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Optogenetic Brain Interfaces

Ramin Pashaie*, Polina Anikeeva*, Jin Hyung Lee*, Rohit Prakash*, Ofer Yizhar*, Matthias Prigge*, Divya Chander*, Thomas Richner*, Justin Williams*

Abstract—The brain is a large network of interconnected neurons where each cell functions as a nonlinear processing element. Unraveling the mysteries of information processing in the complex networks of the brain requires versatile neurostimulation and imaging techniques. Optogenetics is a new stimulation method which allows the activity of neurons to be modulated by light. For this purpose, the cell-types of interest are genetically targeted to produce light-sensitive proteins. Once these proteins are expressed, neural activity can be controlled by exposing the cells to light of appropriate wavelengths. Optogenetics provides a unique combination of features, including multi-modal control over neural function and genetic targeting of specific cell-types. Together, these versatile features combine to a powerful experimental approach, suitable for the study of the circuitry of psychiatric and neurological disorders.

The advent of optogenetics was followed by extensive research aimed to produce new lines of light-sensitive proteins and to develop new technologies: for example, to control the distribution of light inside the brain tissue or to combine optogenetics with other modalities including electrophysiology, electrocorticography, nonlinear microscopy, and functional magnetic resonance imaging.

In this paper, the authors review some of the recent advances in the field of optogenetics and related technologies and provide their vision for the future of the field.

Index Terms—Optogenetics, Brain Interface, Micro-ECOG, Optrode, 2-photon Neurostimulation, Optogenetic fMRI.

I. INTRODUCTION

Over the last few decades, the dominant hypothesis in treating neurological and psychiatric diseases has been the *chemical imbalance paradigm* which assumes any mental disorder is the result of imbalance in the concentration of chemicals in the central or peripheral nervous system. Based on this hypothesis, such diseases are curable if we invent mechanisms to control and monitor the concentration of the

corresponding chemicals by using appropriate pharmacological substances. This approach has been somewhat successful in treating several mental disorders such as depression or anhedonia that are caused by the reduction in the concentration of the monoamine neurotransmitter serotonin [1].

Recent advances in the development of brain interface instrumentation and prosthetic devices has opened a new line of research to treat mental disease by speaking the electrical language of neurons, usually known as *interventional psychiatry*. *Deep-brain stimulation* (DBS) [2], [3] is an example of this approach which has been reasonably successful in treating some neurological diseases including Parkinson's. Nonetheless, most interventional therapeutic procedures lack specific-cell-type targeting capabilities and potentially cause serious side effects. For instance, implanted electrodes in Parkinsonian patients stimulate most cells in their reach with no preference to target any specific cell-type, and as a result, cause several side effects including depression, mood alteration, or sensory and motor control problems which are all suppressed by turning off the stimulations pulses.

To address this challenge, a new neurostimulation technique, known as optogenetics, was invented by combining the tools of molecular genetics with recent advances in the fields of optics and photonics. In this technique, a family of light-gated microbial opsin proteins that function as light-activated proteins, are expressed in genetically targeted neurons [4], [5], [6], [7], [8], [9], [11], [12]. Once these light sensitive proteins are expressed in a neuron, the activity of the cell can be increased or suppressed, with millisecond temporal accuracy, by exposing the cell to light with appropriate wavelengths, even without the addition of the exogenous cofactor in mammalian cells.

Some major advantages of optogenetic stimulation are:

- Specific cell-type targeting can be achieved in optogenetics by controlling the gene delivery process to express light sensitive proteins predominantly in the cell-type of interest. This goal can be achieved, for example, by using appropriate promoters [13].

- Bidirectional control of cellular activities is feasible in optogenetics. It is possible to simultaneously express cation channels and anion pumps that are sensitive to different wavelengths in one cell-type of interest. Thus, by exposing the cell to appropriate wavelengths, researchers can depolarize or hyperpolarize the neurons to manipulate their activities [5], [14].

- The inherent parallelism of optics can help to manipulate neural activities in large-scale neural networks of the brain, particularly in the cortex.

Some major applications of optogenetic neuromodulation are:

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-*Cracking neural codes*: Neurons communicate by generating sequences of action potentials while information is embedded in the *mean firing rate* or the *precise timing* of action potentials. As described earlier, optogenetic tools provide a bidirectional mechanism to control neural activities with millisecond temporal resolution. Consequently, by engineering a sequence of light pulses, any firing pattern is producible which, in principle, represents a specific neural code. Then, with a suitable quantitative readout, the effect of the generated codeword on the post-synaptic neurons or the corresponding behavioral phenotypes can be investigated,

- *Interrogating neural circuits*: By providing a discriminative mechanism for controlling the activity of cell-types in a neural network, optogenetics enables the dissection of mental disease circuitries (e.g., Parkinsonian neural circuits [15]) or interrogation of the role of circuit elements in the overall dynamics of the network (e.g., functionality of fast-spiking Parvalbumin inhibitory interneurons in cortical microcircuits of prefrontal cortex [16]),

- *Generating reversible models of neurological diseases*: The mechanism for bidirectional control of genetically designated neural populations gives the opportunity to emulate and study new models of neurological diseases (e.g., optogenetics can be used to reversibly generate patterns of epileptogenic activity [4]),

- *Developing new and efficient therapeutic treatments*: Optical modulation of activity in targeted cell-types can help in the discovery of efficient treatments for mental diseases with minimum side effects, since we can selectively modulate the activity of excitatory or inhibitory neurons separately (e.g., hyperpolarization of glutamatergic neurons in subthalamic nucleus (STN) with expression of NpHR and suitable light exposure has shown to be effective in treating Parkinson's [15] in animal models of the disease).

The advent of optogenetics has opened new lines of research to develop other light sensitive ion channels with faster or slower kinetics or different spectral sensitivities. It also opens new fields of investigation to control the distribution of light inside the three-dimensional structure of the brain tissue or combine optogenetics with other stimulation or imaging modalities including electrophysiology, electrocorticography, nonlinear microscopy, and functional magnetic resonance imaging.

In this paper, the authors review some of the recent advances in the field of optogenetics and related technologies and provide their vision for the future of the field. In the section II, the progress in development of new tools of optogenetics via bioprospecting search or genetic engineering is discussed. Mechanisms of light delivery are explained in section III, while section IV is devoted to probes that are designed and microfabricated for optogenetic stimulation. The recently developed methodologies to combine optogenetics with fMRI are discussed in section V. Multi-photon stimulation is another new development in the field which allows researchers to reach deeper within the cortex and simultaneously stimulate and image neural activities. Multi-photon stimulation is covered in section VI. An example of a hybrid brain interface platform where optogenetic stimulation is combined with electrocor-

ticography (ECoG) is detailed in section VII and applications of optogenetics in the study and treatment of neurological diseases are covered in section VIII. Finally, concluding remarks are summarized in section IX.

II. TOOLS OF OPTOGENETICS AND MECHANISMS OF GENE DELIVERY

A. Light-activated proteins

Optogenetics applies light-sensitive proteins which have been isolated from various microorganisms and plants, to manipulate excitable cells in heterologous systems. Initial work in the field used naturally occurring photosensitive proteins such as channelrhodopsin (ChR) [6] and halorhodopsin (HR) [5] to induce activation or inhibition of neural activity in mammalian neurons and this has opened up a rapidly expanding field utilizing additional genetically encoded actuators in a wide range of applications [7], [8], [9]. Beyond the utilization of such naturally-occurring photoreceptors, protein engineering over the last decade has generated an expanded optogenetic toolbox for more precise and effective manipulation, allowing refined modes of control that are tailored to individual systems.

The most widely used optogenetic tools are proteins of the microbial rhodopsin family [10]. These proteins were first discovered in the 1970s and were already then thought to serve as a "scientific goldmine" for renewable energy [17]. Microbial rhodopsins are single-component units, which can transform light energy into current with efficiency higher than any man-made mechanisms so far. Microbial rhodopsins such as bacteriorhodopsin (BR) and halorhodopsin, were found in the archaea *Halobacterium salinarium* which inhabit extreme environmental surroundings such as the Dead Sea in Israel [18], [19]. In these ancient halobacteria, rhodopsins are believed to use light energy for generating the electrochemical gradient required for ATP synthesis in the absence of oxygen and for withstanding the high osmotic pressure in these extreme surroundings [20]. In 2002, a new member of the microbial rhodopsin family was isolated from the fresh water algae *Chlamydomonas reinhardtii* [21]. Unlike previously discovered microbial rhodopsins, which typically serve as ion pumps and transport ions against electrochemical gradients, channelrhodopsins (ChRs) possess an aqueous ion channel pore, allowing passive conduction of cations across the cell membrane, according to the electrochemical gradient [22].

Therefore, microbial rhodopsins can be grouped into pumps, acting as photodiodes, and light-triggered ion channels which can be considered as light-dependent resistors. Regardless of the mechanism of action or the ion species conducted, all microbial rhodopsins rely on the same fundamental photoreaction comprising the isomerization of a covalently bound retinal co-factor, Figure 1(a). Upon photon absorption, the co-factor isomerizes from so called all-trans to 13-cis conformation. This conformational shift changes the dipole moment of the retinal molecule and initiates pronounced structural rearrangements of the protein, which finally lead to the transport of ions [23]. All these complex rearrangement occur within less than a millisecond whereby the activated rhodopsin cycles through various photochemically distinct states. This series of

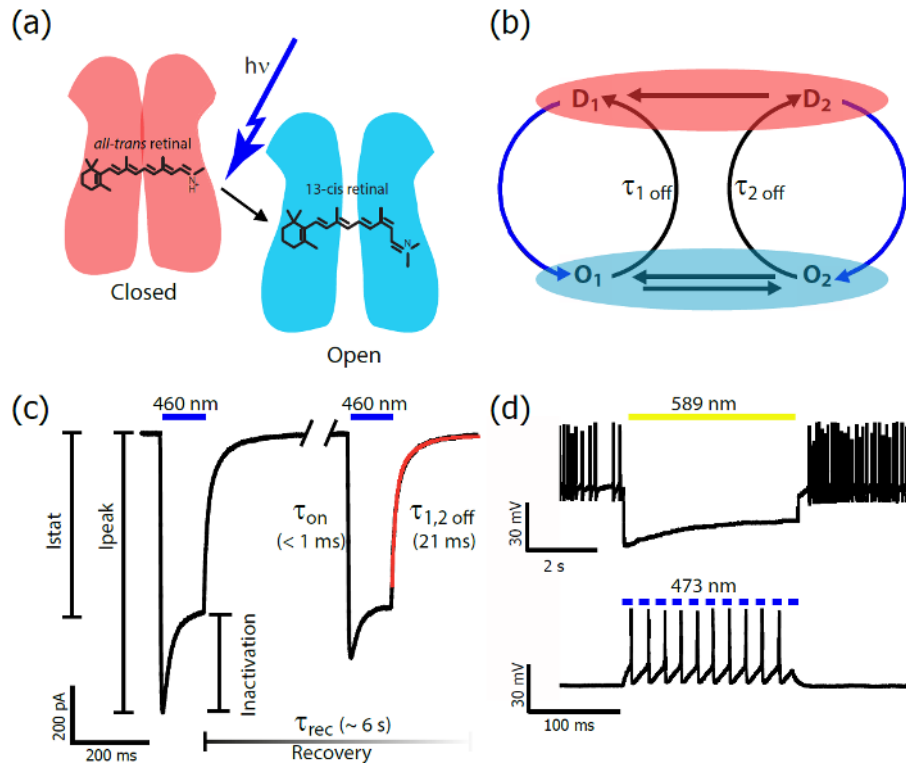


Fig. 1. Photocycle of the wild-type ChR2 and light-induced photocurrents. (A) Schematic drawing of channel opening upon light illumination. In the closed, dark state the light antenna, all-trans retinal, can isomerize after absorption of a photon to 13-cis retinal, thereby triggering opening of the channel. (B) The light-induced changes in the electrical properties of the channels upon opening can be modeled as a 4-state photocycle consisting of two dark states (D1 and D2) and two light-induced open states O1 and O2. (C) Illustrates the electrical response of a single cell expressing ChR2 to two successive light pulses. Upon illumination a transient peak inward current is triggered, which decays to the stationary photocurrent. This drop in photocurrent is commonly referred as inactivation. Upon light-off the photocurrent returns to baseline with an apparent τ of f 21ms. The second light pulse only evokes a smaller peak photocurrent while reaching the same stationary current. The time it takes to return to full peak current size is known as recovery time. (D) Shows current-clamp recordings of neurons expressing NpHR (top) and ChR2 (bottom). Action potentials can be triggered with pulses of blue light in ChR2-expressing cells (473nm) whereas spontaneous activity can be suppressed by activating eNpHR3.0 with yellow light (589nm).

states can be collectively described as a photocycle in various level of detail. Figure 1(b) shows a 4-state electrophysiological photocycle, which explains the major electrical features of channel function, including inactivation, photocurrent kinetics and adaption phenomena as observed in electrical recordings from cells expressing channelrhodopsin (Figure 1(c)) [24]. For example, a fully dark adapted ChR2 molecule activated with a short blue light pulse will first proceed through an open state of high conductance (O1), causing a transient peak photocurrent and then entering a lower conductive state (O2) which leads to lower photocurrent. This drop in photocurrent is commonly referred as inactivation, Figure 1(c) and can be as large as 72% of the initial current for the wild-type ChR2 [25]. The dwell time for the conductive states is estimated to be 10ms [26]. Molecules in the open state can transition to two different dark states with an apparent $\tau_{1,2\text{off}}$ of 21ms [26], [25]. Upon repeated illumination at this stage, molecules re-excited from the second dark state will then only pass through the low conductance state, giving rise to a light-adapted steady-state photocurrent which is smaller than the initial peak current, Figure 1(c). Consequently, a series of 2-ms light pulses will cause attenuation of the photocurrent to a lower equilibrium level [25]. Only after a recovery period of $> 6s$ all molecules will have returned to their initial dark-adapted state, Figure

1(c).

To achieve light-based control of neural function, these bio-electric units have been selectively targeted to different excitable cells such as neurons, heart or muscles cells [6], [27], [28], [29]. In neurons, microbial pumps such as HR or BR induce a square-like hyperpolarization response of the membrane potential upon illumination [5], [30], which can rapidly and effectively silence neuronal activity, Figure 1(d)(upper trace). ChRs perform the opposite function of depolarizing neurons in response to illumination by allowing positively charged cations to passively enter the cell. ChRs therefore serve as light-induced activators. Figure 1(d)(lower trace) shows a pulsed activation of neurons expressing ChR2. Since light naturally penetrates intact biological tissues, the optogenetic approach has been extended to living organisms, including nematodes, flies, zebrafish, rodents and monkeys [28], [31], [32], [33], [34].

Driven by the success of the initial approach with microbial rhodopsins isolated from several species, large genomic screens for new microbial rhodopsins in all kingdoms of life have led to the discovery of several new variants, which show diverse functional properties such as spectral tuning, ionic selectivity and compatibility towards mammalian cell expression [35], [36], [37], [38]. These proteins, encoded by genes from diverse microbial species, were subjected to

molecular engineering which aimed to increase their efficacy as light-driven tools for controlling neural excitability. Generally, two lines of improvement strategies have been applied. The first was aimed toward increasing the biocompatibility to mammalian cells. The second approach was to modify the intrinsic properties of the actuator along one or more functional dimensions. Optimization of membrane targeting was mainly required for the inhibitory microbial rhodopsins, most of which were derived from prokaryotic species in which membrane proteins are produced and transported using mechanisms significantly different from that used in mammalian cells. To improve the proper assembly of these rhodopsins, signaling peptides, which act as a intercellular "zip code", from endogenous ion channels have been added to each end of the microbial rhodopsin [39]. The addition of Golgi- and ER export signals from mammalian ion channels reduced protein aggregation and increased the number of rhodopsin molecules reaching the plasma membrane [12]. This strategy turned out to generalize quite well, and many available actuators used in optogenetic research these days make use of such sequences [40]. As a consequence, the most commonly used tools for neural silencing are the two enhanced versions: NpHR3.0 and Arch3.0 [14].

Whereas the main improvements for hyperpolarising tools were focused on increasing photocurrent amplitude through enhanced membrane targeting, ChR engineering has been very versatile over the last few years. Molecular engineering of ChR variants has yielded numerous tools with distinct spectral and kinetic properties. Two mutations, H134R and T159C, have been shown to increase photocurrent amplitude in pyramidal neurons by a factor of 1.5 to 2 [28], [41]. In both variants, this change corresponds with slower off-kinetics of 30-60ms in neurons corresponding to a prolonged dwell time [26], [14], [25]. One of the key amino acids controlling the protonation state of the retinal chromophore is a glutamate at position 123 in the ChR2 sequence. Substitution of an alanine or threonine at this position generated the E123A/T mutants, in which the photocycle is accelerated to an apparent off-kinetics of 4-6ms [42]. These mutations can increase the fidelity of high frequency evoked action potentials trains. However, since the open state is populated only for shorter times, the amount of ions conducted during this interval is decreased. This is effectively manifested in reduced photocurrent amplitude. For the same reason, the necessary light intensity to reach half saturation is increased from $1mW/mm^2$ to $5mW/mm^2$ [14], placing constraints over *in-vivo* applications in which tissue heating could in fact pose a significant challenge [43].

To address the heating issue and to increase the effective light sensitivity of optogenetic excitation, light switchable variants have been engineered. Mutations at positions C128 and D156 can substantially delay the closing reaction of the pore. The single mutations C128S and D156A slow down the apparent off-kinetics of ChR2 to 120s and 250s, respectively [44]. Combining both mutations generated a variant with a closing time constant of 30 minutes. Spectroscopic data revealed that, as with the wild-type ChR2 [45], yellow light can facilitate the closing reaction [46]. Hence, these mutants can be triggered into the open state by a brief blue light

pulse and can then be flashed back to the closed, dark state with intense yellow light. Due to the off-on nature of the light-driven changes in membrane potential, these tools were named step function opsins (SFOs; [44]). Since activated SFOs accumulate in the open state for the entire duration of blue light illumination, even low light intensities will render all molecules active when pulse duration is prolonged. Therefore, those variants act as photon integrators and are thus 100 times more light-sensitive than naturally occurring microbial rhodopsins [9]. Long low-intensity light pulses can also reduce the variability of activation throughout the targeted cell population *in-vivo* since the full population of opsin-expressing cells, even in a large volume of tissue, could depolarize over time [46].

With respect to ion selectivity, under physiological ionic conditions and membrane resting potential of a neuron, the photocurrent of ChRs is composed out of 65% H⁺, 35% Na⁺ and 1% Ca²⁺ and Mg²⁺ [47]. Only few mutations have been shown to have a significant impact on ion selectivity. Most notably the L132C mutation in ChR2, which was found to increase conductance of the divalent cations Ca²⁺ and Mg²⁺ by a factor of five [47]. The ChR2 L132C/T159C mutants show large photocurrents with an increased conductance of divalent cations. Since Ca²⁺ can trigger intracellular signaling cascades, these variants can potentially be used to manipulate cell activity by changing intracellular ionic composition. Such alterations to the ionic selectivity of ChRs can potentially be very useful, if selectivity can be manipulated to the extent that only single ions (e.g. sodium, potassium or chloride) can be exclusively conducted. Although this is probably a difficult task, it is likely that the recently resolved three dimensional crystal structure of ChR [48] will further boost this type of molecular development.

Following the success of applying microbial rhodopsins as optogenetic tools in mammalian neurons, similar approaches have been applied to engineer a range of light-driven actuators that allow the manipulation of diverse cellular processes. One common approach involves addition or substitution of enzyme domains with photoreceptor units, which exhibit structural changes upon illumination. For this purpose, the most commonly used photoreceptor domains are LOV (light-oxygen-voltage domain), BLUF (blue light sensor using FAD), cryptochromes and phytochromes. These photoreceptors all bind to organic co-factors such as flavins and different porphyrin molecules which essentially perform a similar role to that played by the retinal chromophore in the rhodopsins [49]. Here, most notable is a photoactivatable adenylate cyclase bPac, which was found in the bacteria *Beggiatoa* sp. *Habitus* hydrogen sulfide rich environments. This cyclase is light-sensitive via a BLUF domain. In *Drosophila*, bPac expression increases the level of signaling molecule cAMP by a factor of 10 upon blue light illumination [50]. Recently, the toolbox was extended by a very promising member - a light-inducible transcriptional effector (LITE) for endogenous gene expression. Here a light inducible dimerization of a cryptochrome called Cry2 with its native truncated binding partner CIBN [51], was fused to a transcription effector domain and a DNA recognition domain, respectively. Upon blue light illumination the DNA

bound CIBN recruits the Cry2-fused activator domain and can thereby increase the transcription of target genes [52]. These and other tools have extended the range of optogenetic manipulation beyond the simple control of neuronal excitability and are likely to contribute significantly to a range of experimental applications.

Despite the increased number of applications, all present optogenetic tools utilize light in the visible range. New tools, sensitive at the longer range of the electromagnetic spectrum (e.g. infrared or radio frequency), are desirable since they would allow deeper tissue penetration and perhaps even facilitate non-invasive approaches. Promising candidates for this approach, for example, are the infrared thermal sensitive ion channels from bats and phytochrome-based tools [53], [54]. Tools which can be specifically switched on and off with minimal cross-excitation would allow consecutive control of different cellular processes without optically cross-activating each other in the same organism.

General speaking ChRs impose another activation pathway on the top of naturally occurring ones. Upon light stimulation, action potentials can be evoked in an all-or-none fashion. From an analytical point of view, genetically-encoded tools rendering specific endogenous lightactivatable proteins would be more valuable, since they would enable researchers to modulate the endogenous cellular mechanisms which drive actions potentials. But the greatest need for improvements in the field of optogenetics are the development of cellular activity reporters. Particularly, fast, non-invasive reporters for membrane potential at the far end of the electromagnetic spectrum would be very valuable. In addition, effective optogenetic reporters at different wavelength for Ca²⁺, cAMP, cGMP or different neuromodulators such as dopamine or acetylcholine are still needed or have room for improvement. Together with the growing range of optogenetic actuators, these tools will allow the characterization of brain dynamics on levels other than electrical activity.

Manipulating cellular processes with light at high spatial-temporal resolution has already been proven as an indispensable method in neuroscience research. Therefore, the preparadigmatic stage, where optogenetic tools are developed just in a proof-of-concept fashion has passed. With the increasing knowledge of the mechanisms of action of these proteins and the complexities of their application in neural systems, new tools should be engineered such that they maximally fulfill their intended function and allow predictable, well-controlled manipulation of neural activity.

B. Gene delivery considerations in optogenetics

One of the most important strengths of optogenetic methodology is the fact that the actuators, microbial rhodopsins or other proteins discussed above, are genetically-encoded. This allows scientists to target specific cell populations for light-based manipulation. These populations can be defined based on genetic signatures called promoters, with DNA sequences that are selectively activated only in particular circuits or cell types. By coupling the microbial opsin gene to a promoter of choice and inserting this DNA into neurons, expression of

the opsin will only occur in cells that selectively express the chosen promoter. Therefore, application of optogenetics *in-vivo* requires the genetic modification of neurons to induce expression of the light-gated tools. Although many methods exist for such gene delivery [55], genetically engineered viruses [56] are by far the most popular means of delivering optogenetic tools. Lentiviral vectors (LV) [57], [58] and adeno-associated viral vectors (AAV) [59] have been widely used to introduce opsin genes into mouse, rat, and primate neural tissues [8]. These vectors allow high expression levels over long periods of time with little adverse effects [59]. AAV-based expression vectors are less immunogenic, and some types of AAV-based vectors allow the transduction of larger tissue volumes compared with LV due to their small particle size and increased viral titers. They are therefore increasingly used both in basic research and in gene therapy trials [60]. Additionally, AAV is considered safer than LV as the currently available strains do not broadly integrate into the host genome and are thus rated as biosafety level (BSL) 1 or 2 agents. Both LV and AAV vectors can be used in conjunction with cell type-specific promoters ([58], [61], [62], facilitating their application in cell-type specific optogenetic manipulation.

III. MECHANISMS OF LIGHT DELIVERY

To study brain microcircuits via optogenetics, we need light delivery mechanisms to control the distribution of light not only on the surface but also inside the brain tissue. Distribution of light on the surface can be controlled precisely by spatial light modulators (SLMs). To deliver light inside the tissue, lightguides, e.g. optical fibers, are implanted in the brain to guide laser pulses with appropriate wavelengths to regions of interest. Recently, more advanced mechanisms such as micro-fabricated channel waveguide arrays or holographic microscopy systems are being developed and tested to control the distribution of light even in the three-dimensional structure of the brain.

A. Waveguiding Systems

Optical fibers are the most popular lightguides that are used to stimulate deep brain objects. In most applications, an aspheric lens couples the beam of a solid-state laser to a multimode step index fiber which guides and deliver the optical power to the region of interest. Superluminescence light emitting diodes (LEDs), which are more stable light sources, are also becoming popular for optogenetic stimulation. Electronically driven LEDs are particularly suitable for integrated systems such as prosthetic devices. However, coupling efficiency dramatically drops when incoherent sources are used and as a result, researchers usually increase the fiber diameter to couple enough light into the fiber. The stereotaxic surgery protocols for implanting fibers are currently well established [63], [11], Figure 2(a), (b). Obviously, optical fibers only deliver light to the area close to the fiber tip. To control the distribution of light in the three-dimensional structure of the brain, more complex light delivery mechanisms are needed.

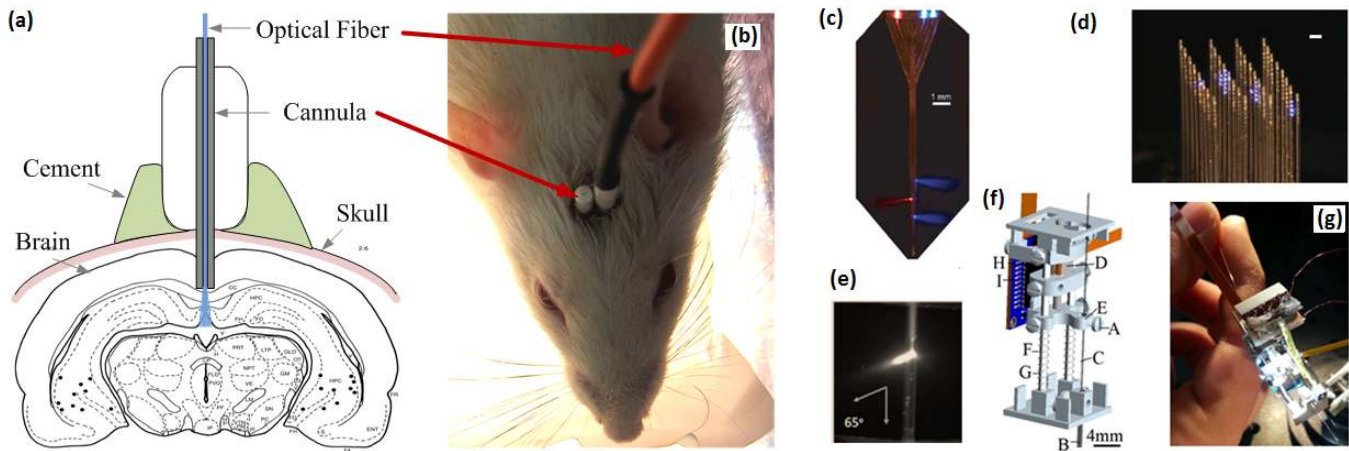


Fig. 2. (A) Optical fiber implanted in the brain tissue to deliver light and stimulate deep brain objects in transfected areas. (B) A rat receiving two implants in the motor cortex on both hemispheres. (D) Structure of a multichannel probe [64], (D) and multichannel waveguide arrays [65]. (E) A side-firing fiber that radiates power almost orthogonal to the optical axis of the fiber [66]. (F), (G) Microactuator assembly that moves a side-firing fiber inside the brain; mechanical design and a prototype [66].

It is possible to make an array of channel waveguides where each waveguide in the array terminates at a different depth [64]. An example of this approach is displayed in Figure 2(c). Here, a 1.45cm-long probe is microfabricated in the form of a 360 micron-wide array of 12 parallel silicon oxynitride ($SiON$) multimode waveguides clad with SiO_2 and coated with aluminum. Each waveguide in the array accepts light from a light source at its input terminal and guides the coupled light down to an aluminum corner mirror which deflects light away from the probe axis. Based on the published data, light losses are relatively small so that each waveguide can almost deliver about 30% of the injected light at the output terminal. The main benefits of this approach are the simplicity of the design and efficiency of light delivery. Moreover, light pulses can be delivered independently at multiple sites following any arbitrary temporal sequence of interest. A new generation of such probes have been recently developed and tested where a matrix of waveguide arrays are integrated on a single substrate to build a prosthetic device which can potentially control the light distribution in different layers of cortex [65], Figure 2(d). The major drawback of this approach is the overall size of the probe, which is relatively large, and the complexity of the optics required to couple light into the channel waveguides in large arrays.

Another more simple approach for developing a distributed light delivery mechanism is to physically move an optical fiber inside the tissue [66]. In this design, a microactuator assembly, which sits on the skull, moves a side-firing fiber inside a glass made capillary that is implanted inside the brain prior to the experiment, Figure 2(e)-(g). Side firing fibers are optical fibers where the tip is precisely angled and polished so that the radiation pattern of the fiber is almost orthogonal to the optical axis of the fiber. It is possible to optimize the parameters of the side-firing fiber, including the tip angle and numerical aperture, to minimize the divergence of the radiating beam and improve the spatial resolution of the device for light delivery. This system can deliver different wavelengths at numerous positions inside the tissue, and the size of the capillary can

be less than 50 micron. The device has spatial resolution of a few microns over a dynamic range of about 4mm and the total weight of the device is less than 2g. The major drawbacks of this design are the limited speed of the system to change the position of the fiber. Also, it is practically difficult, if not impossible, to integrate an array of such side-firing fibers and microactuators into a single prosthetic device.

Another approach remained to be explored in the future is the use of tilted fiber gratings that couple guided modes of an optical fiber to radiation modes [67].

B. Spatial Light Modulator Based Systems

Two types of spatial light modulators are used to produce distributed patterns of light in the brain: microelectromechanical (MEMS) based SLMs and liquid crystals.

The most well-known MEMS light modulator is the Texas Instrument's digital micromirror device (DMD). The DMD system is a light processor consisting of a large array of bistable micro-mirrors. The position of each mirror in the array is under independent computer control. Exposure is controlled by programming the duration each mirror reflects light toward a sample [68]. An example of the optical design of a DMD system which is specifically designed for optogenetic applications is shown in Figure 3(a). In most DMD designs, a high-power incoherent light source, with a wide spectrum, produces the optical power which is filtered to select the appropriate range of wavelengths. The filtered beam is then homogenized by an integrating rod and collimated by telecentric optics to expose the active area of the DMD through a total internal reflection (TIR) prism. The TIR prism, which is placed in front of the DMD, guides the beam of light to illuminate the DMD surface with the right angle considering the specific design of micro-mirrors in DMD chips. This prism also separates the illuminating beam from the modulated reflected beam. Next, the DMD modulates the beam and a telecentric lens projects the pattern on the entrance pupil of an image combiner. The image combiner is an assembly of mirrors and epi-fluorescence optics which makes it possible to simultaneously project a

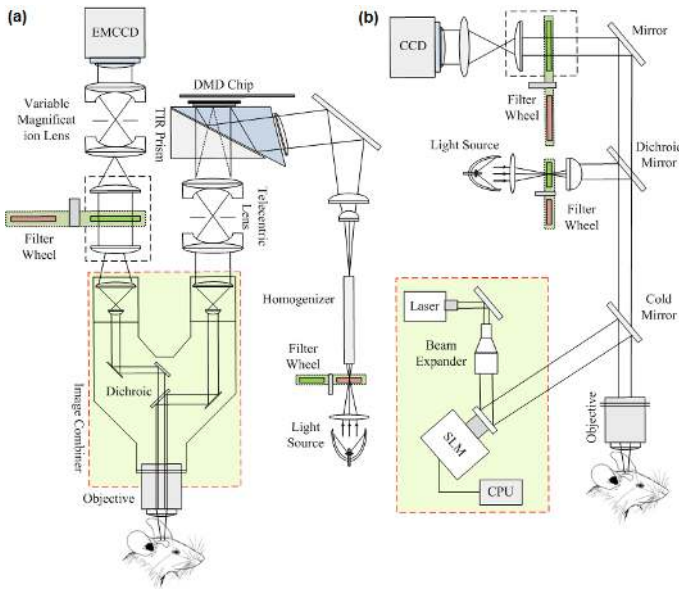


Fig. 3. Spatial light modulator systems for optogenetic stimulation, (a) Basic optical design of a DMD based system for optogenetic stimulation and imaging, (b) Liquid crystal based light delivery mechanism and holographic microscopy.

pattern on the sample and perform bright field or fluorescence imaging.

An interesting example of optogenetic stimulation by a DMD system is the study of the motor circuit in freely moving *Caenorhabditis-elegans* [69]. Since the DMD can produce rapidly changing high-resolution illumination patterns, this system has been used combined with a real-time image processing software to track a freely moving worm and optically target its motor system with single cell resolution. Another application of DMD based system for optogenetic stimulation is discussed later in the discussion of optogenetic electrocorticography recording.

Liquid crystal SLMs are specifically designed to be used with lasers and coherent light sources. A typical design of such systems is shown in Figure 3(b). In this design, a laser source produces a collimated beam of light which is phase modulated by a liquid crystal SLM and the modulated beam is launched into the optical path of a fluorescence microscope [70], [71]. The SLM generates a dynamic mask which reshapes the beam of a laser to control the distribution of light on the sample. From Fourier optics we know that a lens is nothing but a simple phase mask. Therefore, it is possible to emulate the function of any arbitrary lens by a phase modulating liquid crystal to focus the beam of laser at different depths, even in parallel, to produce three-dimensional patterns of light distribution, such as holograms, inside the tissue [71]. Recently, liquid crystals have been integrated into the optical path of multiphoton microscopes, and femto-second pulsed lasers are used to produce three-dimensional patterns of optogenetic stimulation over the entire depth of the cortex in small rodents. In other words, dynamically changing holograms are produced in the cortex to generate complex spatial-temporal patterns of activities in the cortical microcircuits. This topic is discussed further in section IV.

IV. ENGINEERING NEURAL PROBES FOR OPTOGENETIC EXPERIMENTS

From the origins of modern neuroscience direct electronic recording of neural activity has been proven essential to detailed understanding of the dynamics and structure of neural circuits underlying observed behaviors. Consequently, neural probe engineering has evolved into a mature discipline covering a variety of aspects ranging from the electronic design and materials development to tissue characterization and computerized neural data analysis, to name a few.

Since optogenetic approaches to neural control have spread through the neuroscience community, the designers of neural recording devices found themselves confronted with a demand for integration of optical elements into the neural probes. As the majority of currently available technologies for simultaneous electrophysiology and optogenetics are based on previously developed recording-only devices, we will review the progress and the challenges in neural probe design and consider the implications of opto-electronic integration at the leading edge of neural engineering.

Over the course of the past several decades a number of neural probe designs have been proposed, fabricated and applied in basic as well as clinical neuroscience experiments [72]. In this article we will focus on invasive tissue-penetrating probes that allow for recordings of individual action potentials (spikes) from the neighboring neurons (single units) based on the local changes in ionic concentrations in the vicinity of the electrodes. While these devices are primarily employed in research, the recent applications in brain-machine interfaces (BMI) demonstrate the utility of single-unit recordings from motor cortex in neuroprosthetics for paralyzed patients ([73], [74]).

In addition to single-unit recording these devices can provide the temporal profiles of local field potentials (LFPs) in the neighboring networks by simply changing the signal filter settings (from ~ 300 -8000 Hz for single units to ~ 1 -1000 Hz for LFPs). LFP data can, for example, be subject to Fourier analysis to reveal predominant oscillation frequencies within the monitored neuronal network. Changes in LFP frequency spectra are often characteristic to a particular neurological disorder (e.g. Parkinson's disease or epilepsy).

While patch-clamp electrophysiology offers the most precise recording of voltage changes across neuronal membranes, this cell-penetrating technique suffers from relatively low throughput and is not compatible with chronic implantation. The vast majority of neuroscience experiments, require recordings of activity of large numbers of individual neurons during a specific behavioral paradigm and hence single-neuron resolution and recording stability over a course of an experiment (days to years) are often the key requirements for the neural probe design [80].

Currently, in addition to the anatomy of the specific region of the nervous system and the desired resolution of the acquired data, the limitations of the available fabrication approaches dictate the neural probe geometry. Individual metallic microwires ~ 50 -100 micron in diameter are the simplest forms of neural probes, Fig. 4(A). These electrodes are primarily

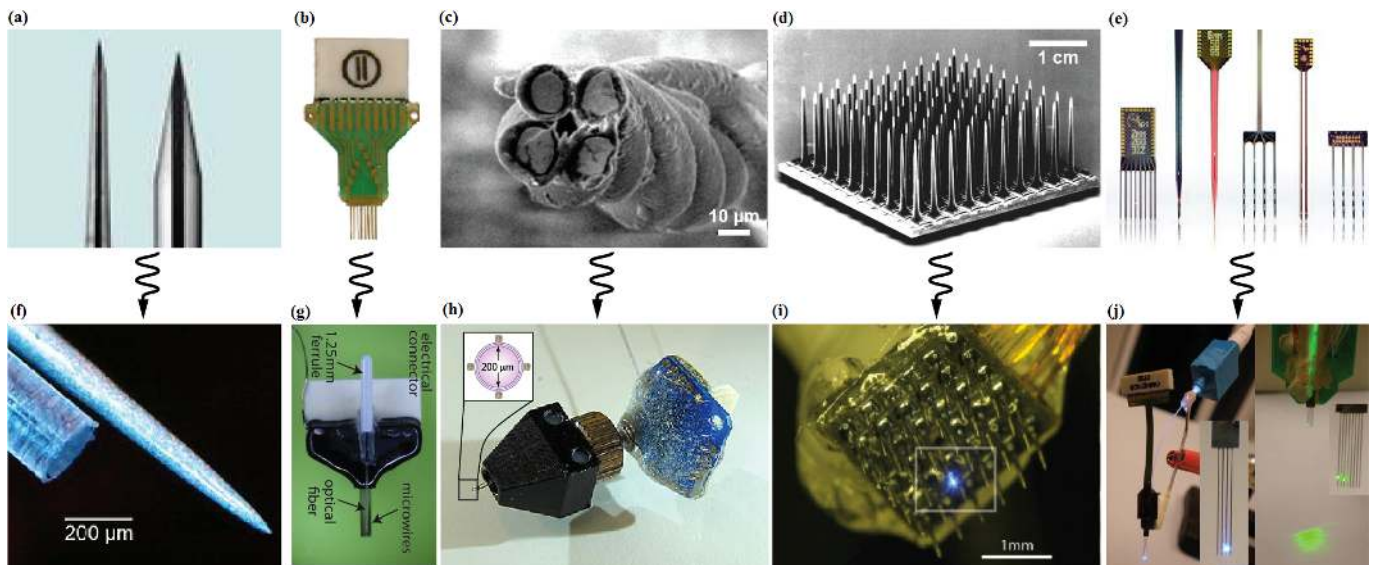


Fig. 4. Examples of commonly used single unit and LFP recording devices and their optogenetics-compatible modifications. (A) Silica-encapsulated tungsten electrodes (Thomas Recording GmbH). (B) Tungsten microwire array (Tucker-Davis Technologies Inc.). (C) Tetrode microwire bundle (University of Queensland). (D) Silicon "Utah" multielectrode array (Blackrock Inc.). (E) Silicon multitrode probes (Neuronexus Inc.). (F) An optrode consisting of a silica fiber and a tungsten electrode [13]. (G) Metal microwire array equipped with a silica fiber [92] (H) Optetrode combining 4 tetrode bundles and an optical fiber (Anikeeva 2011). The inset shows the cross section schematic. (I) Utah array incorporating a tapered silica fiber [94], [101]. (J) Silicon multitrode probes integrated with tapered silica fibers [102].

based on tungsten, steel, gold, platinum and iridium and can be assembled into arrays of up to several tens to increase the number of recorded single units, Figure 4(B). The ability to distinguish between action potential shapes originating from different neurons was shown to be improved by assembling individual microwires into tightly wound stereotrodes (two intertwined microwires) and tetrodes (four microwires) [75], [76], Figure 4(C). These neural probes usually consist of polymer-coated nickel-chromium alloy electrodes ~ 12 micron in diameter, since larger and stiffer electrodes do not lend themselves to the assembly process and produce bulky and tissue-damaging devices. Stereotrodes and tetrodes were originally developed for high-throughput recordings from dense hippocampal layers in rodents, which remains the main application of these devices to date. These wire assemblies are often used in combination with micro-drives that allow individual propagation of each assembly independently thus enabling active search of firing neurons.

Due to the maturity of the processing methods enabled by electronics industry, silicon has become a popular material for the fabrication of neural probes. While numerous silicon-based designs have been produced over the past two decades their basic structure can be approximately divided into multi-electrode "Utah" arrays [78], [77], MEA Figure 4(D) and multitrodes [80], [82], which are sometimes referred to as "Michigan probes", Figure 4(E). The former structures consist of arrays of highly-doped silicon "peaks" created by a combination of direct micromachining and wet etching and insulated from each other by regions of glass. The bodies of the electrodes are coated with a chemical-vapor deposited (CVD) polymer parylene C and the conductive tips are exposed. The size of the individual electrodes within the array is on the order of several tens of microns at the recording tip but 300-400 micron at the

base, which yields a comparatively large footprint for arrays comprised of more than sixteen electrodes (e.g. a 10×10 MEA has dimensions of approximately $4 \times 4 \text{ mm}^2$, not including connector parts). One of the main advantages of these devices lies in their floating design. The MEAs are deposited on the cortical surface attached to the connector mounted on the skull with long flexible loosely bundled wires. As a result these devices require a sensitive implantation procedure during which the MEA is deployed pneumatically into the cortex. Since rodents lack sufficient intracranial space, floating MEAs are limited to applications in primates. Furthermore, the length of the electrodes is restricted to ~ 1 -1.5mm by the fabrication and implantation procedures and hence these devices are used primarily for cortical recordings in primates, facilitating an entire field of brain-machine interfaces (BMI) and, recently, neural control of robotic aids by paralyzed human patients [73].

Multitrode probes employ a thin silicon substrate with lithographically patterned metal electrodes distributed along the shaft. This streamlined geometry and deterministic positioning of the recording sites makes these probes attractive for recordings in deep brain structures with high cell density such as the rodent hippocampus. These probes have recently been extended to devices incorporating logical elements thus enabling on-site signal amplification, which holds the potential to improve the yield of recorded single units. However, the lengths of the silicon shaft is still limited to a few millimeters by the micromachining process restricting the applications to experiments in rodents and cortical recordings in primates. Similarly to stereotrodes and tetrodes these devices can be combined with microdrives. However, due to the device geometry, the entire shaft is propagated through the brain thus advancing all of the patterned recording channels.

Neurotrophic electrodes comprised of a silica pipette with several internal electrodes incorporate neural tissue, which promotes neurite ingrowth into the cone allow for high signal-to-noise recordings. However, to our knowledge they are yet to be combined with optogenetics and hence will not be discussed in detail in this review. Interested readers may refer to [81] for further details.

The majority of opsins, with the exception of step-function opsins, at average expression levels (2-8 weeks of incubation time following viral delivery into the neural tissue) require light power densities of approximately 1 mW/mm^2 in order to robustly evoke or inhibit action potentials *in-vivo*. This poses a requirement of direct light delivery into the area of interest, as the neural tissues are highly scattering and absorptive within the visible range, which currently encompasses the activation/inactivation spectra of all retinal-containing opsins. Consequently the vast majority of optogenetic experiments are performed with implanted optical fibers and the light is supplied by an external source such as a laser or a high-power light-emitting diode (LEDs). The direct implantation of miniature LEDs into the brain is an alternative strategy for light delivery, which will be discussed in the following paragraphs.

While the initial proof-of-concept optogenetic experiments performed *in-vitro* employed intracellular patch-clamp recordings [6], the need for combined light delivery and extracellular electrophysiological readout was recognized as this technology transitioned *in-vivo*. Furthermore, as the state of the awake brain of behaving rodents differs dramatically from activity patterns of the unconscious brain of anesthetized animals, not to mention dissociated neuronal culture, it became essential to observe the electrophysiological outcomes of optogenetic stimulation and correlate them to an observed behavior. As optical fibers are routinely employed in optogenetic experiments, their integration with electrophysiological probes provides the most straightforward engineering strategy. Thus, to date, the majority of commonly used neural probes have been modified to carry externally attached conventional commercially available silica fibers.

The simplest devices "optrodes" comprise of individual metal electrodes and optical fibers held together by an adhesive, Figure 4(F). These devices are primarily employed for recording of single-unit and population (combined signals from multiple units) activity in anesthetized animals [13]. Similarly, an optical fiber can be attached to a metal electrode array and the resulting devices are employed during behavioral experiments primarily for population activity and LFP recordings, Figure 4(G) [92].

Single-unit recording during optical stimulation can be readily achieved with "optetrodes", which comprise of an optical fiber surrounded by several tetrode bundles, Figure 4(H) [93]. These devices allow for recording during free behavior and provide access to multiple recording sites due to a miniature concentric mechanical drive. While these devices insure illumination of all the neurons that can be recorded with the tetrodes they do not allow for independent manipulation of individual tetrodes.

In order to introduce a fiber into a Utah-style MEA one

of the electrode positions can be replaced with an opening, Figure 4(I) [94]. In this case the scattering within the tissue only allows for illumination of cells within the direct vicinity of the fiber. Consequently, the utility of this approach is mainly in exploring the effects of localized stimulation or inhibition on a broader cell population that can be accessed by electrodes distributed in a several millimeter area, as only a few ($\sim 4-9$) electrodes will be in contact with the illuminated tissue. The integration of a fiber with a Utah MEA compromises the otherwise floating design of the device, as the conventional silica fibers are too rigid and, hence, anchor the device to the skull.

Silicon multitrode probes can be outfitted with fibers similarly to optrodes by simply attaching the latter to the former with an adhesive following the fabrication process, Figure 4(J) [102]. These devices often employ tapered fibers, which allow for reduction of the overall device dimensions but significantly increase optical losses and reduce the numerical aperture leading to reduced illuminated tissue volume. The relative position of the fiber with respect to the electrode pads determines, which of the channels will record activity of the illuminated neurons. Positioning the fiber at the top of the array may require very high light powers to reach the electrodes at the tip of the array, while positioning of the fiber tip within the array pads compromises the recording on the upper pads as the tissue may be damaged or pushed away by a relatively large fiber.

Connector design remains one of the main challenges for simultaneous neural recording during optogenetic experiments as optical coupling is required in addition to an electrical connector. This imposes the design constraints, which often lead to devices too large or heavy to be employed during free behavior in smaller mammals (mice, bats) or birds. High fidelity optical coupling (approaching 100% transmission) can now be achieved with knife-edge polish of pigtailed ferrules (stainless steel or zirconia) held together with ceramic sleeves. While ferrule to ferrule connection is not considered stable by photonics standards, its performance is sufficient for behavioral experiments on timescales of minutes to hours. Ferrule coupling, however, restricts the position of the fiber with respect to the recording device, which generally results in a fiber illuminating only a fraction of the recording electrodes.

Note, that all the devices discussed so far are based on rigid materials (metals, silicon, silica) with Young's moduli (e.g., $E(\text{Si})=130-170 \text{ GPa}$, $E(\text{SiO}_2)=73 \text{ GPa}$, $E(\text{W})=411 \text{ GPa}$, $E(\text{Pt})=168 \text{ GPa}$) that are many orders of magnitude higher than that of neural tissues ($E \sim \text{kPa-MPa}$) [79], [83]. It is thought that this mismatch in stiffness contributes to tissue damage and subsequent decay of the recording quality [84], [85]. It is reasonable to assume that the insertion of the implant into the tissue causes neuronal death and displacement, however, the initial inflammatory response usually ceases within approximately 2 weeks following the implantation, which yields improved signal-to-noise ratio (SNR) and the overall number of single units recorded. However, the SNR deteriorates and the number of monitored units decays over the course of following weeks to months [97]. While the origins of neural probe failure are being actively investigated,

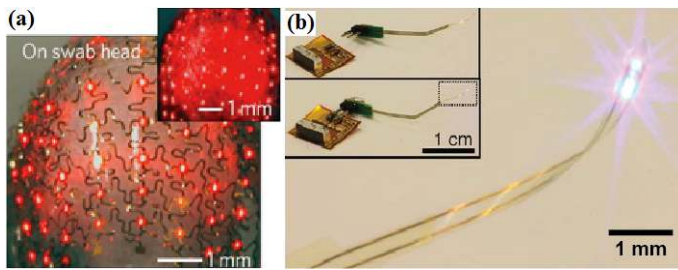


Fig. 5. (A) Contact printed AlInGaP LED on a PDMS substrate [87]. (B) Releasable GaN micro-LED for deep-structure optogenetics [90].

several complimentary hypotheses have been proposed. During normal movement, the brain undergoes several tens of microns shifts (micromotion) with respect to the skull [98], [99]. Consequently, the tissue surrounding the hard fixed implant is subject to chronic damage, which is manifested in glial and astrocytic activity resulting in device encapsulation. If the latter were a dominant mechanism of device failure, the floating Utah arrays would be immune to failure, which is not the case. The disruption of glial networks by the implants significantly exceeding the dimensions of individual cells has also been suggested as a potential trigger of glial and astrocytic response. This is supported by the reduced glial scarring around miniature implants with cross sections approaching single cell size. Finally, recent work suggests that the disruption of blood-brain barrier (BBB) is yet another factor associated with the tissue response to the implanted devices [82]. The latter findings imply that the sharper devices such as Utah MEA or multitrode probes produce a greater BBB damage and yield more severe gliosis as compared to the metal electrode arrays consisting of relatively blunt cylindrical wires [95].

As the majority of optogenetic neural probes incorporate rigid silica fibers of 50-400 micron in diameter, it is reasonable to expect that the reliability of the neural probes will be further compromised by such opto-electronic integration. Furthermore, so far this review has focused on the devices designed for applications within the brain, while the recent interest in the peripheral nervous system as a potential early therapeutic target for optogenetics emphasizes the need for devices tailored to highly mobile and flexible nerves. Consequently, flexible, biocompatible platforms are needed for the advancement of combined long-term optogenetic and electrophysiological studies.

Over the past several years a number of technologies have spurred in attempt to address the elastic modulus mismatch between neural probes and neural tissues. Silicone resins (predominantly poly(dimethyl sulfoxane), PDMS) have been successfully employed as substrates for metal electrode arrays by Lacour and Fawcett among others [96], [100]. This allowed for the development of chronically implanted peripheral nerve cuffs capable of single-unit recordings from the nerve surface. This technology however, has not yet been integrated with light delivery, as silica fibers are rigid, and hence an alternative solution such as an LED array is required. Furthermore, this technology is not yet compatible with applications beyond the

nerve or cortical surface (micro-ECoG) as it has limited resolution due to the fabrication constraints and the implantation surgery into the deep tissue is yet to be developed.

Similarly, parylene C has been employed as a substrate for thermal molded soft cone-electrodes incorporating recording sites within the cone. These devices are potentially intended to operate akin to neurotrophic silica-cone electrodes and may yield improved SNR and stability. However this technology is not currently scalable to high-density recordings, nor does it lend itself to the incorporation of optical fibers [86] thus facing the challenges of the silicone-backed devices.

Micro-contact printing process developed by Rogers and colleagues has been recently employed in fabrication of sophisticated integrated structures on highly flexible silicone, polyimide and silk fibroin substrates, Figure 5(A) [87], [88], [89]. This innovative approach takes advantage of the mature chip-scale semiconductor processing and combines it with designer pre-strained interconnects, which enable the release and transfer of the devices fabricated on wafers onto flexible substrates. Consequently, these devices can be designed to integrate a variety of electronic components enabling on-site data processing as well as micro LEDs based on proven group III-V semiconductors. The utility of these devices lies primarily in the cortical LFP recordings as the resolution of the printing process limits the channel size. The devices, integrating a single recording channel with LEDs, photodetectors and thermal sensor (resistor) have been recently tested in deep brain structures of mice, Figure 5(B). The insertion into the depth of the tissue in this case was enabled by silicon microneedles adhered to the devices by resorbable silk fibroin layers [90]. However, the mechanics of the printing process would yield comparatively large structures (several millimeters in area) if the number of recording sites were to increase to several tens or hundreds, hence these structures are currently limited to a small number of electrodes.

A more traditional approach to flexible neural probes borrows fabrication strategies developed by micro-electro mechanical systems (MEMS) industry and produces devices resembling multitrode probes, in which silicon substrates have been replaced with polyimide [91]. In this case optogenetics can be enabled by integrated polymer waveguides based on photoresists poly(methyl methacrylate) (PMMA) or SU-8, Figure 6. The resulting structures are very flexible and require temporally stiffening encapsulation in order to penetrate deep into the tissue. A number of encapsulation strategies such as coatings with sugars, poly(ethylene glycol) (PEG) and, more recently, tyrosine-based polymers allow to tune the time of the implant softening into the neural tissue. One the main challenges of this approach is relatively low transmittance of the polymer waveguides (>1 dB/cm), which translates into the need for high input light powers (>100 mW sources) in order to enable opsin-facilitated optical neural interrogation at the tip of fairly short (length < 5 mm) polymer waveguides.

Currently, MEMS-style fabrication and the micro-contact printing processes suffer from the relatively low yield, which drives the high cost of these devices reducing the number of animals that can be employed in a given optogenetic behavioral study and hereby increasing the variability of the results.

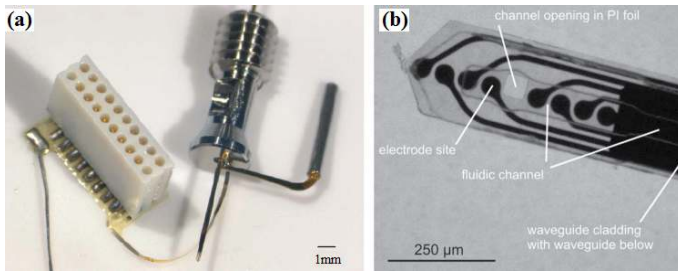


Fig. 6. A photograph (A) and a tip close-up (B) of a MEMS-processed polyimide-backed device equipped with a SU-8 waveguide and drug delivery channel [91].

Furthermore, the wafer-scale processing limits the device dimensions to the applications in rodents and the primate cortex. Applications such as deep brain areas or the peripheral nerves in primates or humans currently remain inaccessible with these state-of-the-art technologies for combined optical stimulation and recording.

A robust fabrication approach is needed to enable integration of optical components (LEDs and/or waveguides) and high-density single-unit electrophysiology. The development of such integrated devices may require exploration of new materials systems such as conductive polymers and polymer composites, which have been shown to improve the recording SNR and stability. Electrospinning and chemical vapor deposition (CVD) enable processing of polymer probes with diameters as low as 10 micron [103], [105]. However, the application of these methods to integrated multi-channel devices will require further development as the number of materials investigated to date remains limited.

Fabrication methods allowing for processing polymers beyond photoresists may allow to reduce the losses in flexible waveguides. The photonics industry may provide insights into high-throughput fabrication of high-quality optical waveguides. For example, thermal drawing process, routinely used to produce conventional silica fibers, can actually be applied to processing of multiple materials such as polymers, metals and semiconductors [104]. Initial attempts to employ thermal drawing in the design of combined optical and electrical neural probes demonstrate the promise of this approach, however, the scaling to high-density electrophysiology as well as combined optical and electronic connectorization would need to be addressed before this fabrication method can be adopted at large.

Nanowires of III-IV semiconductors provide highly intimate interfaces with individual neurons and can also be designed to incorporate p-n junctions necessary for LEDs and field-effect transistors enabling high SNR recordings [106], [107]. The majority of nanowire devices are designed on flat rigid substrates and are primarily used for studies of individual cultured cells. However, flexible substrates have recently enabled integration of nanowire electronics with hydrogel scaffolds creating three-dimensional hybrids of neural tissues with embedded electrophysiological probes [108]. This approach provides a particularly promising strategy for optogenetic neural regeneration devices, granted that challenges associated with scalability and connectorization are overcome.

The design and implementation of future intimate and integrated optogenetic electrophysiological probes now depends on the collaboration between materials scientists, chemists, electronics, photonics and packaging engineers as the solutions to the opto-electronic integration challenges would also need to address the biocompatibility and reliability issues stemming from the fragile and inhomogeneous structure of the neural tissue.

V. OPTOGENETIC STIMULATION COMBINED WITH MAGNETIC RESONANCE IMAGING

The optogenetic functional magnetic resonance imaging (ofMRI) [109], [110], [111] technology combines optogenetic control with high-field fMRI readout. This enables cellular level precision in control while whole brain network function can be readout *in-vivo*. Systematic control of the brain with specificity in the cellular level combined with whole brain dynamic imaging has been a fundamental tool missing in the neuroimaging and neuroscience fields; and arguably has been a key missing tool to link findings from the more precise cellular level investigation to the overall brain function. While standard fMRI provided important clues and insights into brain function including mapping of visual, sensory, motor areas, and association of brain activation changes to neurological diseases, they were limited in fidelity due to the brain's densely interconnected network of heterogeneous cellular populations. OfMRI addresses this problem by combining genetically targeted control of neurons with a method capable of whole brain functional readout.

In the first study, it was shown that cell types specified by genetic identity, cell body location, and axonal projection target can be specifically excited while the whole brain function associated with the stimulation can be monitored with spatiotemporal precision. In this study, it was first shown that ofMRI could reliably detect local activities resulting from cell type-specific control of neurons. For example, upon stimulation of the Chr2-expressing excitatory neurons in the motor cortex, a positive blood oxygen level-dependent (BOLD) signal was observed at the site of stimulation. In addition, the temporal shape of the optogenetically induced BOLD responses matched the conventional BOLD signals that are typically evoked during sensory stimulation, suggesting that excitation of a single population is sufficient to drive the conventional BOLD response.

Then, to demonstrate the global imaging capabilities of fMRI, the authors measured the BOLD response at the thalamus, which has direct axonal projections from the motor cortex. At the thalamus, robust BOLD signal was measured, demonstrating the global imaging capability. However, an unexpected difference in the BOLD response shape was also observed. While largely similar in shape as the motor cortex hemodynamic response function (HRF), there was a marked reduction in HRF signal rise slope. To identify the source of such difference, electrophysiology recordings were made at the motor cortex stimulation site and the location within the thalamus where the ofMRI signal was observed. The electrophysiological recording revealed a delay in spike-rate

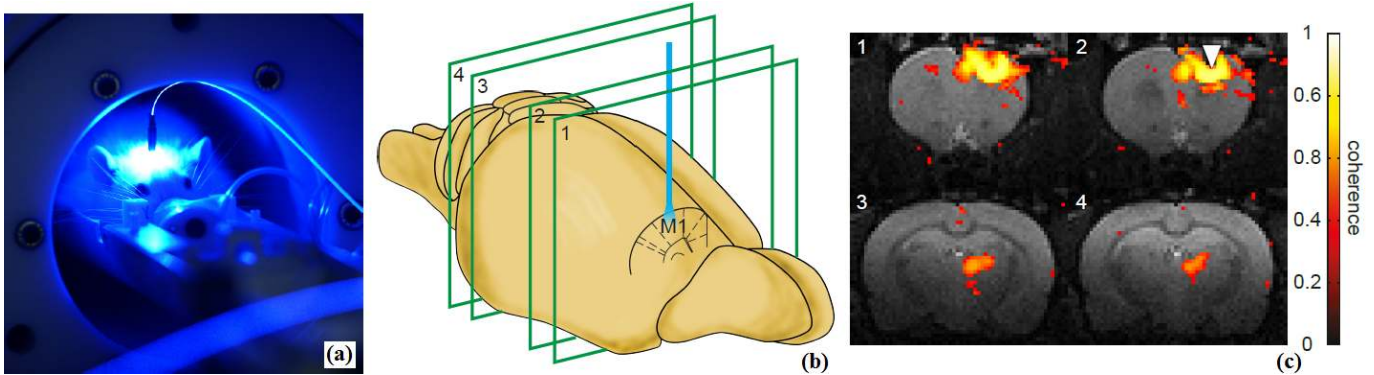


Fig. 7. ofMRI with stimulation of excitatory neurons in the primary motor cortex. (A), During the ofMRI scan, the subject is intubated and lightly anesthetized. The respiration rate, body temperature, and expiratory CO_2 are monitored and controlled. A surface coil (not shown) is mounted and centered over the region of interest on the skull to maximize the signal-to-noise ratio. The subject's eyes are also covered (not shown) to avoid unintended visual stimulus. (B), Six cycles of 20s-on-40s-off optical stimulation are delivered to the primary motor cortex of the animal during the scan. The gradient-recalled-echo (GRE) BOLD sequence is used and 23 slices are collected for full brain coverage with 3 s temporal resolution. (C), BOLD signals are detected at both the stimulation site (primary motor cortex, image 1,2) and downstream areas (thalamus, image 3,4). The bottom tip of the white triangle indicates the optical stimulation site.

increase in thalamic neurons compared to cortical neurons, matching the smaller HRF slope observed with ofMRI. This opened up a possibility for the ofMRI technology to enable not only spatially resolved neural activity mapping but also to temporally resolve underlying activity patterns.

Next, to further expand the ofMRI technology's capability to map responses associated with specific modulations, the authors visualized the brain's response to light stimulation delivered to the thalamus with anterograde viral injection in M1 cortex. With anterograde viral injection in M1 cortex, thalamic regions that receive direct axonal projection will have opsin expression in the axons of neurons projecting from M1. With the light stimulation of these axons, robust activities were observed at both the thalamus and motor cortex, demonstrating the feasibility of mapping the causal influence of cells defined not only by genetic identity and cell body location, but also by connection topology. Finally, the authors showed that ofMRI could be used to trace the different global responses to activation of two distinct thalamic nuclei that matched predictions from literature, demonstrating the feasibility of the *in-vivo* circuit function mapping capability of ofMRI. These findings laid the basic foundation for how the ofMRI technology can be a potent tool that can enable systematic *in-vivo* circuit analysis.

Since the original publication, several studies have built upon these results, further demonstrating the utility of the ofMRI technology. For example, ofMRI implementation was demonstrated in awake mice [112] that showed that a larger BOLD response was evoked in awake animals compared to anesthetized conditions during optical stimulation of the primary somatosensory cortex. The study also showed that the degree of functional connectivity between active regions was strengthened. Therefore, under the following conditions, ofMRI studies could preferably be performed without anesthesia: animal does not experience significant levels of stress due to restraint and noise and importantly, large motion is that is not compatible with the imaging setup is not evoked by the stimulation.

The ofMRI technology have also been demonstrated at

a number of different stimulation locations including motor and sensory cortex [109], [112], [113], ventral tegmental area [114], and hippocampus [115], further demonstrating its utility. Looking forward, demonstrating the ofMRI technology's capability to identify dynamic network function across multiple synapses also be a key next step.

Advances of the optogenetics technology [8] will also enable diverse ofMRI experiments to be performed. For example, with optogenetics now becoming feasible in non-human primates [61], [116], future ofMRI experiments may turn to monkeys as an animal model for studying more complex brain circuits that is difficult to study with rodents. Improvements in optogenetics may also enable precise control of subcellular compartments. With such a capability, ofMRI could provide a unique bridge between subcellular perturbation and the resulting global brain function. This could provide a new platform to study the specific role of axons and dendrites in the context of overall brain function, which have been implicated in many neurological diseases and disorders including Alzheimer's disease [117], multiple sclerosis [118], Autism [119].

In addition to advances in ofMRI's capability that is expected to come with the development of new optogenetic tools, the development of advanced imaging technologies is of critical importance to accelerate scientific discovery. For example, while fMRI uniquely provides the readout of the global brain function, the image quality is often a concern. To accurately register neural activity to anatomical locations, it is necessary to have high quality, distortion-free images. Solutions to this problem include the passband SSFP fMRI [120] method that was demonstrated in the original ofMRI publication. Distortion-free passband SSFP fMRI combined with optogenetics was shown to result in more robust, and spatially accurate registration of the activities.

Another important technical development that needs to take place is to increase spatiotemporal resolution of ofMRI images. While fMRI is unlikely to deliver single cell level, millisecond temporal resolution, it is quite possible that advanced imaging technology development would enable corti-

cal layer and sub-nuclei specific imaging. Furthermore, the temporal resolution of the imaging could be improved to a sub-second range. Note however that, for the improvement of the spatiotemporal resolution, it is important to maintain the whole brain volume coverage. In MRI, temporal and spatial resolution can be easily traded off with smaller spatial volume coverage. Therefore, without the large volume coverage, high spatiotemporal resolution is of limited value. For example, taking several slices or even single slice images at high spatiotemporal resolution, might be of interest for a dedicated study looking at that particular spatial location, it does not allow the key advantage of ofMRI, which is the global imaging capability, to be fully utilized. A method that can fundamentally improve spatial and temporal resolution without sacrificing the volume coverage therefore is of critical importance.

With the large degree of freedom in neuronal cell population control including the type of cell targeted, the temporal pattern of stimulation, and the type of response elicited (e.g. excitation or inhibition), methods for robust, fast, and high-throughput data acquisition in a closed-loop system will greatly accelerate scientific discovery utilizing the ofMRI technology. As a first step in achieving a real-time ofMRI system, Fang et al. [121] developed a highly parallelized method for performing image reconstruction, motion correction, and activation detection in a fraction of the typical MRI acquisition repetition time. Combined with methods such as compressed sensing [122] and multiple coil arrays [123], cryo-cooled coils will for signal-to-noise ratio (SNR) improvement, real-time image acquisition and activation detection can be achieved. With such a system is in place, it will be possible to interactively perturb specific brain circuits and immediately visualize the network-level activity that results. The simplest form of closed-loop ofMRI would rely on manual inspection of the evoked patterns of brain activity and corresponding change of stimulation parameters. To enable a more robust and high-throughput system, automated analysis techniques that can identify activations and quantify their spatial and temporal characteristics could also be combined. Integration of such automated analysis methods with real-time image acquisition will considerably accelerate the discoveries made with ofMRI technology.

Optogenetic fMRI enables the study the causal global effect of perturbing specific brain circuit elements in live animals. This has great potential to providing insights into the mechanisms that underlie normal and diseased brain function, and driving the development of new therapies. For example, ofMRI may be used to track how the functional interactions within the brain progress over time. Specifically, ofMRI may be used to identify which brain regions fail to communicate with another region properly, how they fail, and when. Studies have shown that global structural and functional changes can predate clinical symptoms of neurological diseases [124], [125] and ofMRI will provide an important new opportunity to systematically assess changes during a disease's progression. Unlike standard neuroimaging techniques, ofMRI can attribute these changes to the modulation of selective neuronal elements and temporal parameters of stimulation (i.e. temporal encoding). This will illustrate the highly dynamic nature of brain circuits [110],

and provide information about the manifestation of a disease not obtainable with other techniques.

Just as ofMRI can be used to visualize how neurological diseases affect the brain network function, it can also be used to determine how a diseased brain will respond to therapy. For example, optogenetic control may be used to selectively simulate separate elements of neuromodulation therapeutic targets currently in use in humans, such as in deep brain stimulation and spinal cord stimulation therapies. Monitoring how the brain responds to different parameters of stimulation with ofMRI could then be used to predict which parameters optimize therapeutic benefit.

Given the extensive library of literature that has used fMRI to study neurological diseases, a subtle yet significant contribution of ofMRI experiments to the clinical domain will also come from an improved understanding of the blood oxygenation level dependent (BOLD) responses. Despite two decades of fMRI research in humans, the nature of the BOLD signal remains poorly understood, due to an incomplete understanding of the relationship between neural activity and haemodynamic processes in the brain [126], [127], [128]. Optogenetic fMRI provides a unique opportunity to probe these systems and to determine the role that different neuronal populations play in generating these responses. The original ofMRI paper [109] was the first to demonstrate this principle, showing that causal activation of a specific single population of neurons could lead to BOLD responses with classical kinetics. Since then, a number of other ofMRI experiments have begun to disentangle the convoluted relationship between neural activity and the BOLD signal. For example, one study compared the temporal characteristics of the ofMRI BOLD response with different electrophysiological measurements, finding that it correlated best with neuronal spiking activity [129]. Another study showed that the BOLD signal summates in response to closely spaced trains of stimulation, confirming that the BOLD signal provides a linear approximation of neural activity levels [113].

To further link the BOLD response evoked by optical stimulation with the fMRI responses typically measured during sensory or cognitive tasks, quantitative comparison of the two will also be important. It has already been shown that optical activation of ChR2-expressing neurons can lead to local changes in blood flow while the normal activation of neurons through glutamatergic receptors would be blocked under the same condition [130]. Future integration utilizing combinatorial optogenetics with ofMRI will allow the contributions of distinct cell populations to the BOLD signal to be even further dissected. In current ofMRI experiments, the optically driven population likely recruits or inhibits other neuronal types and glial cells in generating the measured fMRI signal. With multiple wavelengths of light control used to prevent additional populations from being excited or inhibited, resulting signal would reflect the change solely caused by the initially targeted population. This information could be used to interpret fMRI results by identifying the types of neuronal elements involved. A simple example of this includes understanding the effect of suppressing neural activity compared to activating inhibitory neurons. Since the BOLD response only provides an indirect

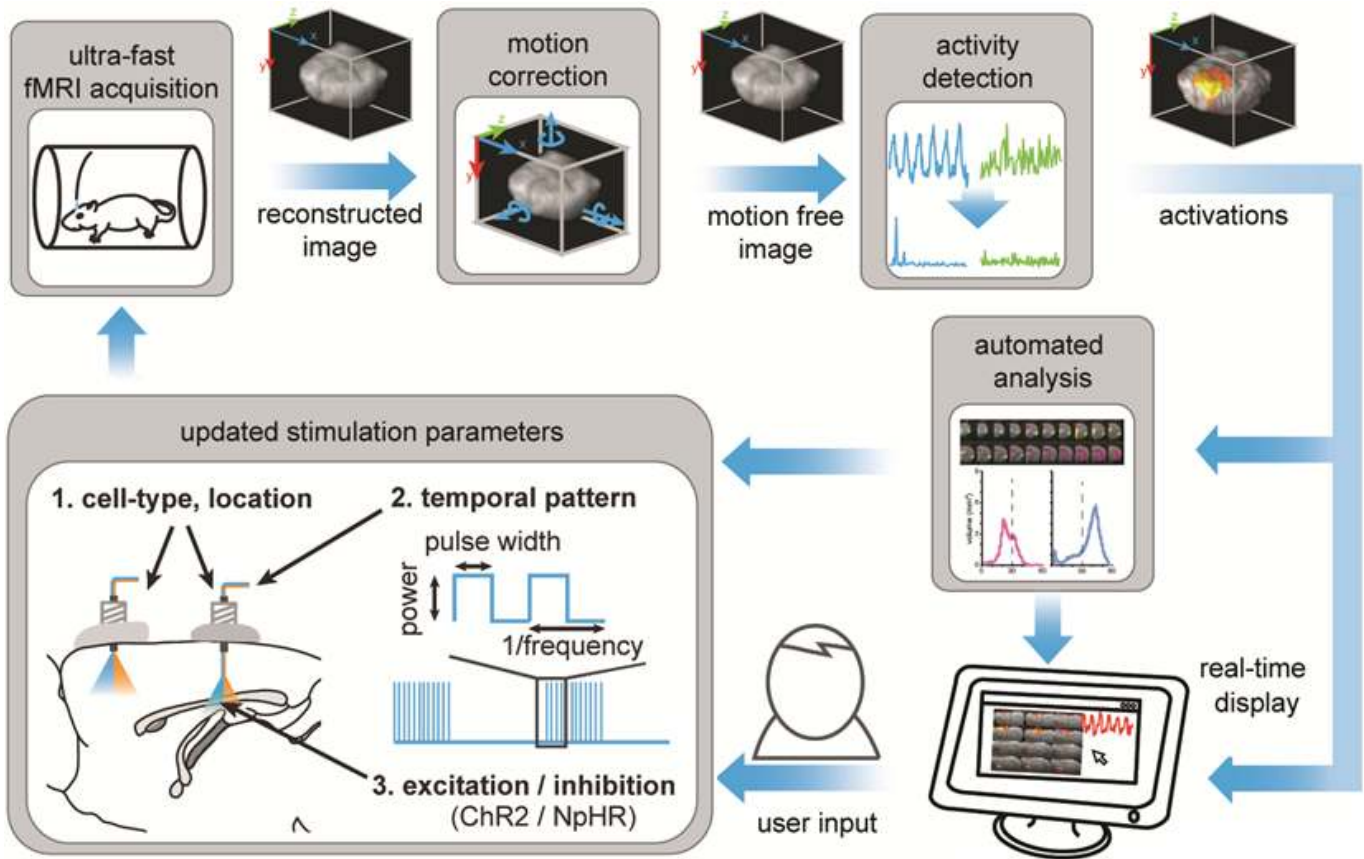


Fig. 8. Schematic of a high-throughput, closed-loop ofMRI system. fMRI images are acquired using ultrafast acquisition techniques during optogenetic stimulation in awake or anesthetized animals. High-throughput, real-time image reconstruction, motion correction, and activation detection are performed in series. The resulting patterns of activity can be analyzed with automated algorithms or inspected manually to decide how to update stimulation parameters. Degrees of freedom available include the location and type of cell targeted, the temporal pattern of stimulation, and the type of modulation (e.g. excitation versus inhibition). (Modified Figure modified from [121]).

measure of neural activity, without a proper tool to control the neural elements with precision while measuring fMRI signal, how these two distinct processes which both reduce the level of neural activity affect the measured signal has remained elusive.

The significance of ofMRI lies in its ability to map global patterns of brain activity that result from the selective control of precise neuronal populations. Currently, no other existing method can bridge this gap between whole-brain dynamics and the activity of genetically, spatially, and topologically defined neurons. While a number of studies have already started to exploit the unique capabilities of this technology, ofMRI will likely experience significant growth and development over the next decade. Both the advances in the molecular toolbox of optogenetics, and improvements in imaging technology hold important roles in bringing ofMRI closer to its full potential. In particular, the integration of ultra-fast, high-resolution data acquisition and analysis, and combinatorial optogenetics will enable powerful closed-loop ofMRI that can real-time visualize brain activity with real-time feedback control. Further research uncovering the nature of the BOLD signal will also make it possible to extract more detailed information about the neural activity measured in fMRI studies. Finally, the application of ofMRI to translational research has the potential to fundamentally transform therapeutic design for neurological

disorders. New targets of therapeutic neuromodulation will likely be discovered, and improved models of neurological disease will be introduced. Taken together, ofMRI is anticipated to play an important role in the future understanding of both normal and diseased brain functions and enable alternative treatment developments.

VI. COMBINING OPTOGENETICS WITH TWO-PHOTON MICROSCOPY

As discussed earlier, methods for guiding spatial delivery of single-photon light excitation have been used to improve the precision of optogenetic modulation [69], [131] - [138]. Light localization with higher precision can be achieved using multi-photon excitation microscopy. In neuroscience, two-photon excitation microscopy has allowed for the direct investigation of the nervous system at different length scales (from synapses to neural circuits) and on different time scales (milliseconds to months) [139], [140]. The physiological importance of such work is highlighted in studies investigating the role of the hippocampus in spatial navigation tasks in rodents. Dombeck and colleagues used two-photon microscopy in combination with genetically encoded calcium indicators in the mouse hippocampus to map the spatial representation of place cells and found that the anatomical proximity of neurons had no

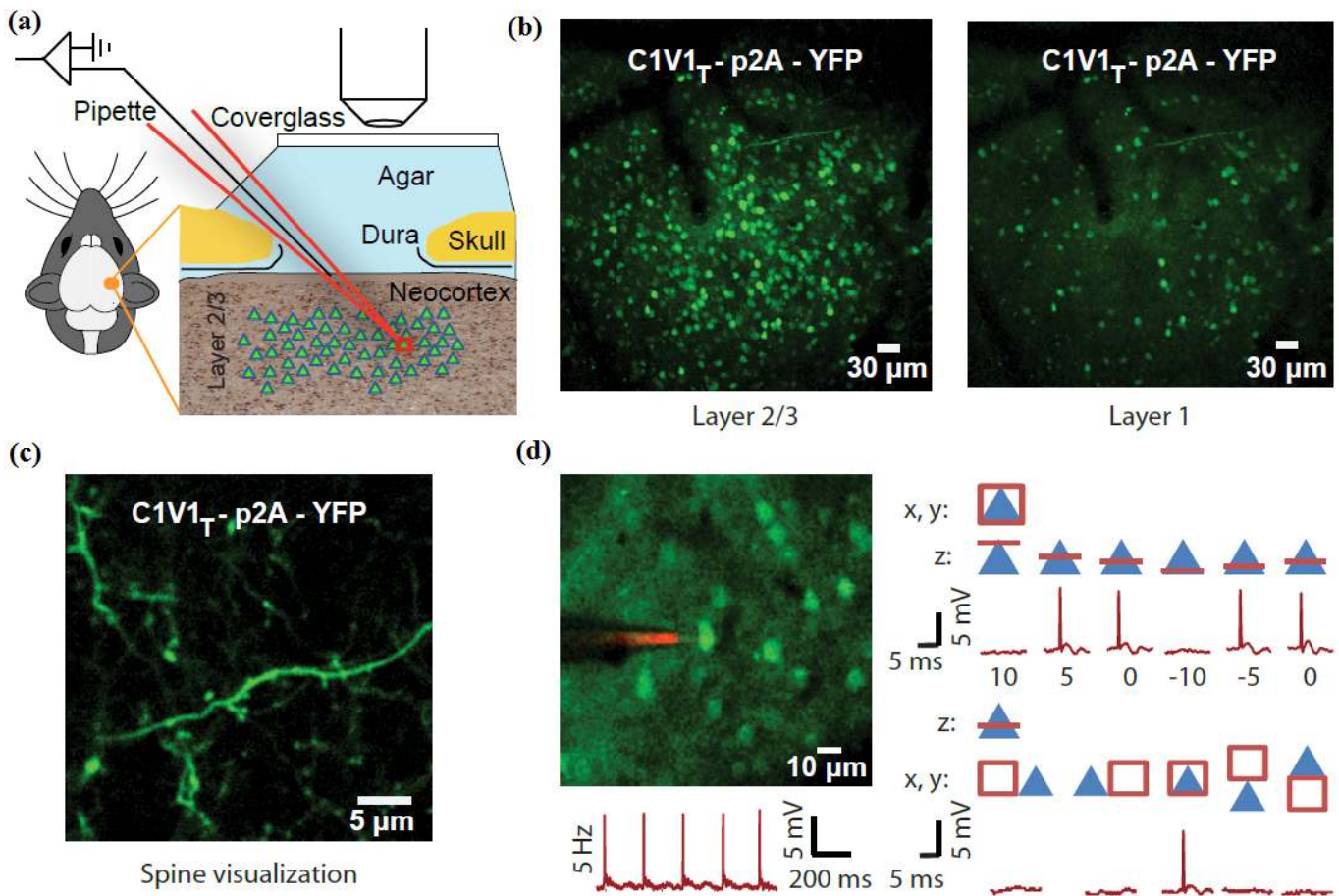


Fig. 9. Two-photon optogenetic control of spike firing *in vivo* in adult mammals. a. Schematic of the experimental setup for *in vivo* two-photon control of layer 2/3 somatosensory neurons transduced with C1V1T-p2A-YFP. b. Left, two-photon image of layer 2/3 pyramidal neurons transduced with C1V1T-p2A-YFP in somatosensory cortex (150-250 micron below pia). Right, two-photon image of layer 1 pyramidal neurons transduced with C1V1T-p2A-YFP in somatosensory cortex (50-150 micron below pia). c. Two-photon image of dendritic spines on pyramidal cells transduced with C1V1T-p2A-YFP in layer 2/3 of somatosensory cortex. d. Upper left, *in vivo* two-photon image of layer 2/3 pyramidal cells transduced with C1V1T-p2A-YFP (imaged under loose patch). Lower left, trace showing precise spike train control with 5 Hz 1040 nm raster-scanning illumination. Upper right, axial resolution of two-photon optogenetic control of spiking *in vivo*. Blue triangles indicate pyramidal neurons and red boxes illustrate ROI positioning. Spiking of layer 2/3 cells as a function of raster scan position is shown. Lower left, lateral resolution of two-photon optogenetic control of spiking *in vivo*.

correlation to the spatial place field they represent [141]. Such observations necessitate the formation of tools that enable researchers to take forward control of the activity of single cells in order that causal inferences can be made on the role of these neural circuit elements to behavior.

An array of techniques have been developed to activate specific cells in a spatially restricted manner but the use of caged-chemical compounds such as MNI-glutamate [142] or CDNI-GABA [143] in combination with two-photon laser scanning microscopy (TPLSM) has been the most popular. Nevertheless, these techniques have inherent limitations including the lack of spatial restriction for single cell activation due to diffusion of the un-caged compounds, temporal imprecision in controlling neuronal firing rates, certainly not suitable for *in vivo* applications, and the inability to be generalized for stimulation of different cell-types in the nervous system [144], [145].

Recent research endeavors were aimed at overcoming these limitations by combining optogenetics and two-photon excitation to manipulate activities in single or multiple genetically and spatially-targeted cells with high temporal resolution over

sustained intervals and within intact tissue volumes. The main challenge in such efforts has been the need for the simultaneous opening of multiple opsins located throughout the cell to overcome the modest conductance of individual light-gated channels (even the more light-sensitive opsin ChR2-H134R). Rickagauer and colleagues were able to overcome this obstacle and produce action potentials in cultured neurons by introducing a complex spiral scan pattern to open sufficient number of channels on individual neurons [146]. In two other studies two-photon optogenetic stimulation were performed in slice preparations but relied on development of innovative hardware and using larger focal spots of laser illumination [147], [148]. While the latter study yielded effective stimulation of single cells, the amount of laser power required to generate action potentials was quite high (>100mW). Consequently this technique is vulnerable to light scattering events which potentially turns to a problem in applications where deep tissue activation is necessary. A recent study has shown activation of neurons more than 200 micron below the surface using temporal focusing in combination with

generalized-phase contrast [149]. While promising, the high optical power per cell requirement still remains as the main limitation specifically in applications where the activities of multiple cells are controlled simultaneously.

The advent of new opsins, particularly the chimeric red-shifted opsin C1V1 family, has allowed alternative methods to be investigated. Prakash and colleagues took advantage of the increased off-kinetics and the high maximal photocurrents of C1V1 (compared to ChR2-H134R) to generate robust sequence of action potentials with TPLSM in cultured neurons, brain slice, and *in-vivo* preparations, Figure 9. The total power required to stimulate an individual cell in this technique is about 20mW where the single action potentials were produced 10-15ms after the initiation of a scan [150]. Moreover, they observed that sub-cellular compartments of a neuron in slice preparation could be activated using the same raster scanning paradigm over the body of a single dendritic spine. Using the same strategy, Packer and colleagues demonstrated that using a spatial light modulator (SLM) in combination with TPLSM C1V1 activation allowed multiple single cells to be activated simultaneously [151]. Two-photon optogenetic inhibition protocols were also developed for the first time by using inhibitory opsins with increased photocurrent and longer off-kinetics [150].

In order to effectively dissect the circuit elements that are significant for neural computation and behavior, it will be important to identify activity patterns within intact tissue. Readouts for neural activity have been developed including activity dependent calcium and voltage indicators. To extend upon the power of two-photon optogenetic applications, a non-invasive all optical method to monitor induced or intrinsic neural activity in parallel is necessary. Two important challenges for these experiments are: First, wavelengths should be chosen precisely to avoid activating optogenetic tools while reading the signal of activity-dependent probes (e.g., voltage sensitive dyes or calcium indicators) and vice versa. This challenge can be addressed via development of spectral variants of optical readout probes and two-photon opsins. Secondly, spatiotemporal patterning of readout and manipulation must be done simultaneously in the same intact volumes with arbitrary patterns and at high speeds.

Arbitrary readout and manipulation requires multiple lasers to be coupled to patterning modalities such as SLMs while maintaining the galvanometer based scanning systems for activating, inhibiting and imaging neural populations. In terms of speed, multiple strategies can be taken to improve the average time to spike seen with two-photon optogenetic activation of neurons. These include improvement in the scan trajectories and developing more sensitive two-photon opsins. As noted in [150], the typical raster scanning paradigm has an extensive "fly-back" time where the galvanometers are repositioning to the first pixel of the next line (~ 4 ms on and ~ 10 ms off per scan). By decreasing the unnecessary fly-back time, the average time to spike can be decreased significantly. Moreover, by engineering more sensitive two-photon opsins, scan times, and thus time to spike initiation, can be decreased.

The non-invasiveness of two-photon optogenetic activation of neurons allows for the development of algorithms for high-

throughput mapping techniques in *ex-vivo* and *in-vivo* preparations. It is known that the local connectivity maps of a neuron depends not only on the type of cell, but also on where it projects [152], [153]. There is evidence that local connectivity between neurons in different regions of the brain are drastically changed in some neurological diseases including autism [154]. Therefore, the high throughput mapping of neural connectivity via two-photon optogenetics in intact tissue potentially opens a new horizon in the study of such diseases.

Two-photon optogenetic stimulation combined with other technologies will help to causally understand the fundamental underpinnings of neural computation and behavior. By recreating neural activity patterns and using both cellular activity and behavioral readouts, the sufficiency can be shown. Meanwhile, by using the same readouts, and inhibiting the same patterns of activity, the necessity can be shown.

Understanding details of the brain's complex patterns of activities that occur within milliseconds and across centimeters of spatial distribution is a formidable task. The traditional mechanisms for optogenetic stimulation, for example by implanting optical fibers within the brain tissue, do not have the capability to elucidate how these micro circuits with a diversity of elements and intricate topologies lead to a myriad of behaviors since several hundreds to thousands of neurons are controlled in a synchronous manner with each light pulse. To causally dissect the spatiotemporal patterns of activity necessary to study the behavior, each circuit element must be controlled independently on the same time and length scales as the brain functions. With the advent of simple and scalable methods for two-photon optogenetic control over individual neurons, we are approaching such goals as we are now able to modulate multiple neurons at the resolution of single cells with millisecond temporal precision.

VII. OPTOGENETIC MICRO-ECOG; EXAMPLE OF A HYBRID BRAIN INTERFACE PLATFORM

A versatile brain interface should be able to control and monitor brain activity across the large-scale networks of the brain. Neural interfaces incorporating both electrode arrays and optogenetic stimulation that reside on the surface of the brain have the potential to fill a niche that exists between intracortical (e.g., optrodes) and minimally invasive approaches (e.g., ofMRI). Optogenetics and electrode array recordings offer millisecond temporal resolution and the ability to spatially modulate and map neural sources.

Over the last few decades, several enabling technologies have been developed to manipulate and record the brain's spontaneous and stimulus evoked activities, and each technique has both strengths and limitations. For instance, fMRI has become a method of choice for *in-vivo* neurophysiological analysis of brain function and has been translated into a powerful clinical tool for diagnosis and treatment planning for patients with brain tumors and other focal pathologies. However, the ambiguity of interpreting recorded BOLD signals plus its low temporal resolution are the main limitations of fMRI. For stimulation, transcranial magnetic stimulation (TMS) is noninvasive but offers very low spatial resolution

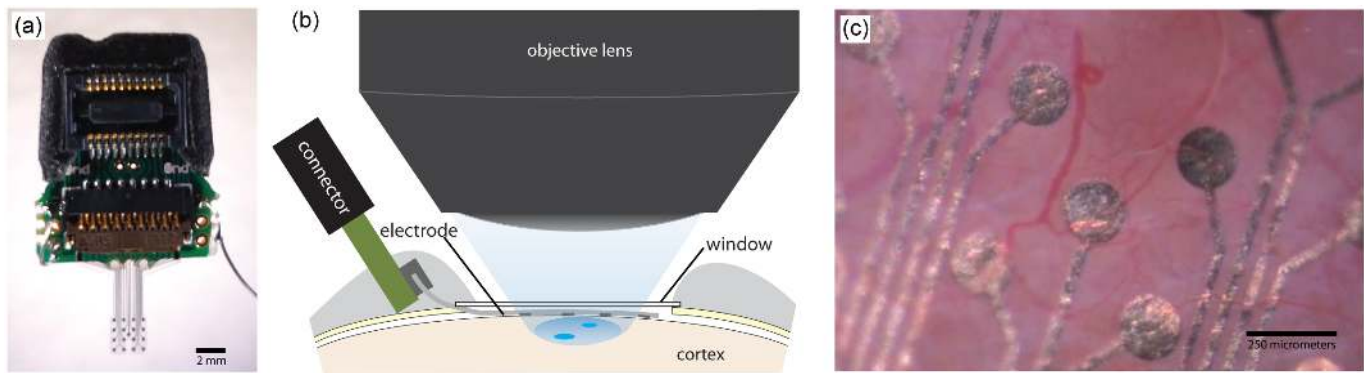


Fig. 10. Microfabricated electricortigraphy array fabricated on a transparent substrate and implanted under a cranial window for simultaneous electrophysiological recordings and optogenetic stimulation. (a) A mouse sized array with connector. A grid of 16 platinum sites (150 micron diameter) were fabricated with 500 micron site-to-site spacing and a nominal 1kHz impedance of 50kOhms. The arrays are implanted onto the dura, under a glass cranial window. (b) A cross-sectional illustration of an implanted array with a photostimulus pattern created with a spatial light modulator is depicted. (c) A brightfield image through a window shows the array resting atop the cortex in a chronically implanted mouse.

and electrical stimulation systems provide no strategy to target specific cell types or to selectively inhibit and excite cell activity. Given the spatial, temporal and cell type advantages afforded, hybrid interfaces incorporating optogenetics should be explored. An example of such a system is the recently introduced optogenetic micro-electrocortigraphy (micro-ECoG) platform.

ECoG arrays are implanted on the surface of the cortex without penetrating into the brain. Compared to electroencephalography (EEG), they offer better spatial resolution and signal to noise ratio [155]. ECoG was first implemented with a set of discrete electrodes to map cortical function and to localize aberrant activity in patients with epilepsy [156]. Over the next half century, large electrode arrays became common clinical technology. In parallel, during the most recent decade, ECoG based brain-computer interfaces (BCIs) were implemented [159], [160], [161], and photolithography was leveraged to create high-density microfabricated ECoG (micro-ECoG) arrays [91], [89], [158]. Micro-ECoG arrays are generally used for electrophysiological recordings, but towards implementing bidirectional neural interfaces, the arrays could be used for electrical microstimulation as well. However, suppressing electrical stimulation artifacts in recorded signals remains significant challenge [162], [163]. Considering all benefits of optogenetics as summarized earlier, optical stimulation is potentially an ideal technique to be combined with ECoG recording to create a new generation of optoelectronic BCI instrumentation.

To create a bidirectional chronic neural interface, micro-ECoG arrays were fabricated on a transparent biocompatible polymer substrate and implanted over the dura, but under a cranial window, in Thy1::ChR2-H134R mice [164], Figure 10. Next, a spatial light modulator and fluorescence imaging system were employed to precisely pattern light onto the cortex through the window and electrode array to stimulate or inhibit neural activities. The micro-ECoG arrays were fabricated by patterning platinum electrode sites on a transparent insulative substrate polymer, Parylene C [164] which remains transparent in the visible and infrared range of spectrum (400nm-1000nm) [165] in contrast to some other commonly used materials in

flexible electrode array fabrication such as polyimide. Parylene C, because of its biocompatibility [166], [167], is a well-known and widely used polymer in neuroengineering particularly in fabrication of flexible implants [168], [169], [170]. The platinum electrode sites and connecting traces occluded a small fraction of the device's area, so optical access to the cortex was maintained through the array [164].

Arrays composed entirely of transparent conductive polymers are potentially the next step in optogenetic micro-ECoG technology. *In-vivo* imaging and photostimulus patterning directly under electrode sites would be possible in completely transparent arrays, and fully polymeric fabrication could increase the flexibility and biocompatibility of the devices while reducing production costs. Indium tin oxide (ITO), a transparent metal layer commonly used for LEDs and photovoltaic cells, were used with PEDOT, a mostly transparent conductive polymer, to make early transparent arrays [171]. However, ITO is brittle. Laminating with PEDOT could bridge potential cracks in the ITO, but forgoing metal all together may be just as good. Conductive polymers can be patterned in multiple ways including spin coating and lift-off, inkjet printing [172], and many can be electropolymerized from their base monomer. Electropolymerization is generally the most durable, but the process requires an existing electrode. Maintaining mechanical durability and electrophysiological recording quality similar to that obtained with noble metals is an ongoing challenge as a wider pallet of transparent materials are used to push a hybrid of optogenetic and electrode array interfaces forward.

A. Source Localization and Intracortical Stimulation

One major application of optogenetic micro-ECoG is neural source localization. Several algorithms exist to solve the inverse problem of localizing neural activity from electrode recordings, but testing and comparing the accuracy of these algorithms is challenging using the existing methods [173], [174], [175]. In most algorithms, the number of neural sources and their spatial extent is set and ideally such assumptions are testable. However, current electrical stimulation methods (e.g., microstimulation or TMS) cannot easily create multiple sources at known locations or replicate diffuse activity.

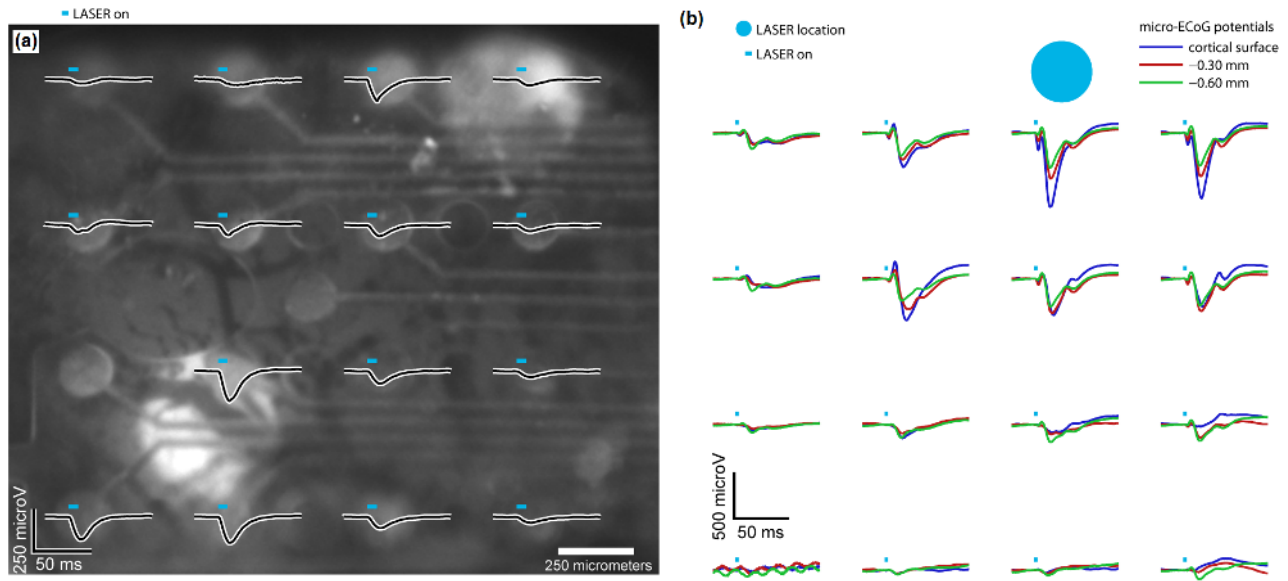


Fig. 11. Mapping of optogenetically induced cortical potentials. (a) Optogenetically evoked cortical potentials driven with a spatial light modulator (SLM) and recorded by a micro-electrocorticography (micro-ECoG) array (4x4 grid of platinum sites, 500 micron site-to-site spacing, 150 micron site diameter, 50 kOhms @ 1 kHz) implanted under a cranial window in a Thy1::ChR2-H134R mouse. Spatial and temporal patterns of light were defined with digital micromirror device (DMD) and projected onto the brain, through the cranial window and the array's transparent substrate. In this example, two areas were illuminated simultaneously (8ms, $4 \text{ mW}/\text{mm}^2$, 445nm, 60 trials averaged). Potentials nearest each photostimulus region had the greatest amplitude. Potentials were recorded at 3 kHz using a high-impedance amplifier (Tucker-Davis Technologies). The photostimulus was imaged through 400-500nm emission filter with an EMCCD camera, (b) Potentials recorded on the cortical surface with micro-ECoG in response to photostimulation at three depths within the cortex with a fiber-coupled laser. A micro-ECoG array was implanted epidurally in a Thy1::ChR2-H134R mouse. A laser-coupled fiber (200 micron, 0.2 NA, 2.4mW, 473nm) was first used to photostimulate at the cortical surface and then stereotactically inserted into the cortex 0.30 and then 0.60mm deep (50 trials at each location).

Optogenetics, together with micro-ECoG and spatial light modulation, creates a platform to empirically test source localization methods over a range of spatially and temporally defined neural sources in experimental models. Using this platform, it is possible to evoke and record localized neural activity by projecting patterns of light onto the cortical surface while simultaneously mapping cortical potentials with a micro-ECoG array, Figure 11(a). With the added precision of a DMD projection system, one can aim around the electrode sites to avoid any potential photoelectric artifact. It is also possible to combine the optogenetic micro-ECoG technology with spatial light modulators to produce a sequence of predefined illumination patterns where the location, area, and distance separating sources are systematically varied to generate a large dataset of micro-ECoG recording and neural activity caused at known locations via optogenetic stimulation. This database could be used to analyze complex signals and identify the corresponding sources that are producing the recorded local field potentials.

Causing and recording neural activity at defined depths below the cortical surface would further improve understanding of the ECoG signal and source localization methods. The DMD projection system enabled precise photostimulation at the cortical system. Holography [71], 2-photon methods [150], and waveguides provide potential methods to photostimulate within the cortex, under an implanted array. Figure 11(b) shows cortical potentials evoked by photostimulating at three depths within the cortex with a stereotactically positioned fiber. The relative amplitude of potential peaks following the stim-

ulus varied with fiber depth, but the largest potentials were still colocalized with the fiber location. Similar experiments with other opsins expressed under different promoters could fill out a cell type-specific study of ECoG.

B. In-Vivo Imaging with Optogenetic Micro-ECoG

Several *in-vivo* optical microscopy methods could be applied through the cranial window and transparent electrode to image neurovascular, metabolic, and neural activity signals. Neurovascular coupling, the causal relationship between neural activity and blood flow, is of great interest given the importance of fMRI and the prevalence of neurovascular diseases. Considerable progress has been made using sensory stimulation [177], and more spatially defined cell type-specific studies could be achieved with direct activation of neurons through optogenetics. Brightfield images of a branch of the middle cerebral artery (MCA) showed a large increase in diameter following photostimulation, Figure 12. Simultaneous potential recordings would help quantify the amplitude of optogenetically evoked activity and complete the neurovascular picture. Further, intrinsic metabolic markers such as reduced nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FAD) could be imaged following optogenetic stimulation, similar to studies using electrical microstimulation and caged glutamate [178]. Miniaturized microscopes [179], optical coherence tomography, and two-photon microscopy are among the methods that could be used in conjunction with optogenetics and microarrays to elucidate the neurovascular system.

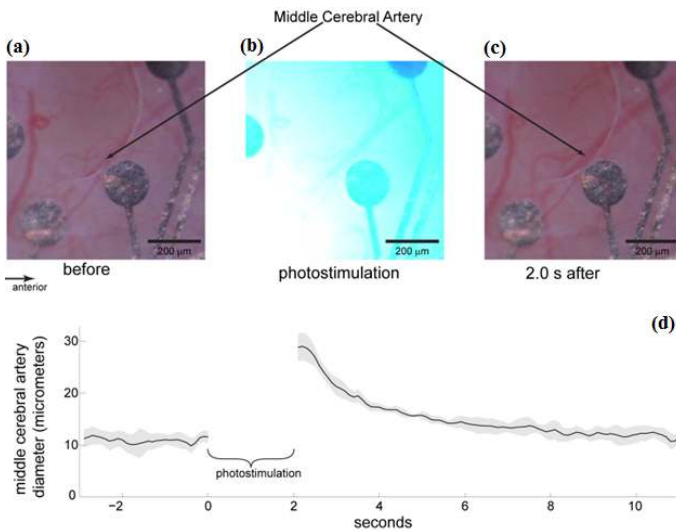


Fig. 12. Diameter of the MCA increased by 20um following optogenetic stimulation. Images were taken in sequence (a) before, (b) during, and (c) after application of a train of blue pulses (133pulses/2s, 5ms, 9mW/mm²).

VIII. OPTOGENETIC APPLICATIONS TO NEUROLOGICAL AND PSYCHIATRIC DISEASE

A. Overview

In the preceding pages, the protein engineering and technical advancements that have made optogenetics such a promising tool have been developed. Ultimately, our goal is to utilize opto-technology to map the nervous system and relieve human suffering. To that end, optogenetics heralds a new era of diagnostic and therapeutic approaches to neurological and psychiatric disorders. Its power to unwrap disease states is multi-fold, and begins first by the ability to selectively perturb and interrogate brain nuclei and circuits in order to determine elements critical to normal function. The second is to determine points at which intervention is possible. Finally, new therapeutic avenues are unlocked through the unique power of optogenetics as a tool to selectively control cell types and circuits. A comprehensive summary of the current state of the field is beyond the scope of this review, and the reader is directed to other reviews on this topic [8], [9], [63], [180], [181], [182]. We focus here on the approach to considering diseases of the nervous system as dysfunction of neural circuits, and then novel ways to combine optogenetics with temporal circuit dynamics to achieve improved knowledge of pathophysiological mechanisms and opportunities for therapeutic interventions. Finally, we conclude by imagining the (near) future in which optogenetic techniques are combined with other powerful tools for disease analysis and therapeutics, to realize its full potential within this realm.

Neurological and psychiatric diseases of the brain were traditionally understood as loss or perturbation of function based on changes in cell mass (neurodegenerative diseases), cell function (loss of memory), neurotransmitter systems (Parkinson's), or disrupted connections (addiction). Unfortunately, most disorders result from a combination of such derangements, and conventional therapies are, by definition, either too limited in their approach or too broad in their side-effect

profile. Furthermore, the finer structure of the cortical and subcortical circuits that serve these input-output loops are incompletely understood, defined by genetically and chemically heterogeneous populations of neurons and their projections. Finally, different disease states may share similar pathological features, such as altered beta or gamma oscillations, but produce distinct phenotypes, such as schizophrenia, autism and Parkinson's disease.

To understand the normal and disrupted function of circuits, optogenetics has been used to great effect to dissect them at different levels in the brain. At the level of the synapse, the functional communication point between neurons, channelrhodopsins have been used to explore both synaptic potentiation and depression [184], [185], [186], providing a deeper understanding of circuit development and pathologies. By stimulating both cell bodies and axonal projections, optical circuit mapping has been initiated to understand local connectivity amongst various neuronal cell types [187], [188] and long-range connectivity between brain regions [187], [189]. Activation and inhibition of these connections locally and at a distance may alter the balance of neuromodulation. For instance, an imbalance in dopamine activity within the indirect and direct pathways of the basal ganglia is thought to underlie Parkinson's disease. By targeting expression of ChR2 to two different classes of dopaminergic neurons (D1, D2), Kravitz and colleagues [190] were able to demonstrate prokinetic and antikinetic pathways in the basal ganglia, by direct photostimulation of each dopamine subtype in normal animals. In an animal model of Parkinson's (6-OHDA) they were then able to rescue the disease phenotype by selectively photostimulating D1-ChR2-expressing medium spiny neurons, providing a potential therapeutic intervention point for deep brain stimulation (DBS). The mix and distribution of local neuronal connections also affects regional inhibition versus excitation. Using targeted expression of the super step-function opsin (SSFO) in both excitatory and inhibitory cells of the medial prefrontal cortex (mPFC), a region that exerts significant top-down control over decision-making, impulse control, and many social behaviors, the balance of excitation and inhibition in this region of the brain could be selectively altered [9]. Mice were tested on a variety of social interaction tests and in a fear-conditioning paradigm. Increasing the balance of excitation over inhibition caused disturbed social interactions and an inability to form appropriate memories for conditioned responses, whereas increasing inhibition did not. Furthermore, these behavioral pathologies were associated with an increase in gamma oscillations in the local field potentials of mPFC neurons. These results are striking because a novel optogenetic tool, SSFO, was utilized to define a mechanism that might link disparate observations of changes in cortical excitatory/inhibitory balance, relative loss or hypofunction of mPFC inhibitory interneurons, altered gamma oscillations, and social and cognitive dysfunction in diseases such as autism and schizophrenia. Two complementary optogenetic studies have provided a more precise link between the loss of inhibitory cortical neurons and the aberrant generation of gamma oscillations in these diseases, potentially linking these to the alterations in observed cognitive flexibility. In an *in-vivo* study,

Cardin and colleagues [191] genetically targeted ChR2 to either inhibitory interneurons or excitatory pyramidal cells of the somatosensory barrel cortex. These populations were then selectively photostimulated over a range of frequencies. Only stimulation of inhibitory (but not excitatory) neurons at gamma frequencies generated local field potentials in the gamma frequency range. Further, they demonstrated that inhibitory interneuron-induced gamma rhythms temporally sharpened the transmission of sensory information from the periphery. In an *in-vitro* study utilizing bidirectional optogenetic control in a brain slice preparation from PFC, suppression of interneurons was found to dampen local gamma band power while feedback inhibition of interneurons on downstream excitatory cells generated emergent gamma oscillations, demonstrating a necessary and sufficient role for these inhibitory cells in the generation of cortical gamma rhythms [16]. Evoked gamma oscillations also increased the gain of the neuronal response to realistic spike trains by increasing mutual information and decreasing noise entropy. Both studies have provided the first support for a causal role for interneurons in the generation of gamma, and their role in sharpening cortical sensory transmission and enhancing mutual information in connected cells. These results imply that loss or aberrant expression of inhibitory cortical cells, often observed in psychiatric disease, may lead to the cognitive derangements seen in patients with schizophrenia and autism.

Optogenetics combining circuit manipulations and behavioral phenotypes has also been used to great success to determine projection-specific targets within normal function and disease. One very successful example is in defining the cortico-brainstem circuitry underlying depressive phenotypes. Most interventions (pharmacological, electroconvulsive therapy, light therapy, transcranial magnetic stimulation) have had incomplete success and multiple side-effects, partly due to the lack of specificity of these treatments and an incomplete understanding of the appropriate circuit sites to intervene. Pharmacological approaches have been particularly non-specific, often globally antagonizing serotonergic signaling systems in the brain, producing unwanted effects. Just as with some of the disorders of social dysfunction described earlier, the medial pre-frontal cortex (mPFC) sends multiple projections to subcortical nuclei implicated in depression. In an elegant demonstration of the power of optogenetics to query projection specificity, Warden and colleagues [192] optically defined two heterogeneous circuits that alternately lead to impulse control disorders and amotivational states, initiated in mPFC. Typically, depressed patients express a degree of hopelessness when challenged with difficult situations. Using a test of learned helplessness in rats challenged with a water environment, selective photostimulation of one set of mPFC projections, terminating in the brainstem dorsal raphe nucleus (DRN), reversibly rescued the depressive phenotype. However, photostimulation of an alternate projection from mPFC to the lateral habenula increased immobility, mimicking more strongly the depressive phenotype. Surprisingly, global stimulation of mPFC primary cortical projection neurons had no effect on behavior. These studies provided a means to dissect depressive circuitry, and identify a point of intervention for

future applications of deep brain stimulation (DBS), taking advantage of this cell-type and projection specificity to limit some of the side effects associated with non-selective electrical stimulation. A similar optogenetic strategy was used to define opposing roles for distinct subregions of the bed nucleus of the stria terminalis (BNST) in anxiety behaviors, both groups of neurons having multiple, distinct projections which independently control individual features of the anxiogenic and anxiolytic response [193].

Optogenetics has been used in this projection specific manner to disentangle the role of brain regions that participate in varied activities. One example is the amygdala, a heterogeneously dense collection of cell types that participates in diverse up- and downstream connections. The amygdala is a deep brain structure implicated in processing and forming memories for emotionally salient or fear-laden experiences. In one set of experiments, by combining projection specificity of opsin expression with directionally-restricted illumination techniques, a causal role for the basolateral to central amygdalar circuit in promoting anxiety was defined [183]. Alternately, a role for the basolateral amygdalar projection to the nucleus accumbens, another deep brain nucleus, was demonstrated for reward seeking and self-stimulation [182]. Though both behaviors can be linked to the development of substance abuse, they are quite distinct, and originate from within the same nucleus, differentiated by their discrete projection patterns.

Combining optogenetics with behavioral read-out methods can also be used to determine up- and downstream effectors of nuclei within a circuit, and the relationship between patterns of activity within these nuclei and behavioral phenotype. One of the first such demonstrations was in the arousal circuit, a series of interconnected subcortical nuclei. Understanding the arousal system is important because disorders of sleep (e.g. narcolepsy) and consciousness (e.g. delayed emergence from anesthesia, coma) arise from derangements in these pathways. Within this pathway, a group of neurons of the lateral hypothalamus (LH) releases peptides known as hypocretins/orexins [194], [195]. Gene deletions in the hypocretins lead to disorders such as narcolepsy [196], and activity and downstream signaling in hypocretin neurons had been correlated but not causally linked to wakefulness. Adamantidis and colleagues [197] achieved this by leveraging a lentivirus strategy to express ChR2 in LH. Direct stimulation of hypocretin-producing neurons within this nucleus increased the probability of sleep to wake transitions, and shortened the latency to wakefulness in a rate-dependent fashion. Until this experiment was performed, it was not known that release of these neuropeptides was sufficient to promote the transition to wakefulness. Stimulation rates of <5Hz produced no effect while those between 5-20Hz shifted latency, demonstrating that different patterns of activity within the same circuit could have completely different behavioral effects. A similar causal role for norepinephrine in arousal was demonstrated by using a Cre-driver line to express ChR2 in the locus coeruleus (LC) [198]. Precise optical activation of LC neurons caused sleep to wake transitions with a shorter time constant than for hypocretin neurons. Using combined photo-modulation of

LH and LC in sequence, they then showed that LC is both a necessary and sufficient downstream effector of the wakeful-promoting effects of the hypocretins. These studies should enable new circuit-specific and rate-dependent targets for the treatment of narcolepsy, other disorders of sleep and arousal, and delayed emergence from general anesthesia.

B. The optogenetic revolution in disease

The range of circuit-specific derangements in neurological disease is vast and complex, and optogenetic tools have provided new methods to break open these circuit codes. However, the reach of optogenetics in diagnostics and therapeutics requires more imagination. Utilizing animal models of disease, optogenetic techniques can be applied to treating these circuits. One such mechanism is by applying modulated patterns of activity. For instance, the selective photoinhibition of cortical excitatory cells [199] or ventrobasilar thalamic cells [200] by halorhodopsin can abort seizure-like bursting activity *in-vivo* and *in-vitro* models of epilepsy. Studies using optogenetics have also been used to define specific nuclei, projections, and rate codes for the therapeutic effect of DBS in Parkinson's disease [15], [190] and depression [201], [203], minimizing side-effects or the cancelation of opposing effects that can be caused by electrically stimulating brain regions with a mixture of diverse cell types. More generally, there is great hope that optogenetics will provide the ability to treat brain diseases that in the past, were ameliorated by surgical removal or lesioning, through a more precise functional resection. Optomodulation of these circuits is certainly a less invasive means of achieving therapeutic effects. Pattern-specific optomodulation could also be used to correct aberrant oscillatory patterns that are the basis of so many diseases [16], [191].

Another possible role for optogenetic therapeutic interventions is to restore or probe newly functional connections. Multiple techniques might exist to achieve this end. As the adage "neurons that fire together, wire together" (the Hebbian rule of plasticity [202]) states, driving pre-synaptic terminals, either to restore transmitter release, or in a temporally precise fashion to strengthen synaptic connections, could re-establish synapses that have been lost (e.g. in neurodegenerative diseases), or engineer synapses that do not currently exist (e.g. to enhance memory or other function). The restoration of functional connections might also be achieved by combining light-sensitive technologies with brain-machine interfaces, to create closed loop systems in which either optical or electrical sensors are used to read circuit activity and then respond with either optical or electrical actuators [199], [200]. Finally, in therapeutic models in which stem cells have been grafted into a host system, optogenetics can be used to assess synaptic to circuit level wiring. Tonnesen and colleagues [204] describe utilizing optogenetic techniques to test synaptic connectivity following dopamine stem-cell replacement in an animal model of Parkinson's disease.

Optogenetics might not only be used to test therapies, but to generate reversible animal models for disease [205]. Such models might be created by introducing opsins (through targeted viral delivery or the generation of transgenic lines)

into a number of potential locations within a circuit, and then activating or inhibiting that circuit with precise temporal dynamics. In this paradigm, dysfunction is induced with light, while multiple pharmacological, electrical or surgical interventions to ameliorate symptoms might then be tried with high throughput in order to determine the most effective therapy.

The optogenetic toolkit has now expanded from the traditional light-gated ion channels, which activate or silence neuronal activity, to opsins that activate intracellular events. This is important as many of the targets of disease are intracellular, and transcriptional and epigenetic events govern the expression of many neuropsychiatric disorders. A new array of photoswitchable molecules that activate G protein-coupled signaling, nucleotidyl cyclases, glutamate receptor signaling, and intracellular trafficking have created the opportunity to use the precise delivery of light to control downstream events [207], [208], [209], [210], [211], [50]. In some cases, light-gated enzymes and transcription factors can be combined to achieve selective gene expression or control inputs and behavior in intact animal [51], [212], [213], [214], [215], [216], [217], [218], [219], [220]. Ultimately, such manipulations affect can affect transcription and epigenetic expression, leading to permanent cell, circuit, and heritable behavioral changes. Immediate early genes such as *c-fos*, *Arc*, *Npas4* and *Zif268* [197], [221], and their upstream transcriptional regulatory elements [222], have been used in combination with photostimulation to identify cells that have been differentially activated or silenced during optomodulation. Together, optical stimulation combined with mapping gene expression changes in up- or downstream elements opens new doors to linking behavior to genetic changes. Novel approaches in which high-throughput techniques to characterize the mRNA transcribed in response to optogenetically-controlled behaviors or circuit activation ("integrated optogenetic-transcriptomics approach") have been described in detail elsewhere [182].

C. Final challenges - human therapeutic applications

While optogenetics has shown great promise in circuit-breaking neuropsychiatric diseases and finding points of intervention within animal models, human therapeutics has proven more elusive. For one, there is considerable light-scattering in brain tissue, with blue wavelengths (473 nm) used to activate ChR2 diminishing to $\sim 1\%$ of their initial power 1mm away from the illumination source [9]. As brain volume scales from rodent to non-human primate to human by a factor of at least $\times 10^3$, the potential stimulated volume in the human brain is cut drastically [223]. Indeed, although there has been some success achieving opsin expression and activation in primate cortex, optogenetically-controlled behaviors are more difficult to achieve than they have been in rodents [34], [61]. This may be related to sparse volumetric photo-excitation of primate cortex. Only a few primate studies, thus far, have achieved optogenetic control of behavior, and these have all been in the visual system [116], [224], [225]. In general, volumetric recruitment of excitable brain tissue has been achieved by using tricks such as pan-cellular promoters (CAG) for expression, task-based fMRI navigation to direct viral injections, and dual

electrodes to achieve greater volumetric excitation. Thus, more precise brain-behavior targeting strategies, more widespread infection, larger volumetric stimulation, or transduction with opsins with higher photon sensitivity or longer wavelength absorption for deeper light penetration will be required to achieve tissue activation in primates.

It may be that the typical optical stimulation will have to be complemented by even newer non-photostimulating techniques [226], or methods that utilize an optical window (e.g. transparent silicone artificial dura) to guide injections and track expression in such a way that the underlying cortex is not excessively damaged or deformed [228]. Such an optical window may also permit bulk volume illumination of superficial layers of cortex. The second is genetic specificity and targeting strategies unique to non-human primates and humans. Tropism, vector packaging, and cross-species variations in promoter specificity are limitations requiring new testing.

Further, the efficiency of gene expression in non-human primates and humans is currently a poorly defined variable. Early CNS gene therapy reveals successes with both lentiviral and AAV transduction, though AAV may be the more promising vector secondary to decreased immunogenicity [229]. Unfortunately, different serotypes of AAV do not uniformly transduce heterologous brain regions, and this suggests that expression efficiency will have to be tested on a serotype and region-by-region basis. The volumetric light sensitivity of a given volume of brain will be related to expression efficiency of the opsin, potentially in a non-linear fashion, but both acute and chronic overexpression might also be neurotoxic. In some of the first studies in which opsins were expressed in macaque cortex, injections of opsins packaged within AAV5 [61] or lentivirus [34] vectors under control of hSyn- and hThy-1 or α -CaMKII led to expression efficiencies of 60-80% within 1mm of the site of injection (viral titer of 10^9 - 10^{10} particles/ml) after 4-5 months. Longitudinal studies in non-human primates are crucial to determining the duration of stable opsin expression, the duration of maximal recruitment and light sensitivity, and the cross-over point into neurotoxicity.

In order to enable the evolution of delivery technologies, new optogenetic toolkits are being developed in non-human primates [34], [61], [227], [228]. In the case of viral gene delivery, human safety is also an issue. The AAV family is less pathogenic and immunogenic than lentiviruses, decreasing the probability of host cell mutagenesis after infection. Invasive techniques for modulation are also less desirable than non-invasive techniques for human photomodulation. Delivery of drugs and/or viruses across the blood-brain barrier (BBB) is typically difficult. One option for gene delivery might be direct injection into the brain parenchyma or ventricles/cisterna to circumvent such difficulties [230]. There is recent evidence that some serotypes of AAV may cross the BBB, and could therefore be delivered via intravenous injection [231], [232]. Newer, less invasive technologies to deliver small designer drugs or molecules have been proposed using conjugated nanoparticles or specific enzyme-substrate pairs, cleaved after crossing the BBB [233], [234], [235], [182]. In addition, toolboxes of cellular and regional mini-promoters in the brain are being defined [236] that can be utilized for targeting

applications. Ultimately, optogenetics may exploit genetics, not simply for targeting cell types for expression, but to create personalized toolkits to treat patients with a particular DNA makeup, utilizing opsin therapeutics aimed at their genotype, not phenotype.

IX. CONCLUSION

Optogenetics, as a new methodology for neurostimulation, has become a popular technique that is widely used in the study of the nervous systems in different animal models. The specific cell-type targeting capability of optogenetics has opened new horizons in dissecting the circuitry of neurological disorders and the parallelism of optics has made it possible to study the dynamics of large-scale neural networks particularly in the cortex. The advent of optogenetics has opened new lines of research including the development of new light-sensitive proteins, the realization of technologies for light delivery and detection in brain tissue, the study of diseases, and the combination of optogenetics with other established brain stimulation and recording systems such as electrode arrays and magnetic resonance imaging.

Optogenetics has also opened a new line of thinking among neuroscience and bioengineering communities. For instance, one question is whether it is possible to use other forms of energy, instead of light, to manipulate neural activities. Using acoustic waves instead of light can potentially help to non-invasively reach deeper inside the tissue with the cost of reducing the spatial resolution. The tools of optogenetics were originally adopted from nature. Further searching in the nature to find new forms of energy-gated ion channels is potentially a new research endeavor for next few years which can benefit from recent advances in biotechnology and bioinformatics. Extending the scope of optogenetics may be possible though the development of other light sensitive proteins (e.g., enzymes that are dysfunctional until exposed to light).

The success story of optogenetics particularly highlights the importance and value of basic-science research. Perhaps, the scientists who were studying the metabolism of algae and bacteria few decades ago could not predict that their discoveries will eventually help to develop one of the most powerful tools for the study of the brain at least in early the 21st century.

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