literature and in the popular media, but its usage has not yet crystallized around a common acceptance.

Etymologically, “optogenetics” simply refers to the combination of optical and genetic approaches and implicitly designates all strategies using genetically addressable light-sensitive tools to study biological systems. As a consequence, the term should seize on 20 years of utilization of FPs, including for simply labeling cells and proteins. More reasonably, optogenetics can designate the use of genetically addressable photosensitive elements not as inert dyes but as environmentally sensitive fluorophores (in which light emission is affected by identified factors) and/or as active agents (which can transduce optical energy into biophysical effects). This definition encompasses

both monitoring and control strategies. We believe that a narrower acceptance of the word is unjustified. Just like “optoelectronics” designates the use of both light sources and detectors, “optogenetics” should encompass the use of both control tools and reporters.

Which control tools and which reporters should be included in this definition? In the broadest sense, optogenetic tools do not need to be fully genetically encoded but only genetically “addressable.” This means that proteins requiring an exogenous cofactor to function can also be considered as “optogenetic” as long as their expression can be restricted to certain groups of cells. This definition includes a range of photochemical approaches where proteins are engineered to bind to a given photochromic ligand. It also encompasses the use of photoreceptor proteins in organisms lacking their specific chromophore. In such cases, the chromophore molecule has to be added exogenously (e.g., retinal in invertebrates for channelrhodopsin-based applications and bilin in nonplant organisms for phytochrome-based applications).

Finally, to what areas of biology should the term optogenetics apply? There is no valid reason to restrict its use to neuroscience only. Current developments even tend to show an accelerated expansion of optogenetic approaches toward general cellular and molecular biology. Overall, we wish to conclude that a comprehensive definition of optogenetics might be the following: optogenetics is the combination of optical and molecular strategies to monitor and control designated molecular and cellular activities in living tissues and cells using genetically addressable photosensitive tools.

Combining the tools

Optogenetic control tools clearly made their breakthrough in neuroscience with manipulations at the level of cell populations while observing the consequences at the systems and behavioral levels (Carter and de Lecea, 2011). However, it is also

clear that a detailed mechanistic analysis and understanding of brain function will require simultaneous observation and/or manipulation of various neuronal types at the same cellular or circuit level. This can be partially implemented by using optogenetics in conjunction with existing techniques like electrical recordings. But we believe that this methodological challenge will be eventually more perfectly met by combining optogenetic tools within the same experiment, an important step which will unleash the full power of optogenetics.

“See it, block it, move it”

To understand and demonstrate how a biological phenomenon works ultimately requires using a canonical scientific methodology often summarized by the formula “see it, block it, move it.” The first step is to identify the conditions for this phenomenon to occur (see it); the second step is trying to find out which of these conditions are necessary using loss-of-function experiments (block it); the third step is to test the sufficiency of one or more conditions through gain-of-function experiments (move it). The first step aims at establishing a correlation, while the two others aim at demonstrating causation. Correlation in neuroscience has been investigated in particular using invasive electrical recordings in order to match neuronal activity with behavior. Although such recordings can provide hints on possible causal relationships (e.g., when identified electrical events precede or follow behavioral events), causation is traditionally approached using genetic (KOs and overexpression), electrical (stimulations), surgical (lesions), or pharmacological (agonists and antagonists) interventions. None of these techniques alone provides both high temporal and spatial (cellular) specificity. Electrical stimulations and recordings of neuronal firing display exquisite microsecond-scale temporal resolution but are usually unable to discriminate between neurochemical cell types. In addition, electrical

stimulations do not discriminate between axons and cell bodies, which seriously limits their interpretative value. Conversely, pharmacological interventions are hampered by their poor temporal resolution although they can provide very good neurochemical specificity.

Optogenetics is considered a true technological breakthrough because it makes it possible to implement the “see it, block it, move it” approach with both high temporal resolution and high cellular (even subcellular and molecular) resolution. Thus compared to standards of the past decade, modern optogenetic studies might bring more definitive answers and allow biologists to form stronger interpretations. More remarkably, combining optogenetic tools will offer the possibility of implementing this approach in the same experiment, which will dramatically increase the yield of individual studies.

Combining optogenetics with electrical recordings

Microelectrode recordings are still the golden standard for measuring neuronal firing, surpassing by far optical sensors at least at the single cell resolution and millisecond time scale. But electrical recordings can be very advantageously combined with optogenetic tools, in general, and light-gated actuators, in particular.

First of all, electrical recordings are and will probably remain the ultimate readout of the efficiency of optogenetic activation and inactivation protocols. Indeed, the reliability of optogenetic control depends on a series of important parameters which can be preparation specific, such as the electrophysiological properties of the target cell type, the optical properties of the tissue, or the expression level of the optogenetic tool (which depends on the time postinfection when using viral vectors). Assessing photoevoked changes in firing during optogenetic control experiments might even become systematic practice with the use of “optoelectrodes” which

integrate light guides and electrodes in the same device.

Second, optogenetic control tools can elegantly replace stimulating electrodes in circuit mapping experiments. Classically, these experiments consist in probing functional connections between neuronal types and brain regions using pairs of recording and stimulating electrodes. Light-gated actuators can substitute for electrical stimulations in order to control efferent and afferent connections in isolation based on their origin, destination, or neurochemical identity. For example, labeling a group of neurons anterogradely with ChR2 or NpHR allows the experimenter to photoexcite or inhibit specifically its axonal projections in distant areas, even when cut from their soma (Atasoy et al., 2008; Cruikshank et al., 2010; Kaneda et al., 2011; Petreanu et al., 2007, 2009; Varga et al., 2009). Conversely, expressing these tools using retrograde transsynaptic activators (Gradinaru et al., 2010) or retrogradely transported viruses (Lima et al., 2009) offers the opportunity to control specifically cells projecting to a particular region. These dual electrical-optogenetic strategies can even be paired with targeted illumination and scanning techniques to refine and accelerate mapping processes.

Third, optogenetics can help overcome the fact that extracellular electrical recordings do not easily distinguish spikes from different neuronal populations. This has been a central issue and a great source of debates in the study of neuronal firing *in vivo*. Traditionally, neuronal types are identified during recording based on electrophysiological criteria like spike shape and firing patterns. But this approach fails in the case where two different populations have overlapping properties. Labeling a group of neurons with ChR2 offers the possibility to confirm their identity online. As shown by Lima et al. (2009), ChR2-tagged neurons can be identified *in vivo* by their reliable and short latency response to brief flashes of blue light, a strategy called PINP (photostimulation-assisted identification of neuronal populations).

Combining optogenetic tools: Toward multicolor interrogation of neural circuits

Multicolor control of neuronal populations

Combining several light-gated actuators in the same experiment requires the ability to recruit one with minimal cross-excitation of the others. Maybe because they were isolated from organisms living in very different ecosystems, microbial opsins display a great diversity of spectral sensitivities. A few of them can be excited almost separately using different wavelengths. The best example so far is the association of ChR2 and NpHR which allows bidirectional control of firing of the same cells using blue- and yellow light (Zhang et al., 2007) opening the possibility of performing loss-of-function and gain-of-function experiments (block it and move it) on the same preparation.

Other “optically compatible” opsin pairs include the blue- and yellow light-gated channelrhodopsin variants ChR2 and VChR1 (Zhang et al., 2008) and the blue- and red-light drivable ion pumps Mac and NpHR (Chow et al., 2010). In theory, these tools allow multicolor control of separate populations of neurons simultaneously. A new generation of red-shifted actuators includes novel channelrhodopsins such as MChR1 from *M. viride* (Govorunova et al., 2011) and C1V1s, a family of ChR1/VChR1 chimera displaying large photocurrents and minimal cross-activation with ChR2 (Yizhar et al., 2011a,b,c) as well as new light-driven pumps such as Halo57, a naturally occurring halorhodopsin displaying larger photocurrents than NpHR when excited in the far red (Klapoetke et al., 2010). These new opsins are expanding the catalog of compatible actuators for multicolor control of neural circuits. Optogenetic control tools can also be combined physically as a unique protein. Recently a tandem gene fusion strategy was proposed for co-localized and stoichiometric expression of opsin pairs (Kleinlogel et al., 2011). This approach has a number of potential applications. Precise bidirectional control of firing with low cell-to-cell variability of the

excitation-to-inhibition ratios can be achieved by fusing a ChR variant and a light-driven pump. This strategy is also a useful way of creating new tools with new properties: for example, ChR variants with different excitation spectra can be combined to create a hybrid tool with a wider action spectrum.

Multicolor probing of neuronal activity

Contrary to microbial opsins, most genetically encoded reporters were not isolated from ecologically diverse species but were engineered based on a very limited number of FPs (GFP or YFP for single FP sensors and CFP and YFP for FRET sensors). However, the color palette of available FP variants has been continuously expanding for the past 10 years, and available FPs now span almost the entire visible spectrum (Chudakov et al., 2010; Day and Davidson, 2009). This opens the door for a new generation of genetically encoded probes with diversified and minimally overlapping spectral characteristics. These novel tools will be used to visualize the activity of distinct neuronal populations in parallel or to image multiple parameters in the same cells.

FRET sensors were the first category of optical reporters to be spectrally diversified. Indeed, grafting a new pair of FPs in an existing FRET sensor scaffold is relatively straightforward since it does not require major modifications of the FPs. In contrast, updating single FP sensors can require more work since they often incorporate modified versions of the FP (e.g., circularly permuted FPs). New blue- and red-shifted spectral variants were already produced for several FRET sensors including the voltage sensors VSFP2s (Akemann et al., 2010), sensors of cyclic nucleotides (Niino et al., 2009), reporters of enzymatic activities (Ai et al., 2008; Grant et al., 2008; Ouyang et al., 2010), or protein translocation (Piljic and Schultz, 2008). Some of these variants were used to demonstrate the feasibility of double and triple FRET measurements (Ai et al., 2008; Grant et al., 2008; Niino et al., 2009; Ouyang et al., 2010; Piljic and Schultz, 2008).

FRET sensors have the advantage of enabling ratiometric measurements but the inconvenience of using two FPs (one donor and one acceptor). For this reason, combining more than two or three spectrally nonoverlapping FRET sensors is very challenging. A smart workaround is to free up one color channel by using a nonfluorescent (dark) acceptor which acts as a dynamic quencher for the donor fluorescence (Ganesan et al., 2006; Niino et al., 2010). Still, single FP sensors provide a simpler and more flexible solution to the problem of spectral crossover. Single FP sensors are still almost exclusively based on GFP or YFP variants except for the blue-shifted kinase activity sensor Cyan Sinphos (Kawai et al., 2004) and the red-shifted monochromatic voltage sensors VSFP3s (Perron et al., 2009). However, the attractiveness of multicolor imaging should promote the construction of additional spectral variants of single FP sensors in the near future. Recent efforts have focused on mutating the calcium indicator scaffold introduced as GCaMP (Nakai et al., 2001) to obtain hue-shifted variants. Using a “molecular evolution strategy” (iterative rounds of mutagenesis and screening of bacterial colonies), Zhao et al. (2011) have engineered a new set of GCaMP mutants called GECOs, comprising blue and red variants. Another initiative which will accelerate the development of new calcium sensors is the GECI project from the HHMI Janelia Farm research campus (<http://www.janelia.org/team-project/geci>). This project uses a high-throughput, mammalian neuron-based imaging platform to screen through libraries of variants. Current lead variants include blue, cyan, and yellow versions of the GCaMP scaffold (BCaMP, CyCaMP, and YCaMP) as well as a red version (RCaMP) which was engineered from scratch using the red FP mRuby (Loren L. Looger, personal communication).

Combined optogenetic monitoring and control of neuronal activity

The next big step remains the association of optical reporters and control tools within the same

experiment to allow all-optical interrogation of neural circuits. To date, only one study has employed this type of strategy: the work by the team of Sharad Ramanathan described how ChR2 and GCaMP can be combined to map functional connections between groups of neurons in *C. elegans* (Guo et al., 2009). Because ChR2 and GCaMP have highly overlapping excitation spectra, the authors had to separate the excitation channels of the two proteins both temporally and spatially. Similar experiments should be greatly simplified by the use of red-shifted activity reporters such as RCaMP and VSFP3s (Perron et al., 2009) or alternatively by the combination of red-shifted opsins with blue-shifted reporters.

Given the current rate of expansion of the optogenetic toolkit, the number of possible tool combinations might soon become overwhelming, giving unprecedented latitude for the experimenter’s imagination. Most important, the analytical power of “all-optogenetic” approaches is potentially mind-blowing: combining monitoring and control will allow researchers to establish correlation and causation in the same experiment. This should increase the yield of individual experiments and raise the standards in many fields of neurobiological research.

New challenges for old technologies

Optogenetics did not evolve as a stand-alone approach but rather emerged at the crossroads of several independent technologies. These technologies include methods for gene delivery on the one hand and for light delivery and collection on the other. By constantly setting new technical requirements, optogenetics is regularly challenging these parent technologies and driving technical innovation. Several key techniques for optogenetics are reviewed in the following chapters. Here, our intention is to provide an overview of the emerging optogenetic know-how in neuroscience, with a strong focus on mammalian models.

Current and future challenges for gene delivery approaches

Optogenetic tools are genetically addressable, which means that all or parts of them are genetically encoded. Thus, the starting point of any optogenetic experiment consists in choosing a particular optogenetic tool and a method to deliver it to a target system. The main objective of this step is to achieve expression in a functionally and/or genetically well-defined set of neurons. Depending on the time and resources available as well as experimental requirements, one can choose to build transgenic lines (germline transgenesis) or to acutely transfer the gene of interest to a particular organ, region, or group of cells in individual animals (somatic gene delivery). Here, we review the current advantages and limitations of these strategies (see also [Zhang et al., 2010](#)).

Somatic gene transfer in the central nervous system

Acute gene transfer can be performed using viral vectors, the two most popular agents currently being retroviruses (which include lentiviruses) and adeno-associated viruses (AAVs) ([Aronoff and Petersen, 2006](#); [Davidson and Breakefield, 2003](#); [Monahan and Samulski, 2000](#); [Teschemacher et al., 2005](#); [Wong et al., 2006](#)). Virus injection into the brain can be performed at all stages of life through a simple surgical procedure ([Cetin et al., 2006](#); [Lowery and Majewska, 2010](#); [Pilpel et al., 2009](#); [Puntel et al., 2010](#)). Electroporation is another method for quick gene delivery which works by forcing expression plasmids into single or groups of cells using an electric field ([Judkewitz et al., 2009](#)). When performed on mouse embryos *in utero*, this technique can provide large numbers of transgenic animals in a short time frame ([Walantus et al., 2007](#)). Electroporation techniques are reviewed in greater details in Chapter 9 of this issue. Acute gene transfer offers a number of advantages over transgenic lines. First, it offers the possibility to test new genetic constructs rapidly

(in several weeks), allowing researchers to keep up with new optogenetic tools. Second, it can provide higher expression levels than transgenic lines, a feature which can be particularly important when working with actuators with low unitary photocurrents such as microbial opsins.

Viral vectors are currently the most popular method for rapid gene delivery mainly because of the versatility that they offer. Viral strategies can be designed to yield both high levels and high cell-type specificity of expression. Cell-type specificity can eventually be empirically achieved through viral serotype-specific tropism but most commonly relies on the use of specific gene promoters. Promoters can be included in the encapsidated transgene to allow autonomous specific expression. In case this approach yields insufficient expression, transcriptional amplification strategies can help enhancing the expression of the transgene ([Liu et al., 2008](#)). A popular alternative is to inject viruses containing a Cre-responsive expression cassette into the brain of a Cre-expressing line ([Kuhlman and Huang, 2008](#)), a technique which was perfected with the flip-excision (FLEX) switch system ([Atasoy et al., 2008](#); this system is also referred to as DiO for doublefloxed inverse open-reading-frame). In the FLEX/DiO system, cell-type specificity is provided by the expression of the Cre recombinase, while high transcription rate of the optogenetic tool is guaranteed by a strong ubiquitous promoter present in the Cre-responsive cassette. This method was a godsend to optogenetics because it made the hundreds of well-characterized Cre-expressing mouse strains generated over the past decade amenable to optogenetics. In particular, FLEX/DiO constructs offer the possibility to quickly test several optogenetic tools on the same type of neurons using the same mouse strain. In theory, FLEX/DiO viruses can also be coinjected with custom Cre-expressing viruses to implement a transcriptional amplification strategy. This approach might reveal useful in species with still very limited catalogs of Cre-expressing transgenic lines such as rats ([Witten et al., in preparation](#)).

Viral approaches can also provide multiple levels of spatial specificity. First, stereotaxic viral injections can be optimized in order to restrict the expression of one or several optogenetic tools to one or more anatomically identified brain regions. Second, strategies for retrograde trans-synaptic expression can be used to target neurons projecting to a particular brain area. Such strategies can employ retrogradely transported viruses like the herpes simplex virus (Berges et al., 2007; Lima et al., 2009), rabies, and pseudo-rabies viruses (Osakada et al., 2011; Wickersham et al., 2007a,b) or certain AAV serotypes (Masamizu et al., 2011). An elegant alternative consists in using a dual-virus approach in which one virus expresses WGA-Cre, a fusion between the Cre and the transcellular tracer protein wheat germ agglutinin (WGA), while the other expresses an optogenetic tool under the control of a FLEX/DiO cassette. The method (described in Gradinaru et al., 2010) follows a three-step process: (1) the first virus is used to infect a particular brain region (region A), while the other is injected into an upstream structure (region B); (2) WGA-Cre is produced in neurons of region A and traffics transsynaptically into their presynaptic neurons; and (3) WGA-Cre activates the transcription of the tool of interest only neurons of region B projecting to region A.

Despite their appreciable flexibility, viral approaches have a number of limitations. First, infection efficiency is usually spatially inhomogeneous, with expression decreasing away from the injection point. Even within the site of injection not all potentially targeted cells express the same amount of the protein depending, for example, on the number of viral copies incorporated into the cells. Overall infection rates are also highly dependent on the quality/titer of the virus preparation which can vary from one batch to the other and introduce variability in the experiment outcome. This important issue has only been addressed and discussed on very few occasions (Aponte et al., 2011; Haubensak et al., 2010; Lin et al., 2011). Inhomogeneous expression can be

partially overcome by performing viral injections in neonates, a method which can yield more widespread expression (Passini and Wolfe, 2001; Pilpel et al., 2009).

Another limitation is the potential toxicity of proteins expressed at high levels using viral gene delivery. High transcription rates may rapidly lead to toxic accumulation of the protein, thus reducing the time window for experimentation. This issue has not been clearly addressed yet in the literature. Other issues include potential immunogenicity of viral particles and DNA packaging limitations of viruses. Indeed, viral capsids can only accommodate exogenous DNA fragments up to a certain limit. This limit (around 5kb for AAVs and 10–15kb for lentiviruses) is not an absolute one in the sense that viral particles can still be produced with larger inserts but with lower titers. Viral gene delivery approaches are reviewed in Chapters 9 and 11 of this issue.

Germline transgenesis

Most of the drawbacks of viruses can be overcome by germline transgenesis. This approach aims at establishing lines of transgenic animals expressing the protein of interest stably and constitutively, eliminating the need of delivering the gene of interest on a single animal basis. Available methods for germline transgenesis are detailed in Chapter 9 of this issue. When compared to viral approaches, the main issue of “optogenetic” transgenic lines so far has been their lower expression levels. This limitation can be problematic for optogenetic tools requiring high expression levels such as microbial opsins. Nevertheless, a number of mouse strains expressing optical reporters (GCaMPs, VSFPs, synaptopHluorin and Clomeleon) or control tools (ChR2, VChR1, ChETA, NpHR, eNpHR3 and Arch) have been generated. These lines express the optogenetic tool under the control of either a specific promoter (Thy1, ChAT, VGAT,

TPH2, VGluT2, PV) or a Cre-activated cassette. The latter type of strain can be crossed with any existing Cre driver line to achieve targeted expression through cell-type-specific recombination. Available mouse lines for optogenetic applications are described in Chapter 10 of this issue.

Although transgenesis clearly saves time and money on the long run, its implementation can be costly and time consuming. While this is true for classical transgenesis techniques (pronuclear microinjection and ES integration into blastocysts), new techniques such as testis electroporation (Dhup and Majumdar, 2008), lentivirus-mediated, and zinc finger nucleases-mediated transgenesis (Le Provost et al., 2010) might hold the keys for rapid and efficient germline transgenesis in various mammalian species.

Cell-subtype specificity through intersectional genetic strategies

Current optogenetic approaches achieve cell-type specificity through the use of single promoters, but discrete cellular subtypes are often defined by the selective coexpression of several markers rather than just one. This is the case for cortical circuits in which functionally distinct subtypes of inhibitory interneurons express specific combinations of calcium-binding proteins, neuropeptides, enzymes, and receptors (Ascoli et al., 2008; Kubota et al., 2011). To target neuronal subpopulations, future optogenetic approaches might employ intersectional strategies to restrict the expression of a transgene to cells coexpressing a particular set of genes. In the mouse, intersectional gene activation was implemented using a dual-recombinase method in order to refine fate mapping studies (Dymecki et al., 2010). In this method, the transcription of a transgene is dependent on the removal of two STOP cassettes by two independent recombinases (e.g., Cre and FLPe) expressed under the control of different promoters. A similar intersectional strategy could

easily be transposed to the FLEX switch system, which already requires two recombination events to produce stable transgene inversion (Atasoy et al., 2008). Intersectional approaches might become more and more attractive with the increasing number of FLPe driver lines and the use of novel site-specific recombinases (Nern et al., 2011). Intersectional expression strategies are reviewed in detail in Chapters 9 and 10 of this issue.

Light delivery and collection

Optogenetics builds on an experimental hardware that blends standard technologies and recent innovations in optical imaging, digital microscopy, and photonics. While much of today's instrumentation is inspired by widespread applications of light microscopy in biology and other disciplines, optogenetics poses new challenges that are likely to expand the technical platform in bioimaging and biophotonics.

Breaking new grounds in microscopic imaging of neuronal activity

Before the advent of modern genetically encoded optical sensors, the use of nonprotein reporters of neuronal activity had already prompted significant advances in microscopic imaging techniques. Optogenetic probes should prolong this momentum by opening new possibilities such as deeper imaging over longer periods of time.

Classically, single cell-resolved fluorescence images are obtained using conventional mono- or multiphoton microscopy combined with laser-scanning techniques. This approach has been used extensively in combination with organic dyes to image neuronal activity in thin preparations (small animals, cultured cells, or brain slices) or superficial brain structures in head-fixed animals. Because dyes can report neuronal activity with relatively high temporal precision (several milliseconds), one major improvement in the past

decade was to achieve high scan rates using new scanning schemes (Saggau, 2006). Fast-scan optical imaging allowed researchers to follow the activity of neuronal networks with combined high temporal and spatial resolution. Genetically encoded optical probes offer a new ground for further developments in the field by allowing multiscale imaging (from large cortical areas to subcellular compartments) over longer periods of time (weeks vs. hours in the case of organic dyes).

Today's challenges for light microscopy consist in accessing deep structures (>1 mm) and imaging neuronal activity in unrestrained animals, with the long-term goal of combining the two. By eliminating the need of a dye-loading step and allowing long-term imaging, genetically encoded activity reporters have dramatically increased the attractiveness of such approaches. Several options have already been investigated. On the side of deep-brain imaging, thin (<1 mm of diameter) gradient refractive index microlenses can be inserted into the brain to increase the reach of laser-scanning microscopy, acting as an optical relay or "microendoscope." Microendoscopy can also be implemented by scanning through high-density optical fiber bundles implanted into deep-brain structures, with each optical fiber behaving as an individual pixel (Vincent et al., 2006). On the side of freely behaving animals, prototypes of lightweight (1–4 g) portable optical microscopes have been designed but their usability is still limited (see Wilt et al., 2009 for review).

Optogenetics might also influence the evolution of light sources and detectors. Because the sensitivity of genetically encoded reporters still lags behind the one of nonprotein sensors (Knöpfel et al., 2006), increasing the quantum yields of detectors and the stability of light sources will represent critical improvements. One solution for low-noise illumination consists in adapting semiconductor light sources like light-emitting diodes (LEDs) to optical microscopy (Albeanu et al., 2008).

Light delivery techniques for optogenetic control of brain activity

Compared to the challenges of optical microscopy, delivering light into the brain to control neuronal activity can seem almost trivial. But this is probably the field where optogenetics is driving the strongest innovation. The main concern is to supply light at a sufficient intensity to a defined volume of brain tissue. Illuminating spatially restricted areas in superficial brain structures or thin preparations can be done using conventional laser-scanning techniques, digital micromirror devices (Jerome et al., 2011), holographic patterned light (Papagiakoumou et al., 2010, this technique is reviewed in Chapter 7), or LED microarrays (Grossman et al., 2010). Alternatively, head-mounted LEDs offer a simple way of delivering light to the surface of the brain in unrestrained animals (Huber et al., 2008; Iwai et al., 2011). Along this line of work, the team of Ed Boyden recently engineered a wirelessly powered and controlled LED system for brain surface illumination weighing only 2 g (Wentz et al., 2011).

Illuminating deep brain areas requires the use of light guides such as optical fibers, an approach which has become standard in the past couple of years. Fiberoptic light delivery can be implemented easily in freely behaving animals. A number of accessories have been developed for this purpose, including implantable optical fiber pieces with miniaturized connectors as well as fiberoptic rotary joints to allow free rotations of the fiber connected to the animal (Gradinaru et al., 2007; Kravitz and Kreitzer, 2011; Yizhar et al., 2011a,b,c). Recent commercial versions of these products can incorporate an independent channel for liquid delivery. Laser beams offer convenient light sources which can be easily manipulated and focused (launched) into the fiber core (typically <200 μm of diameter). LEDs offer a cheaper alternative and can also be coupled to optical fibers (pigtailed) but with lower coupling efficiency than lasers. Alternatives to

optical fibers are under investigation such as waveguide materials which can be deposited onto thin layers of silicon to create multipoint light delivery probes (Zorzos et al., 2010). Chapter 11 of this issue reviews some important technical considerations to take into account for fiberoptic light delivery into the brain.

Optoelectrodes: New devices for combined light delivery and electrical recording

One new requirement of optogenetics is the possibility of delivering light and recording electrical activity with the same implantable device. These “optoelectrodes” (or optrodes) are particularly useful to assess the efficiency of photostimulation and -inhibition *in vivo*. Many different optoelectrode designs have been introduced in the past 5 years. Optical fibers can be simply glued onto or bundled with existing single and multielectrode systems (Anikeeva et al., 2011; Diester et al., 2011; Gradinaru et al., 2007, 2009; Halassa et al., 2011; Kravitz and Kreitzer, 2011; Royer et al., 2010; Zhang et al., 2010) or gold metalized to behave as electrodes (Zhang et al., 2009). Alternatively, 2D multielectrode silicon probes can be modified to integrate waveguide materials (Cho et al., 2010). In the near future, electrical wires might be embedded into the structure of optical fibers. Wires can be added at the fabrication stage by inserting them in a large-diameter optical fiber “preform” and then pulling out a thin string from the heated preform.

One problem facing the use of optoelectrodes is direct interaction between light and metal electrodes when immersed in brain tissue (or saline), a phenomenon which causes light-induced electrical artifacts that can obscure local field potential and spike recordings. These artifacts are most likely due to a photovoltaic effect (also referred to as photogalvanic or Becquerel effect) but have not been investigated and discussed in detail, except on a few occasions (Ayling et al., 2009; Cardin et al., 2010; Han et al., 2009). Efforts

are underway to develop “light-proof” electrodes using, for example, indium tin oxide coating (Zorzos et al., 2009). Glass electrodes are devoid of such light-induced artifacts but are mostly suited for recordings in isolated preparations or immobilized animals. Recently, LeChasseur et al. (2011) introduced a thin (<20 μ m at the tip) fiberoptic microprobe containing one core for light delivery and one electrolyte-filled hollow core for neuronal recording. This strategy allows combined artifact-free single unit recording and photocontrol with minimal damage to the tissue. The issue of light artifacts on metal electrodes is covered in Chapter 11.

Collecting bulk fluorescence in vivo

Monitoring neuronal activity optically often rhymes with imaging the activity of single neurons. But a simpler approach consists in retrieving global (bulk) fluorescence signals rather than single cell-resolved microscopic images, in order to obtain information on population activity. This approach was implemented on anesthetized mice expressing optical reporters (GCaMP2 or synaptopHluorin) in order to map responses elicited by sensory or local electrical stimulations (Diez-Garcia et al., 2007; Fletcher et al., 2009; Petzold et al., 2009). Bulk fluorescence measures are also easy to implement in freely behaving animals using the same fiberoptic hardware used for light delivery. This approach has been pioneered in particular by the team of Matthew E. Larkum using nonprotein calcium-sensitive dyes (Murayama and Larkum, 2009; Murayama et al., 2007). Since genetically encoded probes have raised the relevance of bulk fluorescence measurements by providing cell-type-specific signals, this methodology might soon become more widely adopted. As an example, Lütcke et al. (2010) recently reported the use of optical fibers to measure large-scale sensory-evoked cortical activity in GECI-expressing freely moving mice.

Another potential application of bulk fluorescence measurements is to map and assess transgene expression *in vivo* following viral infection. Most optogenetic tools are indeed either fluorescent or fused with FPs in order to facilitate histological examination. Optical fibers can be used to localize the core of an infection, where optogenetic signals and effects are expected to be higher, prior to recording or stimulation. Chronic measurements of fluorescence signals can also track the expression of a transgene and help define optimal time windows for experimentation. This approach was used recently to guide optogenetic stimulations in nonhuman primates (Diester et al., 2011). In the future, fiberoptic deep-brain fluorescence measurements might even be adopted to assess transgene expression in viral therapy approaches in humans, including potential optogenetic therapies.

Spreading the knowledge and tools

Optogenetics is a field with nearly unprecedented momentum. Optogenetic approaches are becoming standard practice in neuroscience and optogenetic tools are evolving at a frenetic pace. While the vitality and creativity of the field can be nothing but warmly acclaimed, it has its flip side: it is simply becoming challenging for researchers to keep up with it.

Acknowledging this glaring situation naturally leads to the question of how scientific and technical knowledge should be optimally spread among researchers. Review articles, which are the traditional way of summarizing the current state of the art on a particular topic, are intrinsically not adapted to a rapidly evolving field because of their relatively long periodicity, limited article lengths, and incompressible publication delays. The snapshot that they provide, although potentially comprehensive at the time of publication, often loses some of its representativity within the next months.

Keeping up with optogenetics through Web collaboration

On the use of wikis in science

Collaborative Web-based solutions are a weapon of choice to tame a rapid flow of information and hold a constantly up-to-date body of knowledge. Among them, wikis have proven their efficiency for sharing and organizing many bits of information across large communities of users. They provide key advantages which are worth enumerating. First, they are extremely reactive since they can be rapidly edited from anywhere and by anyone. Second, they are extraordinarily dynamic since their content can be modified and corrected at will by a potentially unlimited number of users. Third, the content of a wiki is very rich since it can incorporate much more than just text and pictures by storing virtually any type of file. And last but not least, the access to the large majority of them is totally free. However, the success of a wiki is never guaranteed from start and resides in its ability to attain the critical mass of users necessary for its effectiveness and durability. Without this critical mass, it usually falls into disuse.

Inspired by the remarkable success of the online encyclopedia Wikipedia, scientific wikis have started to proliferate within the past few years with the common aim of sharing protocols, tricks, and ideas (Butler, 2005; Pearson, 2006; Waldrop, 2008). As a result, many different fields of biological research of various sizes and focuses have been “wiki-fied.” For example, OpenWetWare (<http://www.openwetware.org>), created in 2005 by students from the Massachusetts Institute of Technology, is hosting information about protocols in biochemistry and molecular genetics, gathering around 9000 users from more than 100 laboratories. These efforts participate in promoting the general concept of “open research” where researchers make clear and exhaustive accounts of their methodology unlike in many peer-reviewed articles.

As new genetically encoded optical tools are greeted by an irresistible wind of enthusiasm and excitement among neurobiologists, it seems that a similar effort for sharing the emerging optogenetic know-how is very timely. Such a project exists: a wiki platform called OpenOptogenetics was launched during the summer of 2010 (<http://www.openoptogenetics.org>). This platform is fully supported by researchers and aims at providing up-to-date technical information about all aspects of optogenetics.

OpenOptogenetics, an open wiki about optogenetics

Pioneer laboratories in optogenetics have started to share their protocols and experience online through dedicated Web pages such as the Optogenetic Resource Center of the Deisseroth lab (<http://www.stanford.edu/group/dlab/optogenetics/>) or the section “Protocols and Reagents” of Edward Boyden’s Synthetic Neurobiology Group (<http://www.syntheticneurobiology.org/protocols>). By adopting a fully open framework, OpenOptogenetics aims at pushing the collaboration a step further. The wiki is fully supported and administered by researchers and allows anyone to create and edit pages through a simple registration step. OpenOptogenetics is committed to a number of missions: (1) maintaining an inventory of all available optogenetic tools, their properties, and where to obtain them or the transgenic lines expressing them; (2) providing detailed protocols related to common procedures (e.g., gene or light delivery into the brain); (3) describing and comparing the available hardware (light sources, fiberoptic components, optoelectrodes), as well as guides on how to build economical setups for most common applications; (4) linking to books, reviews, or other documentations about optogenetics; (5) keeping an up-to-date list of scientific events (workshops, conferences) related to optogenetics; (6) providing a news feed tracking the latest technical developments in the field.

Promoting the diffusion of an emerging technical know-how is especially meaningful. Facilitating and democratizing the implementation of new techniques has a positive impact on how quickly and how widely they are adopted. OpenOptogenetics will help newcomers take the right decisions on how to best set up their experiments according to their needs and resources. The information available in the wiki should, for example, help people pick the appropriate channel-rhodopsin variant or decide which light source to buy, a key question for laboratories with tight budgets. OpenOptogenetics should not just benefit to the end users. By channelizing the demand for information and guidance, it should help technology makers deal with an increasing number of inquiries about their resources and protocols. Finally, an efficient horizontal transfer of information can be expected to raise the profile of methods by spreading subtle and usually unpublished (but nonetheless important) methodological tricks.

Distributing the tools

Sharing sequences

Knowledge is not the only thing that can be shared. One of the pillars of modern molecular biology is the management and sharing of the millions of nucleotide sequences isolated every year across the world. These sequences are made publicly available and searchable by institutional databases such as the International Nucleotide Sequence Database Collaboration (<http://www.insdc.org/>). At a smaller scale, biologists also share recombinant DNA under “ready-to-use” forms such as bacterial plasmids. Synthetic plasmids used routinely in genetic engineering are called vectors. These vectors have become essential tools to store and multiply genes and to perform common cloning procedures. Several initiatives are exploiting the power of Web-based

strategies not only to better share vector information but also to physically distribute them. These existing solutions are most appropriate for a rapid and fair diffusion of optogenetic tools.

Several laboratories and companies are hosting and maintaining their own online vector database but only two platforms have emerged as popular and potentially comprehensive vector repositories: PlasmID (<http://plasmid.med.harvard.edu/PLASMID/>) and Addgene (<http://www.addgene.org>). PlasmID was established in 2004 to facilitate the search and request of plasmids from the DNA Resource Core of the Dana-Farber/Harvard Cancer Center and is currently supported by several institutions (Zuo et al., 2007). Addgene was started the same year as a nonprofit organization and was initially funded by private donations and small business loans (Fan et al., 2005). Both platforms are backed up by solid plasmid storage and sequencing facilities and distribute their samples internationally at low cost.

PlasmID's strategy is oriented toward gathering large existing collections, whereas Addgene is very proactively working with individual laboratories. Addgene was created with the objective of reducing the burden on researchers to store, maintain, and distribute plasmid clones and supporting information. Its financial model is to set prices such that revenues from requests (around \$65 per plasmid) are just enough to cover the operating costs. This point of financial self-sustainability was reached in 2007, only 3 years after the creation of the organization. Addgene's catalog currently has over 15,000 plasmids from around 1000 laboratories and keeps on growing. Searching this catalog online is particularly user-friendly: most plasmids are linked to the corresponding articles, allowing scientists to quickly find the actual experiments performed with them, and a color code tracks their popularity (how many times they were requested). Addgene was born just on time to catch on with the recent boom of optogenetics. The major players in optogenetic engineering have already deposited over 60 constructs in only a few years.

It is thus relatively safe to predict that Addgene will become a major platform for the distribution of optogenetic tools.

Sharing transgenic animals

Distribution channels similar to Addgene also exist for transgenic animals. The Jackson Laboratories (<http://www.jax.org/>), one of the biggest world's source of genetically modified mouse strains, already distributes more than 20 lines expressing optogenetic tools under the control of specific promoters or a Cre-activated cassette (<http://research.jax.org/grs/optogenetics.html>). Both reporter strains and FLEX/DiO viruses allow the use of the hundreds of available Cre driver mouse lines for optogenetic applications. Data repositories for Cre transgenics are starting to emerge, such as Cre-X-Mice (http://nagy.mshri.on.ca/cre_new/index.php), and large-scale efforts are under way to expand the catalog of Cre lines through the GENSAT project (Geschwind, 2004; Heintz, 2004, <http://www.gensat.org>), the Cre Driver Network (<http://www.credrivermice.org/>), and the Allen Institute for Brain Science (<http://www.brain-map.org/>).

Concluding remarks

From the beginning, optogenetics has been more than just a toolbox. It represents the answer to the experimental difficulties in bridging the complexity and diversity of molecular and cellular neurophysiology with systemic functions. This is a necessary step for several large-scale projects aimed at reverse engineering the cerebral cortex, including the Blue Brain Project (<http://bluebrain.epfl.ch>) at EPFL (Ecole Polytechnique Fédérale de Lausanne) that is currently running for a very large European Commission research grant and the ambitious goal to deciphering the neuronal code at the Allen Institute for Brain Science (www.alleninstitute.org). Optogenetics

can be seen as a revolutionary new field, but at the same time, it evolved over decades propelled by visionary expectations and a pedigree of scientific discoveries and inventions. The perspective presented in this introductory chapter of the *Progress in Brain Research* issue on optogenetics sets the scene for the subsequent chapters dealing with specific optogenetic tools and their application to brain research.

References

- Ai, H. W., Hazelwood, K. L., et al. (2008). Fluorescent protein FRET pairs for ratiometric imaging of dual biosensors. *Nature Methods*, 5(5), 401–403.
- Airan, R. D., Thompson, K. R., et al. (2009). Temporally precise in vivo control of intracellular signalling. *Nature*, 458(7241), 1025–1029.
- Akemann, W., Mutoh, H., et al. (2010). Imaging brain electric signals with genetically targeted voltage-sensitive fluorescent proteins. *Nature Methods*, 7(8), 643–649.
- Albeanu, D. F., Soucy, E., et al. (2008). LED arrays as cost effective and efficient light sources for widefield microscopy. *PLoS One*, 3(5), e2146.
- Anikeeva, P., Andalman, A. S., Witten, I., Warden, M., Goshen, I., Grosenick, L., et al. (2011). Optetrode: a multi-channel readout for optogenetic control in freely moving mice. *Nature Neuroscience*, advance online publication.
- Aponte, Y., Atasoy, D., et al. (2011). AGRP neurons are sufficient to orchestrate feeding behavior rapidly and without training. *Nature Neuroscience*, 14(3), 351–355.
- Aronoff, R., & Petersen, C. C. (2006). Controlled and localized genetic manipulation in the brain. *Journal of Cellular and Molecular Medicine*, 10(2), 333–352.
- Ascoli, G. A., Alonso-Nanclares, L., et al. (2008). Petilla terminology: Nomenclature of features of GABAergic interneurons of the cerebral cortex. *Nature Reviews Neuroscience*, 9(7), 557–568.
- Atasoy, D., Aponte, Y., et al. (2008). A FLEX switch targets Channelrhodopsin-2 to multiple cell types for imaging and long-range circuit mapping. *The Journal of Neuroscience*, 28(28), 7025–7030.
- Ayling, O. G., Harrison, T. C., et al. (2009). Automated light-based mapping of motor cortex by photoactivation of channelrhodopsin-2 transgenic mice. *Nature Methods*, 6(3), 219–224.
- Banghart, M., Borges, K., et al. (2004). Light-activated ion channels for remote control of neuronal firing. *Nature Neuroscience*, 7(12), 1381–1386.
- Bartels, E., Wassermann, N. H., et al. (1971). Photochromic activators of the acetylcholine receptor. *Proceedings of the National Academy of Sciences of the United States of America*, 68(8), 1820–1823.
- Beja, O., Aravind, L., et al. (2000). Bacterial rhodopsin: Evidence for a new type of phototrophy in the sea. *Science*, 289(5486), 1902–1906.
- Berges, B. K., Wolfe, J. H., et al. (2007). Transduction of brain by herpes simplex virus vectors. *Molecular Therapy*, 15(1), 20–29.
- Boyden, E. S. (2011). A history of optogenetics: The development of tools for controlling brain circuits with light. *FI000 Biology Reports*, 3, 11.
- Boyden, E. S., Zhang, F., et al. (2005). Millisecond-timescale, genetically targeted optical control of neural activity. *Nature Neuroscience*, 8(9), 1263–1268.
- Bulina, M. E., Chudakov, D. M., et al. (2006). A genetically encoded photosensitizer. *Nature Biotechnology*, 24(1), 95–99.
- Bulina, M. E., Lukyanov, K. A., et al. (2006). Chromophore-assisted light inactivation (CALI) using the phototoxic fluorescent protein KillerRed. *Nature Protocols*, 1(2), 947–953.
- Butler, D. (2005). Science in the web age: Joint efforts. *Nature*, 438, 548–549.
- Cardin, J. A., Carlen, M., et al. (2010). Targeted optogenetic stimulation and recording of neurons in vivo using cell-type-specific expression of Channelrhodopsin-2. *Nature Protocols*, 5(2), 247–254.
- Carter, M. E., & de Lecea, L. (2011). Optogenetic investigation of neural circuits in vivo. *Trends in Molecular Medicine*, 17(4), 197–206.
- Cetin, A., Komai, S., et al. (2006). Stereotaxic gene delivery in the rodent brain. *Nature Protocols*, 1(6), 3166–3173.
- Chambers, J. J., Banghart, M. R., et al. (2006). Light-induced depolarization of neurons using a modified Shaker K(+) channel and a molecular photoswitch. *Journal of Neurophysiology*, 96(5), 2792–2796.
- Cho, J., Won Baac, H., et al. (2010). A 16-site neural probe integrated with a waveguide for optical stimulation. In: *IEEE 23rd International Conference on Micro Electro Mechanical Systems*, (pp. 995–998). Wanchai, Hong Kong, IEEE.
- Chow, B. Y., Chuong, A. S., et al. (2011). Synthetic physiology strategies for adapting tools from nature for genetically targeted control of fast biological processes. *Methods in Enzymology*, 497, 425–443.
- Chow, B. Y., Han, X., et al. (2010). High-performance genetically targetable optical neural silencing by light-driven proton pumps. *Nature*, 463(7277), 98–102.
- Chudakov, D. M., Matz, M. V., et al. (2010). Fluorescent proteins and their applications in imaging living cells and tissues. *Physiological Reviews*, 90(3), 1103–1163.
- Cohen, L. B., Keynes, R. D., et al. (1968). Light scattering and birefringence changes during nerve activity. *Nature*, 218(5140), 438–441.

- Crick, F. H. (1979). Thinking about the brain. *Scientific American*, 241(3), 219–232.
- Crick, F. (1999). The impact of molecular biology on neuroscience. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, 354(1392), 2021–2025.
- Cruikshank, S. J., Urabe, H., et al. (2010). Pathway-specific feedforward circuits between thalamus and neocortex revealed by selective optical stimulation of axons. *Neuron*, 65(2), 230–245.
- Davidson, B. L., & Breakefield, X. O. (2003). Viral vectors for gene delivery to the nervous system. *Nature Reviews Neuroscience*, 4(5), 353–364.
- Day, R. N., & Davidson, M. W. (2009). The fluorescent protein palette: Tools for cellular imaging. *Chemical Society Reviews*, 38(10), 2887–2921.
- Deisseroth, K. (2010). Controlling the brain with light. *Scientific American*, 303(5), 48–55.
- Deisseroth, K. (2011). Optogenetics. *Nature Methods*, 8(1), 26–29.
- Deisseroth, K., Feng, G., et al. (2006). Next-generation optical technologies for illuminating genetically targeted brain circuits. *The Journal of Neuroscience*, 26(41), 10380–10386.
- Denk, W., Strickler, J. H., et al. (1990). Two-photon laser scanning fluorescence microscopy. *Science*, 248(4951), 73–76.
- Deuschle, K., Okumoto, S., et al. (2005). Construction and optimization of a family of genetically encoded metabolite sensors by semirational protein engineering. *Protein Science*, 14(9), 2304–2314.
- Dhup, S., & Majumdar, S. S. (2008). Transgenesis via permanent integration of genes in repopulating spermatogonial cells in vivo. *Nature Methods*, 5(7), 601–603.
- Diester, I., Kaufman, M. T., et al. (2011). An optogenetic toolbox designed for primates. *Nature Neuroscience*, 14(3), 387–397.
- Diez-Garcia, J., Akemann, W., et al. (2007). In vivo calcium imaging from genetically specified target cells in mouse cerebellum. *Neuroimage*, 34(3), 859–869.
- Dymecki, S. M., Ray, R. S., et al. (2010). Mapping cell fate and function using recombinase-based intersectional strategies. *Methods in Enzymology*, 477, 183–213.
- Fan, M., Tsai, J., et al. (2005). A central repository for published plasmids. *Science*, 307(5717), 1877.
- Fenno, L., Yizhar, O., et al. (2011). The development and application of optogenetics. *Annual Review of Neuroscience*, 34, 389–412.
- Fletcher, M. L., Masurkar, A. V., et al. (2009). Optical imaging of postsynaptic odor representation in the glomerular layer of the mouse olfactory bulb. *Journal of Neurophysiology*, 102(2), 817–830.
- Fortin, D. L., Dunn, T. W., et al. (2011). Optogenetic photochemical control of designer K⁺ channels in mammalian neurons. *Journal of Neurophysiology*, 106(1), 488–496.
- Frommer, W. B., Davidson, M. W., et al. (2009). Genetically encoded biosensors based on engineered fluorescent proteins. *Chemical Society Reviews*, 38(10), 2833–2841.
- Ganesan, S., Ameer-Beg, S. M., et al. (2006). A dark yellow fluorescent protein (YFP)-based Resonance Energy-Accepting Chromoprotein (REACH) for Förster resonance energy transfer with GFP. *Proceedings of the National Academy of Sciences of the United States of America*, 103(11), 4089–4094.
- Geschwind, D. (2004). GENSAT: A genomic resource for neuroscience research. *Lancet Neurology*, 3(2), 82.
- Göbel, W., & Helmchen, F. (2007). In vivo calcium imaging of neural network function. *Physiology (Bethesda)*, 22, 358–365.
- Gorostiza, P., & Isacoff, E. (2007). Optical switches and triggers for the manipulation of ion channels and pores. *Molecular BioSystems*, 3(10), 686–704.
- Gorostiza, P., & Isacoff, E. Y. (2008). Optical switches for remote and noninvasive control of cell signaling. *Science*, 322(5900), 395–399.
- Govorunova, E. G., Spudich, E. N., et al. (2011). New channelrhodopsin with a red-shifted spectrum and rapid kinetics from *Mesostigma viride*. *MBio*, 2(3), e00115–11.
- Gradinaru, V., Mogri, M., et al. (2009). Optical deconstruction of parkinsonian neural circuitry. *Science*, 324(5925), 354–359.
- Gradinaru, V., Thompson, K. R., et al. (2007). Targeting and readout strategies for fast optical neural control in vitro and in vivo. *The Journal of Neuroscience*, 27(52), 14231–14238.
- Gradinaru, V., Zhang, F., et al. (2010). Molecular and cellular approaches for diversifying and extending optogenetics. *Cell*, 141(1), 154–165.
- Grant, D. M., Zhang, W., et al. (2008). Multiplexed FRET to image multiple signaling events in live cells. *Biophysical Journal*, 95(10), L69–L71.
- Grossman, N., Poher, V., et al. (2010). Multi-site optical excitation using ChR2 and micro-LED array. *Journal of Neural Engineering*, 7(1), 16004.
- Guo, Z. V., Hart, A. C., et al. (2009). Optical interrogation of neural circuits in *Caenorhabditis elegans*. *Nature Methods*, 6(12), 891–896.
- Gutierrez, D. V., Mark, M. D., et al. (2011). Optogenetic control of motor coordination by Gi/o protein-coupled vertebrate rhodopsin in cerebellar Purkinje cells. *Journal of Biological Chemistry*, 286(29), 25848–25858.
- Halassa, M. M., Siegle, J. H., et al. (2011). Selective optical drive of thalamic reticular nucleus generates thalamic bursts and cortical spindles. *Nature Neuroscience*, 14(9), 1118–1120.
- Han, X., Chow, B. Y., et al. (2011). A high-light sensitivity optical neural silencer: Development, and application to optogenetic control of nonhuman primate cortex. *Frontiers in Systems Neuroscience*, 5, 18.

- Han, X., Qian, X., et al. (2009). Millisecond-timescale optical control of neural dynamics in the nonhuman primate brain. *Neuron*, 62(2), 191–198.
- Haubensak, W., Kunwar, P. S., et al. (2010). Genetic dissection of an amygdala microcircuit that gates conditioned fear. *Nature*, 468(7321), 270–276.
- Hegemann, P., & Moglich, A. (2011). Channelrhodopsin engineering and exploration of new optogenetic tools. *Nature Methods*, 8(1), 39–42.
- Heintz, N. (2004). Gene expression nervous system atlas (GENSAT). *Nature Neuroscience*, 7(5), 483.
- Huber, D., Petreanu, L., et al. (2008). Sparse optical microstimulation in barrel cortex drives learned behaviour in freely moving mice. *Nature*, 451(7174), 61–64.
- Inouye, S., Noguchi, M., et al. (1985). Cloning and sequence analysis of cDNA for the luminescent protein aequorin. *Proceedings of the National Academy of Sciences of the United States of America*, 82(10), 3154–3158.
- Iwai, Y., Honda, S., et al. (2011). A simple head-mountable LED device for chronic stimulation of optogenetic molecules in freely moving mice. *Neuroscience Research*, 70(1), 124–127.
- Janovjak, H., Szobota, S., et al. (2010). A light-gated, potassium-selective glutamate receptor for the optical inhibition of neuronal firing. *Nature Neuroscience*, 13(8), 1027–1032.
- Jerome, J., Foehring, R. C., et al. (2011). Parallel optical control of spatiotemporal neuronal spike activity using high-speed digital light processing. *Frontiers in Systems Neuroscience*, 5, 70.
- Johnson, I., & Spence, M. T. Z. (2010). *The Molecular Probes Handbook: A guide to fluorescent probes and labeling technologies* (11th ed.). Eugene, Oregon: Life Technologies.
- Judkewitz, B., Rizzi, M., et al. (2009). Targeted single-cell electroporation of mammalian neurons in vivo. *Nature Protocols*, 4(6), 862–869.
- Kami, C., Lorrain, S., et al. (2010). Light-regulated plant growth and development. *Current Topics in Developmental Biology*, 91, 29–66.
- Kaneda, K., Kasahara, H., et al. (2011). Selective optical control of synaptic transmission in the subcortical visual pathway by activation of viral vector-expressed halorhodopsin. *PLoS One*, 6(4), e18452.
- Kawai, Y., Sato, M., et al. (2004). Single color fluorescent indicators of protein phosphorylation for multicolor imaging of intracellular signal flow dynamics. *Analytical Chemistry*, 76(20), 6144–6149.
- Kennedy, M. J., Hughes, R. M., et al. (2010). Rapid blue-light-mediated induction of protein interactions in living cells. *Nature Methods*, 7(12), 973–975.
- Khorana, H. G., Knox, B. E., Nasi, E., Swanson, R., & Thompson, D. A. (1988). Expression of a bovine rhodopsin gene in *Xenopus* oocytes: demonstration of light-dependent ionic currents. *PNAS*, 85(21), 7917–7921.
- Kim, J. M., Hwa, J., et al. (2005). Light-driven activation of beta 2-adrenergic receptor signaling by a chimeric rhodopsin containing the beta 2-adrenergic receptor cytoplasmic loops. *Biochemistry*, 44(7), 2284–2292.
- Klapoetke, N., Chuong, A., et al. (2010). Novel classes of optogenetic reagent derived from screening genomic and ecological diversity. In: *2010 Society for Neuroscience Meeting*, San Diego, CA: Society for Neuroscience Program No. 106.1/MMM8.
- Kleinlogel, S., Terpitz, U., Legrum, B., Gökbuget, D., Boyden, E. S., Bamann, C., et al. (2011). A gene-fusion strategy for stoichiometric and co-localized expression of light-gated membrane proteins. *Nature Methods*, 8(12), 1083–1088.
- Knöpfel, T., Diez-Garcia, J., et al. (2006). Optical probing of neuronal circuit dynamics: Genetically encoded versus classical fluorescent sensors. *Trends in Neurosciences*, 29(3), 160–166.
- Kravitz, A. V., & Kreitzer, A. C. (2011). Optogenetic manipulation of neural circuitry in vivo. *Current Opinion in Neurobiology*, 21(3), 433–439.
- Kubota, Y., Shigematsu, N., et al. (2011). Selective coexpression of multiple chemical markers defines discrete populations of neocortical GABAergic neurons. *Cerebral Cortex*, 21(8), 1803–1817.
- Kuhlman, S. J., & Huang, Z. J. (2008). High-resolution labeling and functional manipulation of specific neuron types in mouse brain by Cre-activated viral gene expression. *PLoS One*, 3(4), e2005.
- Lalonde, S., Ehrhardt, D. W., et al. (2005). Shining light on signaling and metabolic networks by genetically encoded biosensors. *Current Opinion in Plant Biology*, 8(6), 574–581.
- Le Provost, F., Lillico, S., et al. (2010). Zinc finger nuclease technology heralds a new era in mammalian transgenesis. *Trends in Biotechnology*, 28(3), 134–141.
- LeChasseur, Y., Dufour, S., et al. (2011). A microprobe for parallel optical and electrical recordings from single neurons in vivo. *Nature Methods*, 8(4), 319–325.
- Lee, J., Natarajan, M., et al. (2008). Surface sites for engineering allosteric control in proteins. *Science*, 322(5900), 438–442.
- Lee, J., Udugamasooriya, D. G., et al. (2010). Potent and selective photo-inactivation of proteins with peptid-ruthenium conjugates. *Nature Chemical Biology*, 6(4), 258–260.
- Levsikaya, A., Weiner, O. D., et al. (2009). Spatiotemporal control of cell signalling using a light-switchable protein interaction. *Nature*, 461(7266), 997–1001.
- Li, X., Gutierrez, D. V., et al. (2005). Fast noninvasive activation and inhibition of neural and network activity by vertebrate rhodopsin and green algae channelrhodopsin. *Proceedings of the National Academy of Sciences of the United States of America*, 102(49), 17816–17821.
- Lima, S. Q., Hromadka, T., et al. (2009). PINP: A new method of tagging neuronal populations for identification during in vivo electrophysiological recording. *PLoS One*, 4(7), e6099.

- Lima, S. Q., & Miesenböck, G. (2005). Remote control of behavior through genetically targeted photostimulation of neurons. *Cell*, *121*(1), 141–152.
- Lin, J. Y. (2011). A user's guide to channelrhodopsin variants: Features, limitations and future developments. *Experimental Physiology*, *96*(1), 19–25.
- Lin, D., Boyle, M. P., et al. (2011). Functional identification of an aggression locus in the mouse hypothalamus. *Nature*, *470*(7333), 221–226.
- Liu, B., Paton, J. F., et al. (2008). Viral vectors based on bidirectional cell-specific mammalian promoters and transcriptional amplification strategy for use in vitro and in vivo. *BMC Biotechnology*, *8*, 49.
- Losi, A., & Gärtner, W. (2012). The Evolution of Flavin-Binding Photoreceptors: An Ancient Chromophore Serving Trendy Blue-Light Sensors. *Annual Review of Plant Biology*, *63*, 1.1–1.24.
- Lowery, R. L., & Majewska, A. K. (2010). Intracranial injection of adeno-associated viral vectors. *Journal of Visualized Experiments*, (45), e2140.
- Lütcke, H., Murayama, M., et al. (2010). Optical recording of neuronal activity with a genetically-encoded calcium indicator in anesthetized and freely moving mice. *Front Neural Circuits*, *4*, 9.
- Markvicheva, K. N., Bilan, D. S., et al. (2011). A genetically encoded sensor for H₂O₂ with expanded dynamic range. *Bioorganic & Medicinal Chemistry*, *19*(3), 1079–1084.
- Masamizu, Y., Okada, T., et al. (2011). Local and retrograde gene transfer into primate neuronal pathways via adeno-associated virus serotype 8 and 9. *Neuroscience*, *193*, 249–258.
- Masseck, O. A., Rubelowski, J. M., et al. (2011). Light- and drug-activated G-protein-coupled receptors to control intracellular signalling. *Experimental Physiology*, *96*(1), 51–56.
- Melyan, Z., Tarttelin, E. E., et al. (2005). Addition of human melanopsin renders mammalian cells photoresponsive. *Nature*, *433*(7027), 741–745.
- Miesenböck, G. (2009). The optogenetic catechism. *Science*, *326*(5951), 395–399.
- Miesenböck, G. (2011). Optogenetic control of cells and circuits. *Annual Review of Cell and Developmental Biology*, *27*, 731–758.
- Möglich, A., Ayers, R. A., et al. (2009). Design and signaling mechanism of light-regulated histidine kinases. *Journal of Molecular Biology*, *385*(5), 1433–1444.
- Möglich, A., & Moffat, K. (2010). Engineered photoreceptors as novel optogenetic tools. *Photochemical and Photobiological Sciences*, *9*(10), 1286–1300.
- Monahan, P. E., & Samulski, R. J. (2000). Adeno-associated virus vectors for gene therapy: More pros than cons? *Molecular Medicine Today*, *6*(11), 433–440.
- Mukohata, Y., Ihara, K., et al. (1999). Halobacterial rhodopsins. *Journal of Biochemistry*, *125*(4), 649–657.
- Muller, V., & Oren, A. (2003). Metabolism of chloride in halophilic prokaryotes. *Extremophiles*, *7*(4), 261–266.
- Murayama, M., & Larkum, M. E. (2009). In vivo dendritic calcium imaging with a fiberoptic periscope system. *Nature Protocols*, *4*(10), 1551–1559.
- Murayama, M., Perez-Garci, E., et al. (2007). Fiberoptic system for recording dendritic calcium signals in layer 5 neocortical pyramidal cells in freely moving rats. *Journal of Neurophysiology*, *98*(3), 1791–1805.
- Nagahama, T., Suzuki, T., et al. (2007). Functional transplant of photoactivated adenylyl cyclase (PAC) into Aplysia sensory neurons. *Neuroscience Research*, *59*(1), 81–88.
- Nagel, G., Ollig, D., et al. (2002). Channelrhodopsin-1: A light-gated proton channel in green algae. *Science*, *296*(5577), 2395–2398.
- Nagel, G., Szellas, T., et al. (2003). Channelrhodopsin-2, a directly light-gated cation-selective membrane channel. *Proceedings of the National Academy of Sciences of the United States of America*, *100*(24), 13940–13945.
- Nagel, G., Szellas, T., et al. (2005). Channelrhodopsins: Directly light-gated cation channels. *Biochemical Society Transactions*, *33*(Pt. 4), 863–866.
- Nakai, J., Ohkura, M., et al. (2001). A high signal-to-noise Ca²⁺ probe composed of a single green fluorescent protein. *Nature Biotechnology*, *19*(2), 137–141.
- Nerbonne, J. M. (1996). Caged compounds: Tools for illuminating neuronal responses and connections. *Current Opinion in Neurobiology*, *6*(3), 379–386.
- Nern, A., Pfeiffer, B. D., Svoboda, K., & Rubin, G. M. (2011). Multiple new site-specific recombinases for use in manipulating animal genomes. *PNAS*, *108*(34), 14198–14203.
- Niino, Y., Hotta, K., et al. (2009). Simultaneous live cell imaging using dual FRET sensors with a single excitation light. *PLoS One*, *4*(6), e6036.
- Niino, Y., Hotta, K., et al. (2010). Blue fluorescent cGMP sensor for multiparameter fluorescence imaging. *PLoS One*, *5*(2), e9164.
- Oh, E., Maejima, T., et al. (2010). Substitution of 5-HT_{1A} receptor signaling by a light-activated G protein-coupled receptor. *Journal of Biological Chemistry*, *285*(40), 30825–30836.
- Okumoto, S. (2010). Imaging approach for monitoring cellular metabolites and ions using genetically encoded biosensors. *Current Opinion in Biotechnology*, *21*(1), 45–54.
- Okumoto, S., Takanaga, H., et al. (2008). Quantitative imaging for discovery and assembly of the metabo-regulome. *New Phytologist*, *180*(2), 271–295.
- Osakada, F., Mori, T., et al. (2011). New rabies virus variants for monitoring and manipulating activity and gene expression in defined neural circuits. *Neuron*, *71*(4), 617–631.
- Ouyang, M., Huang, H., et al. (2010). Simultaneous visualization of protumorigenic Src and MT1-MMP activities with

- fluorescence resonance energy transfer. *Cancer Research*, 70(6), 2204–2212.
- Papagiakoumou, E., Anselmi, F., et al. (2010). Scanless two-photon excitation of channelrhodopsin-2. *Nature Methods*, 7(10), 848–854.
- Passini, M. A., & Wolfe, J. H. (2001). Widespread gene delivery and structure-specific patterns of expression in the brain after intraventricular injections of neonatal mice with an adeno-associated virus vector. *Journal of Virology*, 75(24), 12382–12392.
- Pearson, H. (2006). Online methods share insider tricks. *Nature*, 441, 678.
- Perron, A., Mutoh, H., et al. (2009). Red-shifted voltage-sensitive fluorescent proteins. *Chemical Biology*, 16(12), 1268–1277.
- Peterka, D. S., Takahashi, H., et al. (2011). Imaging voltage in neurons. *Neuron*, 69(1), 9–21.
- Petreanu, L., Huber, D., et al. (2007). Channelrhodopsin-2-assisted circuit mapping of long-range callosal projections. *Nature Neuroscience*, 10(5), 663–668.
- Petreanu, L., Mao, T., et al. (2009). The subcellular organization of neocortical excitatory connections. *Nature*, 457(7233), 1142–1145.
- Petzold, G. C., Hagiwara, A., et al. (2009). Serotonergic modulation of odor input to the mammalian olfactory bulb. *Nature Neuroscience*, 12(6), 784–791.
- Pham, E., Mills, E., et al. (2011). A synthetic photoactivated protein to generate local or global Ca^{2+} signals. *Chemical Biology*, 18(7), 880–890.
- Piljic, A., & Schultz, C. (2008). Simultaneous recording of multiple cellular events by FRET. *ACS Chemical Biology*, 3(3), 156–160.
- Pilpel, N., Landeck, N., et al. (2009). Rapid, reproducible transduction of select forebrain regions by targeted recombinant virus injection into the neonatal mouse brain. *Journal of Neuroscience Methods*, 182(1), 55–63.
- Prasher, D. C., Eckenrode, V. K., et al. (1992). Primary structure of the *Aequorea victoria* green-fluorescent protein. *Gene*, 111(2), 229–233.
- Prasher, D., McCann, R. O., et al. (1985). Cloning and expression of the cDNA coding for aequorin, a bioluminescent calcium-binding protein. *Biochemical and Biophysical Research Communications*, 126(3), 1259–1268.
- Puntel, M., Kroeger, K. M., et al. (2010). Gene transfer into rat brain using adenoviral vectors. *Current Protocols in Neuroscience*, Chapter 4: Unit 4. 24. See: [http://onlinelibrary.wiley.com/doi/10.1002/0471142301.ns0424s50/abstract](http://onlinelibrary.wiley.com/doi/10.1002/0471142301.ns0424s50.abstract).
- Ridgway, E. B., & Ashley, C. C. (1967). Calcium transients in single muscle fibers. *Biochemical and Biophysical Research Communications*, 29(2), 229–234.
- Royer, S., Zemelman, B. V., et al. (2010). Multi-array silicon probes with integrated optical fibers: Light-assisted perturbation and recording of local neural circuits in the behaving animal. *The European Journal of Neuroscience*, 31(12), 2279–2291.
- Ryu, M. H., Moskvin, O. V., et al. (2010). Natural and engineered photoactivated nucleotidyl cyclases for optogenetic applications. *Journal of Biological Chemistry*, 285(53), 41501–41508.
- Saggau, P. (2006). New methods and uses for fast optical scanning. *Current Opinion in Neurobiology*, 16(5), 543–550.
- Schroder-Lang, S., Schwarzel, M., et al. (2007). Fast manipulation of cellular cAMP level by light in vivo. *Nature Methods*, 4(1), 39–42.
- Serebrovskaya, E. O., Gorodnicheva, T. V., et al. (2011). Light-induced blockage of cell division with a chromatin-targeted phototoxic fluorescent protein. *Biochemistry Journal*, 435(1), 65–71.
- Sherrington, C. S. S. (1940). *Man on his nature, O.M. The Gifford lectures, Edinburgh, 1937–8*. Cambridge: Cambridge University Press.
- Shimizu-Sato, S., Huq, E., et al. (2002). A light-switchable gene promoter system. *Nature Biotechnology*, 20(10), 1041–1044.
- Souslova, E. A., & Chudakov, D. M. (2007). Genetically encoded intracellular sensors based on fluorescent proteins. *Biochemistry (Mosc)*, 72(7), 683–697.
- Stierl, M., Stumpf, P., et al. (2011). Light modulation of cellular cAMP by a small bacterial photoactivated adenyl cyclase, bPAC, of the soil bacterium *Beggiatoa*. *Journal of Biological Chemistry*, 286(2), 1181–1188.
- Strickland, D., Moffat, K., et al. (2008). Light-activated DNA binding in a designed allosteric protein. *Proceedings of the National Academy of Sciences of the United States of America*, 105(31), 10709–10714.
- Strickland, D., Yao, X., et al. (2010). Rationally improving LOV domain-based photoswitches. *Nature Methods*, 7(8), 623–626.
- Teh, C., Chudakov, D. M., et al. (2010). Optogenetic in vivo cell manipulation in KillerRed-expressing zebrafish transgenics. *BMC Developmental Biology*, 10, 110.
- Teschemacher, A. G., Wang, S., et al. (2005). Targeting specific neuronal populations using adeno- and lentiviral vectors: Applications for imaging and studies of cell function. *Experimental Physiology*, 90(1), 61–69.
- Tian, L., & Looger, L. L. (2008). Genetically encoded fluorescent sensors for studying healthy and diseased nervous systems. *Drug Discovery Today: Disease Models*, 5(1), 27–35.
- Toettcher, J. E., Gong, D., et al. (2011). Light-based feedback for controlling intracellular signaling dynamics. *Nature Methods*, 8(10), 837–839.
- Tyszkiewicz, A. B., & Muir, T. W. (2008). Activation of protein splicing with light in yeast. *Nature Methods*, 5(4), 303–305.
- Varga, V., Losonczy, A., et al. (2009). Fast synaptic subcortical control of hippocampal circuits. *Science*, 326(5951), 449–453.
- Vincent, P., Maskos, U., et al. (2006). Live imaging of neural structure and function by fibred fluorescence microscopy. *EMBO Reports*, 7(11), 1154–1161.

- Volgraf, M., Gorostiza, P., et al. (2006). Allosteric control of an ionotropic glutamate receptor with an optical switch. *Nature Chemical Biology*, 2(1), 47–52.
- Walantus, W., Castaneda, D., et al. (2007). In utero intraventricular injection and electroporation of E15 mouse embryos. *Journal of Visualized Experiments*, 6, 239.
- Waldrop, M. (2008). Science 2.0—Is open access science the future? *Scientific American*, May 2008, 68–73.
- Waschuk, S. A., Bezerra, A. G. Jr., et al. (2005). Leptosphaeria rhodopsin: Bacteriorhodopsin-like proton pump from a eukaryote. *Proceedings of the National Academy of Sciences of the United States of America*, 102(19), 6879–6883.
- Wentz, C. T., Bernstein, J. G., et al. (2011). A wirelessly powered and controlled device for optical neural control of freely-behaving animals. *Journal of Neural Engineering*, 8(4), 046021.
- Wickersham, I. R., Finke, S., et al. (2007). Retrograde neuronal tracing with a deletion-mutant rabies virus. *Nature Methods*, 4(1), 47–49.
- Wickersham, I. R., Lyon, D. C., et al. (2007). Monosynaptic restriction of transsynaptic tracing from single, genetically targeted neurons. *Neuron*, 53(5), 639–647.
- Wilt, B. A., Burns, L. D., et al. (2009). Advances in light microscopy for neuroscience. *Annual Review of Neuroscience*, 32, 435–506.
- Witten, I. B., Steinberg, E. E., Davidson, T., Tye, K. M., Zalocusky, K., Brodsky, M., et al. (2011). Recombinase-driver rat lines for optogenetics: tools, techniques, and application to dopamine-mediated positive reinforcement. Submitted.
- Wong, L. F., Goodhead, L., et al. (2006). Lentivirus-mediated gene transfer to the central nervous system: Therapeutic and research applications. *Human Gene Therapy*, 17(1), 1–9.
- Wu, Y. I., Frey, D., et al. (2009). A genetically encoded photo-activatable Rac controls the motility of living cells. *Nature*, 461(7260), 104–108.
- Yazawa, M., Sadaghiani, A. M., et al. (2009). Induction of protein-protein interactions in live cells using light. *Nature Biotechnology*, 27(10), 941–945.
- Ye, H., Daoud-El Baba, M., et al. (2011). A synthetic optogenetic transcription device enhances blood-glucose homeostasis in mice. *Science*, 332(6037), 1565–1568.
- Yizhar, O., Fenno, L., et al. (2011a). Microbial opsins: A family of single-component tools for optical control of neural activity. *Cold Spring Harbor Protocols*, 2011(1), top102.
- Yizhar, O., Fenno, L. E., et al. (2011b). Optogenetics in neural systems. *Neuron*, 71(1), 9–34.
- Yizhar, O., Fenno, L. E., et al. (2011c). Neocortical excitation/inhibition balance in information processing and social dysfunction. *Nature*, 477, 171–178.
- Zemelman, B. V., Lee, G. A., et al. (2002). Selective photostimulation of genetically chARGed neurons. *Neuron*, 33(1), 15–22.
- Zemelman, B. V., Nesnas, N., et al. (2003). Photochemical gating of heterologous ion channels: Remote control over genetically designated populations of neurons. *Proceedings of the National Academy of Sciences of the United States of America*, 100(3), 1352–1357.
- Zhang, F., Gradinaru, V., et al. (2010). Optogenetic interrogation of neural circuits: Technology for probing mammalian brain structures. *Nature Protocols*, 5(3), 439–456.
- Zhang, J., Laiwalla, F., et al. (2009). Integrated device for optical stimulation and spatiotemporal electrical recording of neural activity in light-sensitized brain tissue. *Journal of Neural Engineering*, 6(5), 055007.
- Zhang, F., Prigge, M., et al. (2008). Red-shifted optogenetic excitation: A tool for fast neural control derived from *Volvox carteri*. *Nature Neuroscience*, 11(6), 631–633.
- Zhang, F., Wang, L. P., et al. (2007). Multimodal fast optical interrogation of neural circuitry. *Nature*, 446(7136), 633–639.
- Zhao, Y., Araki, S., et al. (2011). An expanded palette of genetically encoded Ca(2) indicators. *Science*, 333(6051), 1888–1891.
- Zorzos, A. N., Boyden, E. S., et al. (2010). Multiwaveguide implantable probe for light delivery to sets of distributed brain targets. *Optics Letters*, 35(24), 4133–4135.
- Zorzos, A. N., Dietrich, A., et al. (2009). Light-proof neural recording electrodes. In: *2009 Society for Neuroscience Meeting*, Chicago, Illinois: Society for Neuroscience Program No. 388.12/GG107.
- Zuo, D., Mohr, S. E., et al. (2007). PlasmID: A centralized repository for plasmid clone information and distribution. *Nucleic Acids Research*, 35(Database issue), D680–D684.