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Optogenetics, the intersection between physics and neuroscience: light stimulation of neurons in physiological conditions

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Deng W, Goldys EM, Farnham MM, Pilowsky PM. Optogenetics, the intersection between physics and neuroscience: light stimulation of neurons in physiological conditions. *Am J Physiol Regul Integr Comp Physiol* 307: R1292–R1302, 2014. First published October 1, 2014; doi:10.1152/ajpregu.00072.2014.—Neuronal stimulation by light is a novel approach in the emerging field of optogenetics, where genetic engineering is used to introduce light-activated channels. However, light is also capable of stimulating neurons even in the absence of genetic modifications through a range of physical and biological mechanisms. As a result, rigorous design of optogenetic experiments needs to take note of alternative and parallel effects of light illumination of neuronal tissues. Thus all matters relating to light penetration are critical to the development of studies using light-activated proteins. This paper discusses ways to quantify light, light penetration in tissue, as well as light stimulation of neurons in physiological conditions. We also describe the direct effect of light on neurons investigated at different sites.

optogenetics; light stimulation; neurons; physiological conditions

OPTOGENETICS is a technique that combines complex principles derived from physics with genetics, cell biology, and neuroscience (19). Its core idea of using light to control cellular activity emerged around the turn of century with key contributions of Miesenboech and Hengeman (3, 4, 6, 23, 40, 41, 78). Technically, the approach involves using genetically modified proteins such as channelrhodopins, halorhodopsins, and other to modify photosensitivity of cellular membranes. These proteins can be specifically addressed by using light of specific wavelengths (such as channelrhodopsin 2, which responds to 480 nm light). The field of optogenetics has been named and pioneered by K. Deisseroth and E. S. Boyden whose leading contributions in using light to control animal behavior are now widely recognized (6, 19, 77). In recognition of its outstanding scientific value, optogenetics has been named the method of the year by *Nature Methods* in 2010 (48a). Because photosensitive proteins are genetically targeted to specific neuron types, they can be used to investigate the function of specific brain regions.

The use of optogenetic techniques has rapidly gained prominence generating a steadily expanding research field (see Refs. 32 and 77 for review). The central idea is to isolate a gene that encodes a light-activated channel from a nonmammalian species and to introduce this foreign gene into a specific site within the nervous system. This causes new, light-sensitive ion channels (such as channelrhodopsins) to be expressed in the membrane of selected cells–typically neurons, although other cells may be affected (6, 36, 43–46). Subsequently, the channel can be activated by application of wavelengths of light that are specific for the receptor. In this way, two separate channels that are sensitive to different wavelengths of light can be investigated in the same experiment.

Typically, stimulation of neurons occurs when external stimuli activate ion channels causing depolarization of the neural membrane, leading to action potential generation, which then propagate along the axon of the neuron. This action potential can be generated chemically, optically, mechanically, or by electrical stimulation in various types of neurons (12, 30, 51, 54, 70, 72). Optogenetics allows light stimulation or inhibition of neurons that normally do not respond to light, and this optical control of neurons may allow us to determine their function.

The key advantage of using optical radiation to excite neurons compared with, for example, electrical stimulation, is its potentially high level of spatial selectivity, up to single micrometers, and the absence of direct contact between the external stimuli and the cells. Thus pulsed light, in particular laser light, can be used for stimulation of neural tissues without damage from optical stimulation. Optical stimulation can also be used in conjunction with other forms of stimulation, for example, electrical or chemical (5, 13, 49, 50, 68). It is important to note that the optically stimulated action potentials produce different responses compared with conventional electrical action.

Light on its own is not entirely innocuous, and it is therefore also essential to understand how different aspects of light, such as wavelength and light intensity (irradiance), may affect cellular function and the extent of light propagation in the tissue. This helps to understand what happens when light illuminates normal neurons, as this invariably occurs in all optogenetics experiments. Thus this review focuses on direct

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light stimulation of neurons in physiological conditions, without any genetic engineering, introduction of foreign gene products, or exogenous light-responsive (e.g., caged) molecular species.

Initial investigations on optical stimulation of such neurons started in 1891 (35). Subsequently, Arvanitaki et al. (2) demonstrated that nerve cells can be inhibited or excited by stimulation with different wavebands. Early work used short, comparatively intense pulses at visible wavelengths that were unsuitable for long-term excitation; because of this limitation these studies were not continued. Over the last few years, researchers have developed methods of using low levels of pulsed infrared (IR) light capable of long-term stimulation of neural potentials. This approach has potential clinical significance. Here, we discuss fundamental principles of such stimulation so that analogies can be drawn between the reasonably well-developed area of IR stimulation (reviewed, for example, in Ref. 57) and the less well-explored effects of visible light excitation. We expect that a clear picture of the general processes of action of optical sources on unmodified neurons and the understanding of light interaction with tissue will improve the design and interpretation of neuronal stimulation experiments.

Fundamental Physical Properties of Light

Light energy and wavelength. Light is an electromagnetic wave and, as all waves are, it is characterized by its wavelength (distance between the troughs or between the maxima). The oscillating quantity is the electric field and associated magnetic field. The electric field oscillates back and forth, and it toggles between pointing in two opposite directions (positive and negative). The average electric field in the wave is zero, but the average of its square is not. The oscillations are very fast, in the order of 10^{15} s^{-1} for visible light. Visible light has wavelengths between 400 nm (purple) and 700 nm (dark red). Wavelengths longer than about 700 nm are invisible and are called infrared. Near IR (NIR) wavelengths are approximately between 700 nm (0.7 μm) to 3,000 nm (3 μm).

Light carries energy, which is measured in energy units [Joules (J)]. Light is also characterized by power. Light power is the rate at which light energy is generated, transmitted, or absorbed. Power is the amount of energy per unit time and is measured in watts (W, 1 W = 1 J/s). These quantities are frequently used in photochemistry and photobiology. In addition, other quantities related to light such as radiant exposure, irradiance, and wavelength are used widely in the field of optical stimulation of neurons. Irradiance (E_e) is the power of light per unit area incident on a surface (at 90 degrees angle), expressed in watts per square meter (W/m^2). This quantity has intuitive meaning, and in common language it is referred to as "intensity" or "brightness," colloquial terms that should be avoided in the scientific literature because they can be misleading. Radiant exposure (H_e) is the accumulated amount of incident light energy per area [in Joules per square meter (J/m^2)], calculated as:

$$H_e = E_e \times t \quad (1)$$

where t is the exposure time of light sources (in seconds).

Key physiological effects such as damage threshold are often expressed in terms of radiant exposure but irradiance also plays a role. In physiological conditions the optimal exposure energy causing responses of neural cells needs to be well below the tissue damage threshold. Wavelength also plays an important role in light stimulation of neurons in physiological conditions. The tissue ablation threshold varies across the visible and IR spectrum. Therefore, a good indicator of optimal wavelength for laser stimulation is the ratio of the radiant exposure at the threshold of ablation versus a physiological indicator of nerve activity such as muscle contraction at a given wavelength. This ratio recognizes the wavelength range where there is an obvious gap between light energy needed to stimulate and damage the neural tissues.

Light in a homogeneous medium such as air, or pure water, propagates along a straight line, but in the tissue it scatters many times so it follows a complicated zig-zag path. We first discuss propagation of light in a homogeneous liquid medium. The most significant effect in this case is losing light energy to the molecules found on the light path. This absorbed light is mostly converted into thermal energy, but it may also be converted into fluorescence light and, as such, be emitted at a different wavelength, and it may also cause photobiochemical reactions. In the case of an absorbing medium, the intensity of light passing (transmitted) through that medium is related to its transmittance (T). Transmittance is calculated by taking the ratio of the irradiance of light leaving the medium (I) to the irradiance of light incident on the medium (I_0) (see Fig. 1). The absorbance (A) is calculated from the transmittance:

$$A = \log(1/T) \quad (2)$$

For media such as dilute solutions of absorbing molecules, the absorbance at a specific wavelength A_λ is directly proportional to the concentration of the solution. This relationship is known as Beer's Law:

$$A_\lambda = \alpha_\lambda dc \quad (3)$$

where α_λ is the molar absorption coefficient of the absorbing molecules at wavelength λ, d is the sample path length in centimeters, and c is the concentration of the absorbing molecule in the sample (molarity, in units mol/l). A Beer's Law plot is a calibration curve of absorbance plotted as a function of molecular concentration. The absorption coefficient is wavelength dependent, and it also depends on the type of absorbing molecule.

In addition, light is more or less strongly reflected by the tissue surface. In this case, for normal incidence, the incident

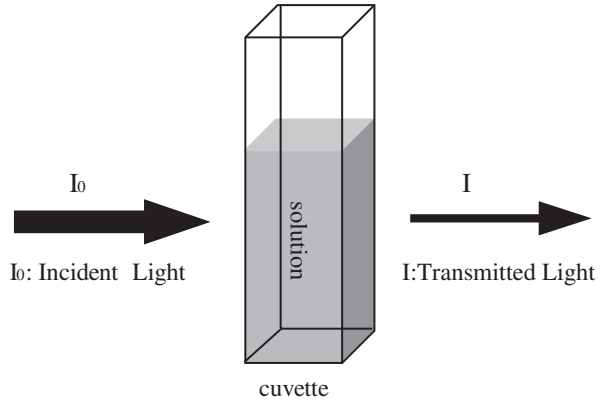

Fig. 1. Transmission of light in a liquid sample.

beam splits into the beam entering the medium and the reflected beam traveling backward. Note that only the beam entering the medium is used to calculate absorbance. The Beer's law in the presence of reflectance takes the form:

$$I_\lambda(d) = (1 - R)I_0 \exp(-\alpha_\lambda d) \quad (4)$$

Light scattering in tissues. Light also interacts with tissue in its path in another way known as scattering. In contrast to absorption, scattering light energy is not lost; however, the direction of individual light rays change. As a result the propagating light beam becomes weaker in the forward direction (as with absorption), and it spreads sideways (contrary to the case of absorption). This effect is usually most pronounced for structures whose size is comparable with the wavelength of light (300–700 nm for visible light). Biological tissue comprises many inhomogeneities, such as cell organelles, at this nanometer scale and hence it scatters light very strongly. As a result, light travels through tissue in a much more complicated way than through a homogeneous sample such as liquid (Fig. 2). To quantify light scattering we use a generalization of the absorption coefficient called the extinction coefficient ε_λ. The intensity of light passing through a scattering and reflecting sample is given by

$$I_\lambda(d) = (1 - R)I_0 \exp(-\varepsilon_\lambda d) \quad (5)$$

Here, the extinction coefficient is composed of the absorption and scattering coefficients,

$$\varepsilon_\lambda = \alpha_\lambda + \alpha_s \quad (6)$$

A useful guide as to how deeply light penetrates into tissue is given by its penetration depth *l*. This single figure describes the depth at which light decays to 1/*e* (about one-third) of its incident irradiance, given by the inverse of its extinction coefficient $l = 1/\varepsilon_\lambda$.

Tissues are characterized by a very wide variation of scattering coefficients, and their absorbance varies greatly as well, both as a function of location in the body and as a function of wavelength. As a result the penetration depth in various areas of the body varies commensurately. As a general rule, light at longer visible wavelength penetrates more deeply. This is due to comparatively lower hemoglobin absorption within this wavelength range. Table 2.1 in Ref 67 lists the values of absorption and scattering coefficients at a selection of wavelengths in a variety of tissues, including the brain (for more recent measurements in rats see Ref. 38). These data additionally show notable differences between measurements taken from different sites. As an example, the values of scattering coefficient in gray matter at 450–460 nm are given as 686 cm^{-1} and 117 cm^{-1}, respectively. This says that penetration depth is no more than ~1/686 cm to 1/117 cm into the tissue, so less than 100 μm. Thus the effective stimulation volume is low, which has an effect on spatial precision for optical action on neural cells and the capability to stimulate the axons within each nerve cluster. (71).

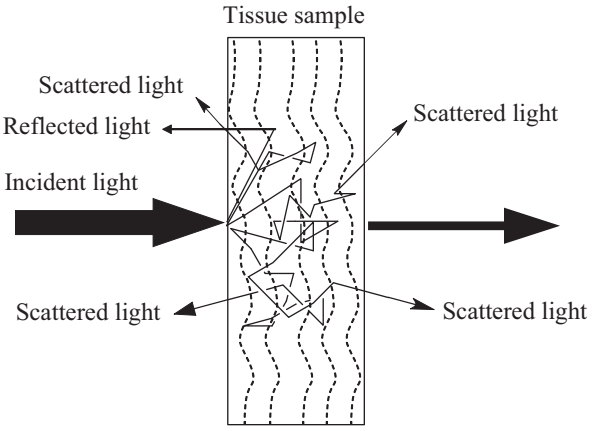

Fig. 2. Schematic illustration of light transmission, reflection, and scattering in a tissue sample.

Light Sources

The most popular light sources used for neuronal stimulation include light-emitting diodes (LEDs) and lasers, especially diode lasers and solid-state diode-pumped lasers. There are no reasons why other lasers or light sources of suitable wavelengths could not be used, but these have the best combination of low price, high enough power, and portability. LEDs are devices where light is produced from a small (<1 mm × 1 mm) chip of semiconductor material packaged in a plastic casing, frequently with a plastic transparent dome. The devices are very robust and can be powered from a battery or battery pack with a resistor in series; they do not require highly specialized power supplies. They produce light within a reasonably narrow wavelength range (about 50–100 nm spectral width). Diodes with a variety of center wavelengths are commercially available; these are made of different materials. LEDs emit light in a certain fairly wide cone, which is determined by their design. This wide spread of emitted light combined with the size of semiconductor chip makes LED light difficult to focus into an optical fiber, a preferred mode of light delivery into the body. Recently, high-power diodes have become available as well with powers reaching as high as 10 W. However, in most cases such high power has been achieved by producing arrays of individual semiconductor chips. These high-power arrays are even more difficult to focus into a fiber than individual LED chips and may offer no extra advantage. A major benefit of LEDs is their very low cost and wavelength flexibility.

Laser diodes and solid-state diode-pumped lasers produce light with a much better defined wavelength and higher overall power from a single device than LEDs. This light also is more coherent than in the case of LEDs. Coherent light means that individual light waves generated by the device exactly follow one another and their wave pattern is matched. As a result of coherence a laser spot is never uniform but shows a strongly spotty speckled pattern that is absent in the case of LEDs. Laser light is also more collimated than LED light, and this makes it easier to focus into an optical fiber. However, lasers have a more constrained set of wavelengths, with some wavelengths, especially yellow, being quite difficult to produce, which increases the cost. Also diode lasers and diode-pumped solid-state lasers require specialized power supplies. In terms of light modulation, both lasers and LEDs can be easily modulated across a wide range of frequencies. Only very high modulation frequencies beyond 10^9 Hz might require special devices.

In many cases, light is delivered to neurons via optical fibers. Multimode optical fibers, mostly made from glass, but also polymers are the work horses of optogenetics. Their diameters may range from 10 micrometers to millimeters. Optical fibers produce a cone of light of which its divergence angle depends on the numerical aperture (NA) and (average) refractive index (n) in the medium

$$\Theta = \sin^{-1}\left(\frac{\mathrm{NA}}{n}\right) \quad (7)$$

According to simple geometry, in free-space conditions, the illuminated radius $R(z)$ increases with distance z as

$$R(z) = z\tan\Theta + r_0 \quad (8)$$

where r_0 is the radius of the optical fiber delivering light, a parameter that is normally small in value and can usually be ignored. As light is strongly, and variably, scattered in the tissue, the above relationship (*Eq. 8*) is only an approximate guide. In such conditions the illuminated region has an approximately conical three-dimensional shape with varying light irradiance. Consequently, the illuminated tissue is not well localized. The irradiance is higher on the symmetry axis and it is the strongest immediately adjacent to the fiber face.

It is also possible to excite tissue with an optical microscope where much better localization and higher irradiances can be achieved, but this requires special animal preparations limiting the scope of investigations.

Direct Effects of Light on Neuronal Function

We now discuss the effects that take place when a neuron is exposed to light. To limit energy (heat) delivery to the tissue such experiments are typically done by using pulsed light. Heat generation is proportional to average absorbed energy, hence, the use of pulsed light at a low duty cycle makes it possible to limit tissue heating while achieving higher peak irradiance levels. In many cases the light source is a laser that provides light that is well collimated and approximately at a single wavelength. This pulsed optical radiation acts directly on neurons (or as close to the neurons as possible, in in vivo situations). Light is then absorbed in neurons or in the surrounding tissue owing to endogenous molecules. For IR excitation the absorption in the neurons is predominantly by water, which has a broad absorption band in the near IR (18). The process of light absorption proceeds according to Beer's law, and one should be aware that the extinction coefficient in the tissue at the illumination wavelength is higher than the absorption of water or solutions of other absorbing molecules. Depending on the geometry of the illuminated tissue surrounding the neuron, light may be significantly or almost completely attenuated before reaching the neuron. However, even an almost complete extinction of light in the tissue before reaching the neuron surface may still produce some effect on the action potential. This is because absorption of light in the tissue produces some expansion, and thus modulated light is able to produce the optoacoustic effect, essentially mechanical sound waves that exert force on neuron surface. The neurons then respond to the pressure of this force. This effect can transduce sound waves into excitation of auditory nerves.

Most neural stimulation work is carried out under conditions where light actually reaches the neuron surface. In this situation light can interact with the nerve cells via one or several of the four physical mechanisms: photochemical, photomechanical, and photothermal tissue effects, and the effect of electric field associated with the light wave (28).

Photochemical reactions of various types may be triggered by the absorption of light illumination; these may happen immediately during light irradiation, but in living cells secondary chemical processes may follow. Theoretically, photochemical phenomena can be practically ruled out because the IR photon energy (<1 eV) is insufficient and the irradiance is too low to induce multiphoton effects.

Photomechanical effects leading to optical stimulation were discussed above, including pressure wave generation created by rapid heating (71). The effects are most pronounced for rapid heating with short laser pulses (in the order of 1 ms), which may ablate tissue and generate pressure waves interacting with tissues and cells (15, 16).

Photothermal effects are due to heat produced when light is absorbed. The resulting temperature increase, if excessive, may lead to the damage of the target tissue: if in excess of 40–45°C for prolonged periods. These effects may be prevented by limiting the (average) power of the radiation. The temperature increase required for stimulation of the peripheral nerve is in the order of 6–10°C and it must be transient.

Effect of Temperature on Neuronal Function

Temperature increases in the nervous system may be due to a number of causes. Clinically, the most common of these is fever due to viral infection. In children, high temperatures may lead to such severe effects on the central nervous system as to cause convulsions. In adults severe temperature changes may occur through exposure to dramatic swings in ambient temperature that exceed the natural ability of the body to maintain normal function. In these situations, coma or seizure may result.

Application of light at any intensity will produce a temperature increase. There is a direct relationship between the intensity (irradiance) of the light applied and an increase in temperature. The extent of the increase in temperature is dependent on the average light intensity (irradiance) for continuous illumination, but also on peak intensity and on the duty cycle for pulsed illumination, on the wavelength of light, which affects the propagation of light in the tissue and, in the end, its ultimate spread.

To the best of our knowledge, the biophysical manner in which transient temperature rise causes activation of nerves is incompletely understood. Tentative explanations of photothermal effects include increased ion flux or creation of new membrane pores, consistent with reports that IR light applied to the neuron body modulates intracellular [Ca^{2+}]. However, current experimental evidence suggests that spatial and temporal temperature gradients directly or indirectly activate ion channels (71).

Action potentials are, in principle, temperature dependent due to several independent effects. First, temperature affects chemical equilibrium of all chemical reactions, including those that underpin the operation of ion channels. Second, the conductance of an open ion channel depends on temperature. Finally, temperature affects the amplitude and length of the potential change (69). However, on a more detailed level, the

physiological mechanisms are not fully known. It is speculated that sodium channels increase conductance with increasing temperature. Once the sodium current toward the cell is established, the membrane potential decreases, affecting the action potential. The current produced by the number of open channels over the irradiated area may be sufficient to induce the localized voltage gradient required for stimulation of sodium channels downstream, and this enables propagation of action potential. This hypothesis is supported by the fact that neural stimulation requires light to be pulsed.

A second potential hypothesis may be attributed to the stimulation of heat-sensitive channels. Their gating mechanism is different compared with the other channel types (11). These heat-sensitive channels are less ordered at increased temperature (10, 47, 65). The experiments with 1.850 μm wavelength stimulation of neurons known to contain specific temperature-sensitive channels reported in Ref. 56 support this (see Fig. 3). The authors used primary sensory neurons from the inferior ganglia of rat vagal nerves. These neurons express the vanilloid transient receptor ion channel (TRPV1), responding to temperature changes (8, 21, 22, 42). This TRPV1 ion channel activated was by 5 W and 2-ms pulses.

The mechanism in other types of excitable cells is likely to have a different origin. For example 1,862-nm pulsed excitation applied to neonatal cardiomyocytes affected mitochondrial Na^+/Ca^{2+} exchanger (mNCX) and mitochondrial Ca^{2+} uniporter (mCU) (see Fig. 4) (14). mCU was rapidly stimulated by light to cause Ca^{2+} influx, whereas mNCX has a slower time scale. In cardiomyocytes, IR-photoactivated fluxes modified cytosolic $[Ca^{2+}]$ (14, 66). The authors suggested that a mitochondrial chromophore plays an important role in optical absorption, resulting in rapid thermal transients localized to mitochondria.

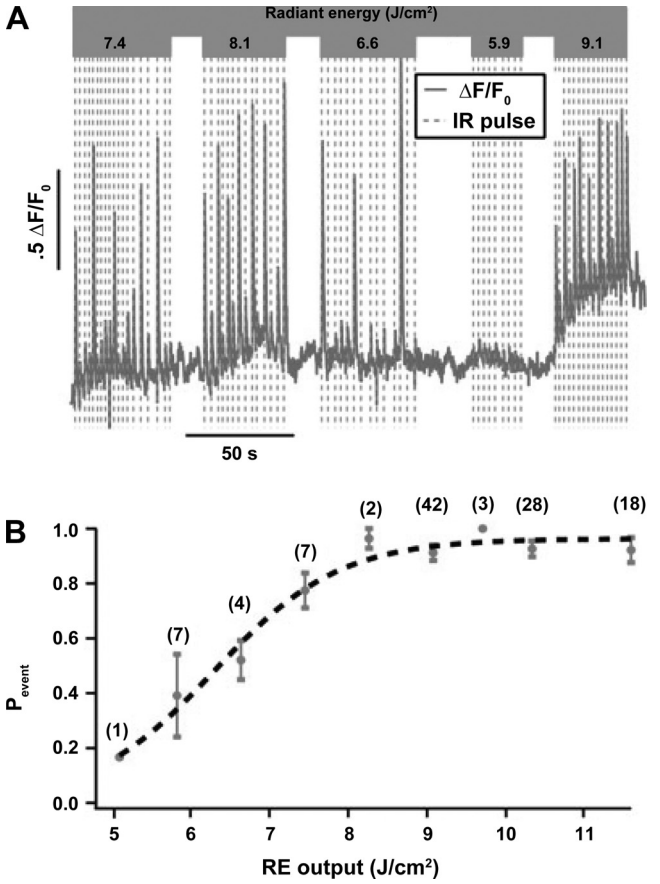

Fig. 4. Activation thresholds for IR-stimulated Ca^{2+} release. *A*: fluorescence intensity trace for a single neonatal cardiomyocyte exposed to varying radiant energy (RE) levels of infrared light. *B*: probability of $[Ca^{2+}]_i$ event (P_{event}) for each IR pulse as a function of pulse RE [From Dittami (14)].

Parameters

Effects of laser wavelength. Although our current understanding of the mechanism of neural stimulation by light is limited, several groups have attempted to establish the range of optical parameters that control the effect. One needs to be aware that parameters required for one application, such as motor nerve stimulation, are not necessarily suitable for other applications viz., stimulation of a sensory system, such as the cochlea.

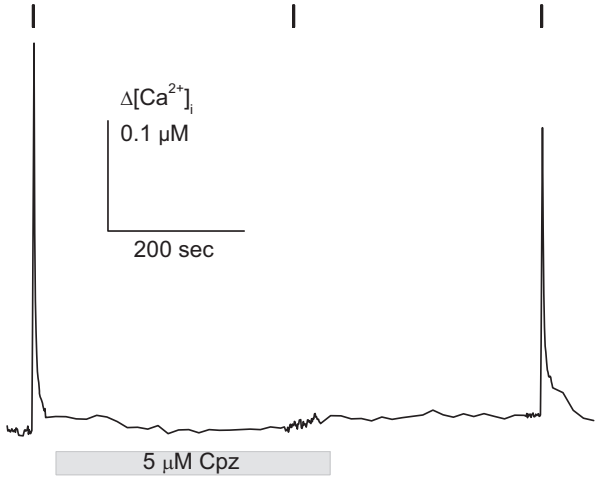

Fig. 3. The infrared (IR) photostimulation-evoked Ca^{2+} response is blocked by capsazepine, a specific antagonist of the vanilloid transient receptor ion channel (TRPV1) channel [From Rhee et al. (56)].

As indicated earlier, laser-tissue interaction is dependent on a thermal or thermomechanical process and the operational parameters of the laser, such as wavelength, pulse duration, and laser radiant exposure, are required to optimize in this process (64). One of the key parameters is the wavelength, as optical properties of the tissue vary greatly. The absorbed power and subsequent temperature rise are related to the extinction coefficient (absorption and scattering), and it significantly affects the results of stimulation. The laser wavelength determines the penetration depth of the light and dictates the spatial distribution of light absorbed in the tissue as well as volume of tissue that is stimulated at a given irradiance. As the absorption coefficient increases, lower irradiance is needed to stimulate the tissue because the absorbed power is higher; however, in these conditions it becomes easier to damage and ablate tissue. Conversely, wavelengths with lower absorption coefficients require more energy to activate neurons, but the potential for thermal damage is then reduced. Typically, excessive laser radiant exposure results in nonreversible thermal or mechanical alterations of the tissue, at lower values the alteration is reversible. To give the idea of length scales, the surface displacement near tissue damage threshold (at 0.4 J/cm^2) was measured to be 300 nm. To optimize the conditions,

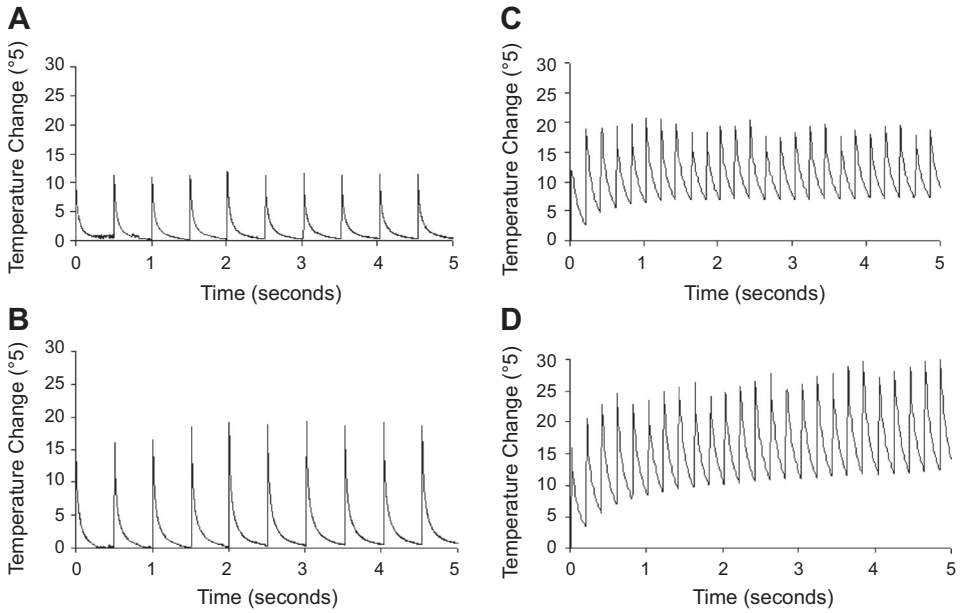

Fig. 5. Steady-state maximum temperature increase in nerve tissue from Ho:YAG laser stimulation. *A*: temperature rise from 0.45 J/cm^2 radiant exposure pulses at 2 Hz stimulation frequency. *B*: temperature rise from 0.65 J/cm^2 radiant exposures at 2 Hz stimulation frequency. *C*: temperature rise from 0.41 J/cm^2 threshold radiant exposures at 5 Hz stimulation frequency. *D*: temperature rise from 0.63 J/cm^2 threshold radiant exposures at 5 Hz stimulation frequency [From Wells et al. (71) with permission from Elsevier].

the efficiency of stimulation must be weighed against the risk of damage.

The wavelengths investigated so far for direct excitation of neurons in physiological conditions included 2.12 μm (from a Holmium: yttrium aluminium garnet laser), 2–6 μm (free electron laser FEL), 1.5 μm (telecommunication Yb-glass laser), 750 nm (alexandrite laser), and 1.87 μm (from a proprietary solid-state laser source) (71, 74). It is possible for optical wavelengths to stimulate neurons if the neural tissue absorbs these wavelengths moderately. The estimated depth of light travelling through the tissue for these wavelengths is 200 to 1,500 μm assuming primarily water absorption at the wavelength of 2.12 μm the depth is 330 μm, at 1.87 μm the depth is 450 μm.

Effects of laser pulse width. Furthermore, we discuss the pulse width of laser pulses. As indicated earlier, pulsed excitation is required to minimize irreversible thermal effects in the tissue and to ensure temporal temperature gradients activating the membrane receptors. Neural stimulation typically uses pulses varying from a fraction of a microsecond (μs) to hundreds of milliseconds (ms). For example, 5-μs to 5-ms pulses have been shown to have similar thresholds for nerve stimulation (70–73).

It needs to be noted that a temperature increase is somewhat delayed with respect to the light pulse. For example, in a typical experiment the maximum temperature rise was reported to occur 350 ms after onset of the laser pulse (71). This means that temperature superposition, or additive temperature effects from multiple pulses, may be observed at higher stimulation frequencies. For example in Ref. 71 the temperature increase and its return to baseline tissue temperature at 2 Hz was independent of laser radiant exposure. In addition, the temperature superposition was also not observed in peripheral nerve tissue at this exposure frequency. While at a frequency of 5 Hz, additive temperature effects were seen with the tissue temperature increase and this temperature did not return to baseline. This quickly led to a much larger maximum temperature in the tissue than seen stimulation at the frequency of 2 Hz (see Fig. 5). This consideration is significant for clinical applications as the peripheral nervous system requires higher repetition rates than the central (59, 61, 62).

Effects of laser repetition rate and laser spot size. The repetition rate is also a relevant parameter in neural stimulation and not just as a determinant of average energy. The reported results show that as the laser repetition rate increased the threshold radiant energy decreased (see Fig. 6) (9). Choosing the optimal spot size of the laser beam is also an important factor. The spot size is crucial to the number of activated neurons since only the neurons present within this spot can be directly stimulated by the IR light. When the spot size reduces, the number of the stimulated neurons would be decreased, resulting in reduced electrical signal generated by them. This makes it more difficult to detect the signal with the conventional electrode (9).

Many authors are trying to establish the relationship of stimulation parameters to light wavelength irradiance and dose in optogenetic experiments (1). The nature of the relationship depends on biological conditions (1, 76), which tend to be

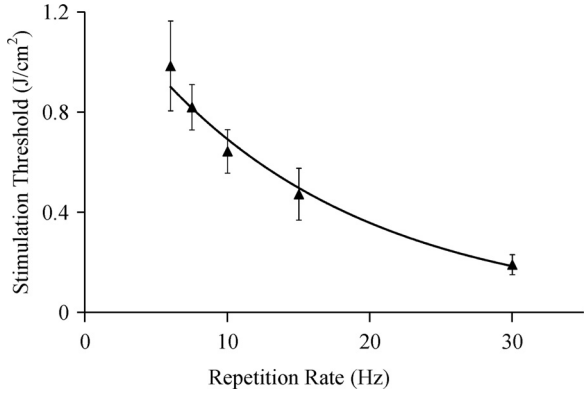

Fig. 6. Increasing pulse repetition rate reduces the stimulation threshold radiant exposure (R^2 = 0.98). Laser parameters: λ = 3.65 μm, 30 Hz, spot size = 320 ± 50 μm [From Cayce et al. (9)].

inadequately characterized especially in in vivo situations, including key characteristics such as the opsin density, which is achieved through genetic engineering. Current genetic engineering methods are not yet fully quantitative, and the research community is yet to achieve the mastery of expression level control. Moreover, the complex architecture of the neuronal populations may play a role because the activation threshold depends on the relative orientation of fiber and neuron (1). Because of these and other circumstances, the values quoted in the literature such as 10–15 mW at the output of the 100-μm optical fiber is required or those action potentials can be evoked at 1 mW/mm^2 (at 470 nm). These values can be regarded as a guide only (see Table 1 in Ref. 76).

Safe Limits

The IR stimulation threshold of unmodified neurons is 2.5 times lower than the tissue damage threshold (0.8–1.0 J/cm^2) (57). This leaves a broad window of light exposures from stimulation threshold to those causing damage in the tissue. It must be noted that radiant exposures in the range of 50 J/cm^2 will likely lead to some thermal damage, resulting in an increase in tissue absorption at this wavelength. Safe short-term laser nerve stimulation can be carried out at radiant exposure levels 0.8–1.0 J/cm^2 (17).

In addition to limitations to radiant exposure there are temperature limitations as well. The relationship between radiant exposure and temperature depends somewhat on the properties of tissue as well as the geometry, so the two limitations need to be treated independently. The literature suggests that thermal changes to mitochondria may begin to occur with temperatures as low as 43°C, whereas protein denaturation begins at tissue temperatures close to 56°C–57°C. The cochlear nerve stimulation experiments show that stimulation threshold radiant exposures are two orders of magnitude smaller than those reported here in the peripheral nerve (25).

Applications

Direct neural stimulation using light has so far been demonstrated in motor nerves, sensory nerves, the central nervous system, and in in vitro preparations. Much of the current work is motivated by the potential use of direct optical stimulation in neuroprostheses. The motivation is to overcome two main limitations of the current devices: detrimental effects of physical contact of the stimulating electrode such as tissue toxicity related to implanted electrodes, spread of the current through the tissue, and degradation of the contact between the stimulator and the tissue. The published investigations were concerned with auditory nerves, vestibular nerves, facial nerves, and excitable cardiomyocytes, and other types of neurons such as retinal and sciatic. Investigations concerned with auditory nerves were motivated by the fact that, unlike electrical stimulation, light can be used for nonoverlapping stimulation of many discrete sites along the cochlea. In addition, the stimulation location in the cochlea (correlated to the acoustic frequency by the tonotopic map) could be modified spatially with the optical fiber moving. It should be noted though that for optical stimulation to be used in a cochlear implant, a nondamaging high-frequency stimulus (200–400 Hz) would be needed to correctly encode sound intensity. Such high repetition rates may lead to thermal damage to neurons.

Light stimulation in auditory nerves. Izzo et al. (25) first reported that auditory nerve stimulation with midinfrared light was another alternative to electrical stimulation. The authors showed that it is possible to stimulate the auditory nerve at 2,012 nm (2.12 μm) with a pulse duration of 250 μs operating at 2 Hz. The stimulation threshold was determined to be 0.018 J/cm^2. No neural damage could be detected even for hours of continual stimulation (see Fig. 7) (26). An increase in radiation energy induced a monotonic increase in the evoked responses. Subsequent experiments with different wavelengths 1,844–1,940 nm (1.844–1.940 μm), 5-μs to 1-ms pulsesm and frequency between 2 and 1,000 Hz showed that the pattern of action potential changed with increasing pulse duration (26, 27).

As we know, some thick layers above the target structure in the cochlea can absorb certain amount of the radiation energy when the light travels through bone structures, preventing auditory neuron stimulation. Hence, the studies are typically carried out with optical fibers irradiating tissue placed on the surface of the cochlear nucleus; Lee et al. (33) reported that neurons of the central auditory system can be stimulated in this way. An optically evoked auditory brain stem response closely resembles that induced by acoustic stimulation. Reproducible signals were obtained above 169 mJ/cm^2, with a 50-μs pulse duration and a 5-Hz repetition rate. The stimulation was stable during continuous optical stimulation for 30 min. In a recent study, a pulsed IR Aculight Capella laser with a radiation wavelength of 1,850 nm (1.85 μm) was used (20). Penetration depth of this optical radiation is ~400 μm. The pulse duration was initially 100 μs and was subsequently increased to 1.6 ms (see Fig. 8).

It is also possible to stimulate auditory nerves indirectly via an optoacoustic effect (75). The authors in Ref. 75 used a green 532-nm laser and 10-ns pulses to activate the cochlea. The stimulation still required surgery, positioning a 50-μm fiber in the round window niche, directed toward the basilar membrane. The nerves response increased with energy level from 0.6 to 23 J/pulse (see Fig. 9). In this work light does not directly excite nerves but creates mechanical waves inducing a physiological response in cochlea.

Light stimulation in facial and other nerves. A frequent complication in skull base surgery is damage to cranial nerves.

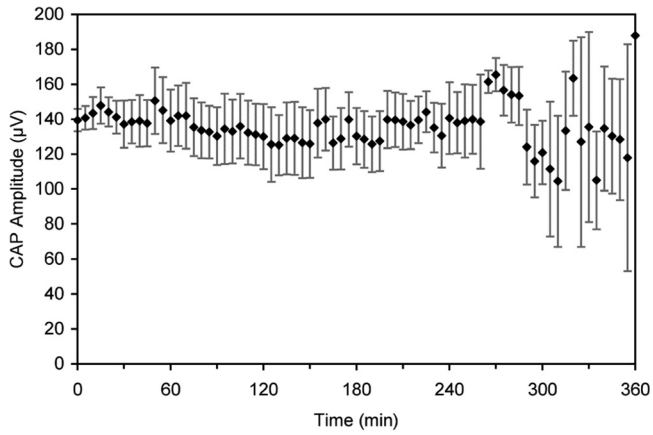

Fig. 7. Extended stimulation of the gerbil cochlea reveals constant evoked response. The compound action potential (CAP) amplitude remains relatively constant over a period of 6 h of continual stimulation [From Izzo et al. (26)].

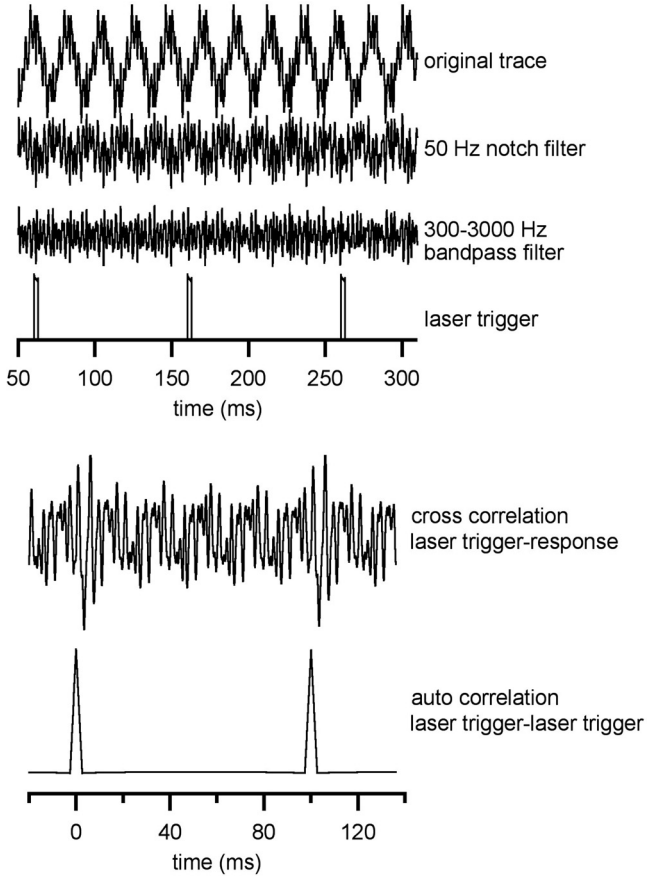

Fig. 8. Recordings from the round window membrane obtained during stimulation with optical radiation [From Fishman et al. (20)].

Currently, devices that stimulate nerves with electric current may be used during surgery to assist in nerve identification. Such intraoperative nerve monitoring can reduce the incidence of nerve damage during surgical procedures. In contrast to electrical stimulation, pulsed IR optical radiation can be used to stimulate neural tissue with a much higher degree of selectivity. The spatial precision demonstrated by Teudt et al. (58, 63) in stimulating the facial nerve with IR light establishes feasibility for optical stimulation to be used to identify neural tissue from surrounding tumour tissue. Recently, pulsed laser light was also shown to be capable of producing action potentials in peripheral nerves and in cultured cardiomyocytes (29, 60, 70–72)

Perspectives and Outlook

It is widely recognized that optogenetics has the potential to transform the discipline of neuroscience, and more broadly, physiology. Hence, intense efforts are focused on the application of existing methods and develop next-generation approaches such as optogenetic inhibition of synaptic release with light (34) or optical control of transcription and epigenetics (31) as well as applications outside the nervous system (7). From these rapid advances many researchers contemplate entering this field, and we hope that the review here will lead to insights on the potential for light to affect cells directly.

Current optogenetic experiments for single excitation with a fiber excite millimeter scale regions comprising a mixture of neurons, some of which have been genetically modified. The same illumination is then delivered into, nominally, the same spatial region in the brain of control animals and the response noted. This approach is methodologically sound and effective if the researchers can be satisfied that the adequately illuminated regions in genetically modified and control animals are similar enough. While physical locations with respect to the anatomical landmarks might be close enough, there needs to be certainty as to anatomical and physiological consistency especially for the regions that are near the fiber tip, where the irradiance is the highest. Figure 10 shows a potential scenario where two highly illuminated regions contain genetically engineered neurons and thermosensitive neurons. In this figure genetically engineered "target" neurons are shown in green, whereas a temperature-

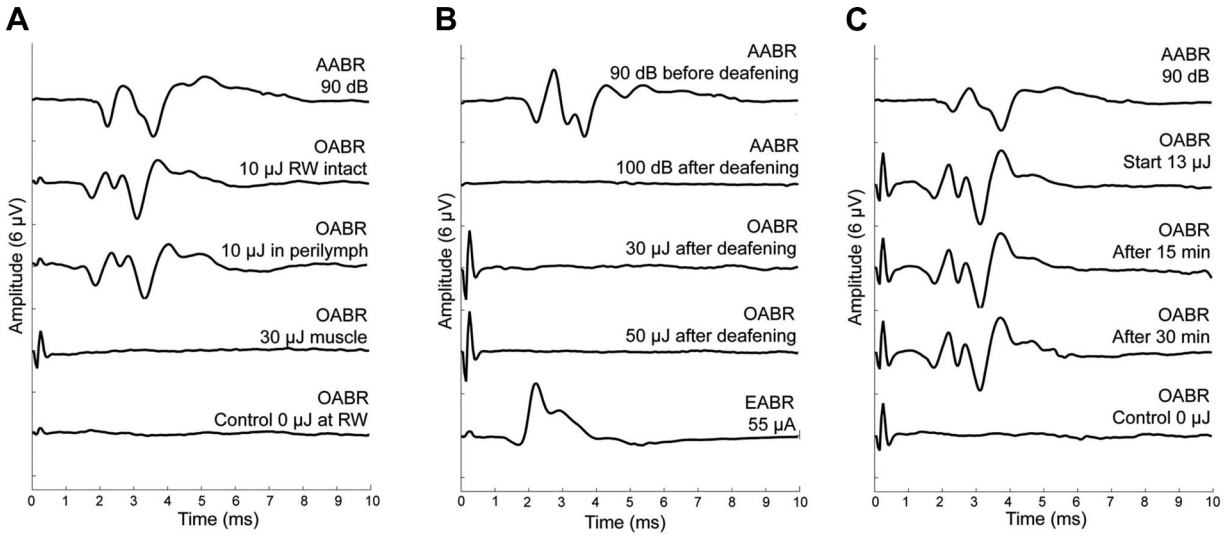

Fig. 9. Auditory brain stem responses (ABRs) to acoustic (AABR), optical (OABR), and electrical (EABR) stimulation in normal hearing and chronically deafened animals. *A*: examples of ABRs and OABR show similar amplitudes between activation at 90 dB SPL and 10 μJ/pulse, respectively. *B*: first and second recordings present responses to acoustic stimulation of the animal before and after deafening, respectively. *C*: stimulation of the cochlea with 13 μJ over 30 min did not cause any electrophysiologically apparent damage [From Wenzel et al. (75)].

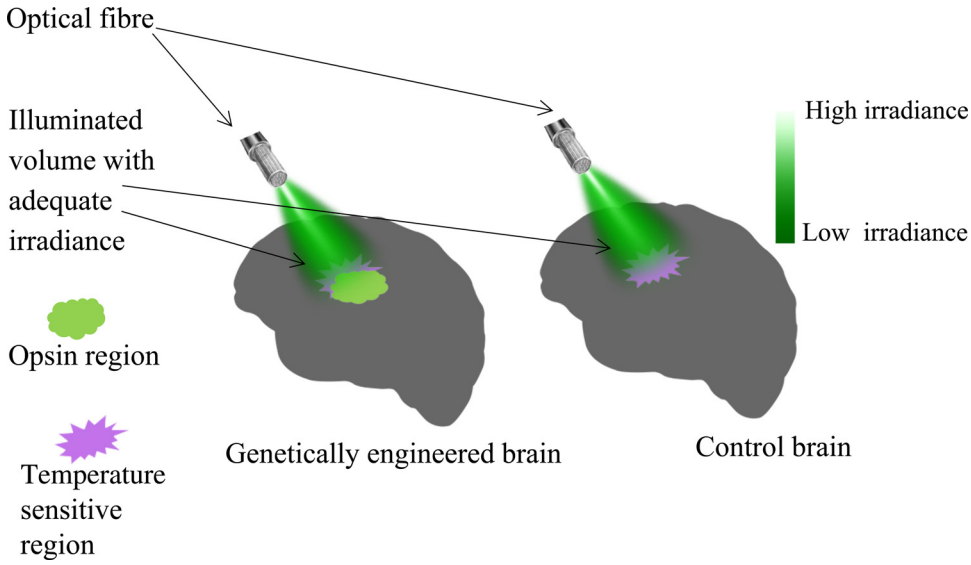

Fig. 10. Schematic illustration of a false positive effect caused by the proximity of genetically engineered neurons and temperature-sensitive neurons in an optogenetic experiment. Green: genetically engineered neurons comprising opsins. Purple: temperature-sensitive neurons. The color scale indicates that the illuminated region has varying light irradiance.

sensitive region is shown in purple. In the genetically engineered brain we observe the signal because the purple, temperature-sensitive region and the green opsin regions are close. Unfortunately the contribution of heat and opsin cannot be determined because of the proximity of the two. This is possible especially when opsin expression is insufficient, or diffuse, because low expression might require commensurately higher irradiance. In the control animal in Fig. 10, the largest part of the response is due to thermal rather than opsin effects. Hence, the experiment shown in Fig. 10 might yield a false positive or a false negative result depending on the proximity of the probe to the two areas, the quality of the opsin transfer, and the degree of temperature sensitivity of local neurons. In practice, this may not be an issue, but it is well known that autonomic neurons can be very sensitive to subtle changes in temperature and pressure. Hosking and colleagues (24) report a dose-dependent doubling in renal and splenic sympathetic nerve activity between 38.5°C and 41.5°C, whereas Nakamura and Morrison (48) report an exquisite temperature sensitivity of sympathetic activity to brown adipose tissue. Clearly, if irradiance increases temperature and opsin expression is low, then the hypothesis may not be adequately tested. Activation of microglia is easily provoked and can potently affect neuronal function; an increase in temperature of as little as 39°C is sufficient to double the release of lipopolysaccharide induced interleukin-10 from microglia in culture (37).

Recent investigations have also unveiled the possibility of false negative optogenetic experiments when excess light stimulation drives genetically modified neurons into a depolarization block, preventing them from producing action potential signals (55). For typical values of 25 mW focused to a 100-μm diameter optical fiber, the peak average irradiance at the tip is 1 W/cm^2 and insufficient to elicit thermal response. However, if the expression of the light-sensitive opsin is low, the researchers may be inclined to increase light irradiance to observe stimulation and to manage average tissue temperature by using a pulsed light with a low duty cycle. With modern diode lasers easily able to provide powers in the order of 500 mW, irradiance of 20 W/cm^2 is achievable. At these irradiance levels, local temperature may become sufficiently high to produce some thermal stimulation. The researchers may also be tempted to increase spatial resolution by using smaller diameter fibers and this will increase irradiance in inverse proportion to fiber cross-sectional area.

A well-pronounced trend is to improve precision of optogenetics toward optical control with single-cell accuracy (52). The currently used optical fibers excite millimeter scale regions, but the complex diverse physiology of the brain requires finer spatiotemporal resolution. To enable more precise guiding and delivery of stimulating light, cell excitation needs to be combined with imaging for better spatiotemporal control. Imaging in cell cultures and similar artificial system has a role to play; however, tissue systems and animal preparations where parts of the living neuronal system are exposed enable physiologically relevant insights in more realistic scenarios. This imaging can be done in the visible, but IR two-photon imaging is even more revealing as IR light has much higher penetration depth (~100 um) and high spatial selectivity due to a nonlinearity intrinsic in two photon processes. Correspondingly, two-photon excitation and inhibition of neuronal cells has been explored, in particular in the Deisseroth group (53). This effect takes advantage of a comparatively high two absorption cross section of purpose-engineered opsins (C1V1), but it still requires high average irradiances in the order of 2×10^6 W/cm^2. At these levels thermal effects become a real possibility leading to opportunities for misinterpretation outlined above. To manage the thermal load excitation is being combined with various forms of scanning, the simplest being a raster scan. However, this then needs to be coordinated with kinetics of the sensitizing proteins. When the kinetics is too slow the speed of the raster scan needs to be slowed down commensurately. This trend toward increased precision may make it more difficult to design appropriate control experiments in optogenetics.

Over the last several years, optogenetics has become a powerful tool in neuroscience and more broadly in physiology. The use of light stimulation in optogenetics is very successful because neural stimulation with light achieves higher spatial resolution compared with electrical activation and better temporal resolution compared with chemical activation. Second, the specificity of neural stimulation obtained with genetic

engineering compared with electrical or chemical stimulation is dramatic. This allows scientists to control precisely the activity of neurons and study their influence on a variety of biological processes. Information provided here suggests that care needs to be taken in any studies that use light activation and describes the physical basis for possible off-target effects. These are especially significant with pulsed light where special precautions are needed to avoid effects that are not related to activation of specific receptors. Despite this challenge, it is expected that the next generation of neural interfaces will use optical stimulation either independently, or in conjunction with other modalities, both in clinical settings and as a neurophysiology research tool. The future for neuronal stimulation control is very bright indeed.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: W.D., E.G., and P.M.P. drafted manuscript; W.D., E.G., M.F., and P.M.P. edited and revised manuscript; P.M.P. approved final version of manuscript.

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