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Cardiac optogenetics

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Entcheva E. Cardiac optogenetics. *Am J Physiol Heart Circ Physiol* 304: H1179–H1191, 2013. First published March 1, 2013; doi:10.1152/ajpheart.00432.2012.— Optogenetics is an emerging technology for optical interrogation and control of biological function with high specificity and high spatiotemporal resolution. Mammalian cells and tissues can be sensitized to respond to light by a relatively simple and well-tolerated genetic modification using microbial opsins (light-gated ion channels and pumps). These can achieve fast and specific excitatory or inhibitory response, offering distinct advantages over traditional pharmacological or electrical means of perturbation. Since the first demonstrations of utility in mammalian cells (neurons) in 2005, optogenetics has spurred immense research activity and has inspired numerous applications for dissection of neural circuitry and understanding of brain function in health and disease, applications ranging from in vitro to work in behaving animals. Only recently (since 2010), the field has extended to cardiac applications with less than a dozen publications to date. In consideration of the early phase of work on cardiac optogenetics and the impact of the technique in understanding another excitable tissue, the brain, this review is largely a perspective of possibilities in the heart. It covers the basic principles of operation of light-sensitive ion channels and pumps, the available tools and ongoing efforts in optimizing them, overview of neuroscience use, as well as cardiac-specific questions of implementation and ideas for best use of this emerging technology in the heart.

channelrhodopsin; light-sensitive ion channels; optical mapping

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Background

Optogenetics refers to the use of light and optics in conjunction with light-sensitive ion channels and pumps to perturb and control cell, tissue, and animal function. Similar to the widespread use of green fluorescent proteins and derivatives as reporters of gene expression and cell function, optogenetics necessitates genetic modification of the cells and tissues of interest, mostly by heterologous expression of microbial opsins. Unlike the green fluorescent protein-based observational/imaging tools, however, optogenetics also offers actuation possibilities and active perturbation of cell function, e.g., change in membrane potential or cell signaling, with high cellular specificity and spatiotemporal resolution, previously not attainable by pharmacological or electrical means.

The term "optogenetics" was coined in 2006 by Deisseroth et al. (32) and is commonly used to refer to specific new developments; the use of microbial opsins as actuation tools, though genetically encoded imaging probes, can also be included (37). There has been a long-standing interest in light-sensitive proteins, such as bacteriorhodopsin (BR), and in

developing optical means for control of biological function, but a breakthrough development came with the discovery of a faster type of microbial opsins that behave more like gated ion channels, and particularly with the cloning of channelrhodopsin1 (ChR1) and channelrhodopsin2 (ChR2) by Nagel, Hegemann, Bamberg, and colleagues (97, 98), which expanded the field beyond microorganisms. A seminal paper in 2005 by Boyden et al. (17) and parallel studies by others (80, 96) offered the first demonstration that such microbial opsins (ChR2, in particular) can generate sufficient photocurrent and can be effectively used to optically stimulate and control mammalian neurons with very high temporal resolution. This set the stage for cross-fertilization between the seemingly unrelated fields of microbial photobiology and neuroscience, resulting in over 1,000 publications with more than 23,000 citations to date for a new field, less than a decade old. Following an explosive rise in the number of publications and outlined opportunities, optogenetics was recognized as "Method of the Year" for 2010 by Nature-Methods (31, 54, 105). Its transformative potential has already been acknowledged in elucidating brain circuitry and function in health and disease, covered by multiple reviews (12, 25, 32, 41, 47, 89, 135, 136, 140, 141, 143, 144), yet expansion of this emerging technology outside neuroscience and into areas like cardiovascular research has surprisingly remained largely unexplored.

Optical sensing and actuation. Successful control of a dynamic system, e.g., the heart or the brain, requires detailed understanding. Conversely, better mechanistic insight is gained by means of active interrogation, i.e., fine control or *actuation*

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in conjunction with passive observations or *sensing*. The desire to "look into" complex systems, such as the brain and the heart, has driven the field of biomedical imaging toward the current state of the art by requiring exceptional spatial and temporal resolution to capture the phenomena of interest. Optical/fluorescence imaging, in particular, spans several orders of magnitude across spatial and temporal scales (124) and has become an indispensable tool in basic research, including the cardiac field, through the use of synthetic voltage- and calcium-sensitive imaging probes as recently reviewed (38, 39, 55). Optical sensing has evolved to capture fast events at the molecular level. For example, monitoring localized calcium flux with two-photon or total internal reflection fluorescence microscopy can be used to indirectly track single (Ca^{2+}) channel activity by optical means (33, 34). Furthermore, optical records of single (K^+) channel conformations are possible with direct fluorescence labeling and total internal reflection fluorescence microscopy imaging to infer membrane voltage changes (87, 113, 116, 117). Certain applications of genetics for development of new imaging probes, i.e., genetically encoded calcium and voltage-sensitive proteins, are considered as part of the broader view of optogenetics (5, 10, 37, 95), despite the lack of an actuation component per se. Such genetically encoded sensors offer cell-specific readout and the potential for long-term in vivo monitoring of electrical activity compared with the widely used organic dyes. Genetically encoded Ca^{2+} sensors (86, 91, 92) have found widespread application; however, their use in vivo has remained limited. Obtaining suitable temporal resolution and reliable performance for genetically encoded optical voltage sensors has been a challenge, mostly due to added capacitive load to the membrane and severe interference with innate cell physiology (90), which is also partially true for calcium sensors, which interfere with intracellular Ca^{2+} buffering. Nevertheless, there are multiple promising developments in this area (5), including the recent atypical use of microbial opsins for voltage sensing (76).

In general, the passive sensing/imaging technology has matured enough to require respective developments on the interrogation/actuation side, optical tools to perturb activity with fine resolution (90). Developments have been under way for over two decades to use light for perturbation and control. A particularly desirable solution is the direct application of near-infrared or infrared (IR) light for stimulation, without the need of genetic or chemical modifications. For example, millisecond pulses of IR light (1.8 μm) have been shown to trigger mitochondrial Ca^{2+} release in myocytes in vitro (34a) and have been used to pace embryonic hearts in vivo (62). The mechanism of stimulation by IR light always involves substantial localized temperature changes and steep gradients (132), which likely trigger depolarizing currents by changes in membrane capacitance (112). Among the limitations of this approach are concerns about the physiological limits of local temperature gradients required to excite, energy needed to reliably achieve such perturbation, insufficient temporal resolution (capacitive effects), and lack of specific cell targeting. Other examples of optical actuation include "caged" compounds, e.g., caged calcium and flash photolysis for understanding the Ca^{2+} control system in muscle by Niggli and Lederer (100), using G protein-coupled signaling and ligand-requiring opsins (chARGe) (138) or light-controlled specialized ion channels (transient receptor potential vanilliod 1 and transient receptor potential melastatin 8) by

Zemelman et al. (139), tools based on synthetic photoisomerizable azobenzene-regulated K^+ channels (SPARK) (13), or light-gated glutamate receptors (LiGluR) by Isacoff and colleagues (120, 125). In general, all of the techniques listed above share relatively high level of complexity and lack of robustness, which determines their limited popularity or their use only within a rather specialized field.

BR: nature's archetypal optoelectric transducer. BR is one of the simplest and the best studied optoelectric transducers from the microbial (class I) opsins, found in archae, eubacteria, fungi, and algae. A different class of opsins (class II) encompasses the mammalian G protein-coupled sensory rhodopsins, which are not among the commonly used optogenetics tools. BR, a protein with seven transmembrane (TM) domains, acts like a light-gated active ion pump; it captures photon energy via its covalently bound chromophore, retinal, and moves protons against the electrochemical gradient from the cytoplasm across the membrane. In its native environment, BR provides energy for ATP synthesis via its light-fueled proton pumping action. Since their discovery (102), the microbial opsins have spurred a lot of interest and have been viewed as potential components for bioelectronics and a new generation of optical memory (16, 126) due to offered ultra-fine spatiotemporal control by light. The latter is of equal interest in control of eukaryotic cells. Structurally and functionally, BR provides good insight for optogenetics as it shares high homology with all (class I) opsins currently in use.

The new generation of optogenetics tools: defining features. Current-day optogenetics was spearheaded by the characterization and cloning of ChR1 and especially of the higher-conductance light-sensitive ion channel ChR2 from green algae by Nagel, Hegemann, Bamberg, and colleagues (97, 98) in 2002 and 2003, followed by the first robust demonstrations of the use of ChR2 to stimulate mammalian cells in 2005 (17, 80, 99). Upon heterologous expression, these microbial ion channels provide excitatory (cation mediated) current with relatively fast kinetics (27, 82) and can effectively trigger electrical impulses (action potentials) in excitable cells upon light stimulation at relevant physiological rates. The demonstrated utility in neuroscience revived interest in other types of microbial opsins, discovered earlier and extensively studied within the microbial photobiology field but never considered for use in mammalian physiology. These include the chloride pump Halorhodopsin (HR) (78, 88) and the BR-like proton pump Archaerhodopsin (AR) (59). Both were proven capable of mediating inhibitory/hyperpolarizing action on membrane voltage in mammalian cells (30, 45).

What makes the recent optogenetic tools (several types of microbial opsins) more practical compared with earlier systems are the following distinguishing characteristics: *1*) simplicity of expression and operation without exogenous cofactors, offering the attraction of a single-component system; *2*) apparent minimal interference with endogenous function (much less than genetically encoded voltage and calcium sensors) and remarkable reliability of use on a large scale, in vitro and in vivo; *3*) offered specificity, i.e., selective cell-type targeting; *4*) very high spatiotemporal precision of manipulation not achievable by other known actuation techniques; *5*) robustness and range of action within the same paradigm, i.e., excitatory and inhibitory effects can be encoded; and *6*) relatively low-light energy required for activation compared with IR stimu-

lation, for example. All of these features contributed to the instant widespread biomedical application of this new technology.

ChR2 Biophysics and Operation

ChR2 from *Chlamydomonas reinhardtii*, cloned by Nagel et al. in 2003 (98), is the prototypical and currently most widely used optogenetic tool. Like BR, it belongs to class I microbial opsins, all of which use retinal as a chromophore (light-sensing element). Unlike BR, ChR2 is a classical ion channel (not an active pump) and upon opening it conducts cations along the electrochemical gradient.

The chromophore, all-*trans*-retinal, is covalently bound to the ion channel, and the complex does not undergo dissociation seen for class II mammalian rhodopsins, where the retinal-opsin complex is reassembled/disassembled upon each stimulus (52). Upon interaction with a photon, all-*trans*-retinal undergoes isomerization to 13-*cis*-retinal, thus triggering the ion channel opening. All-*trans*-retinal, derived from intake of vitamin A containing nutrients, is only present in small amounts in nonretinal and nonembryonic tissues (<0.5 nmol/g) (68). It was a serendipitous finding that in most vertebrate cells and systems, there is enough all-*trans*-retinal naturally to form functional ChR2 complexes. This is even more surprising in cell culture, where the source of vitamin A must be from serum/cell culture impurities. To date, there are no systematic studies demonstrating whether and how retinal availability varies between cell and tissue types and whether it can be a limiting factor in the light responsiveness of different cell types modified with ChR2.

Similar to BR, ChR2 has seven TM domains. It has molecular masst of 77 kDa and a total of 737 amino acids, ~300 of which located at the amino-terminus fully define its photocurrent generation (69). The crystal structure of ChR2 was recently solved (69, 94): it revealed that the conductive pore is defined by TM1, -2, -3, and -7 and that TM7 is critical for the interaction with retinal, whereas TM2 determines channel selectivity and conductance. ChR2 has higher energy barrier for excitation compared with BR; its spectral response peaks at around 470 nm (570 nm for BR).

ChR2 conducts cations with differential selectivity in the following order ($H^+ > Na^+ > K^+ > Ca^{2+}$ . . .) (82) (Fig. 1). Thus, for physiological membrane potentials, ChR2 provides exclusively inward current and has a reversal potential close to 0 mV with prominent rectification properties, i.e., minimal outward current (27, 40, 48, 64, 82, 96). Several competing theories have been put forward about the mechanism of rectification, including it being a single-channel property defined by an asymmetric barrier (40) or macroscopic property resulting from the kinetics of multiple ion species interacting with the channel (48).

Upon delivery of a light pulse with proper wavelength (470 nm) and of sufficient irradiance (in mW/mm^2) for excitation, ChR2 generates photocurrent with a fast peak and relaxation to a steady-state component (Fig. 1). Higher irradiance and more negative voltages speed up the onset of the peak and the relaxation to steady state. Even at room temperature, all time constants are under 20 ms (27, 64). The single-channel conductance for the wild-type ChR2 is relatively small, and the few reported values vary widely: from 40–90 fS (40, 145) to
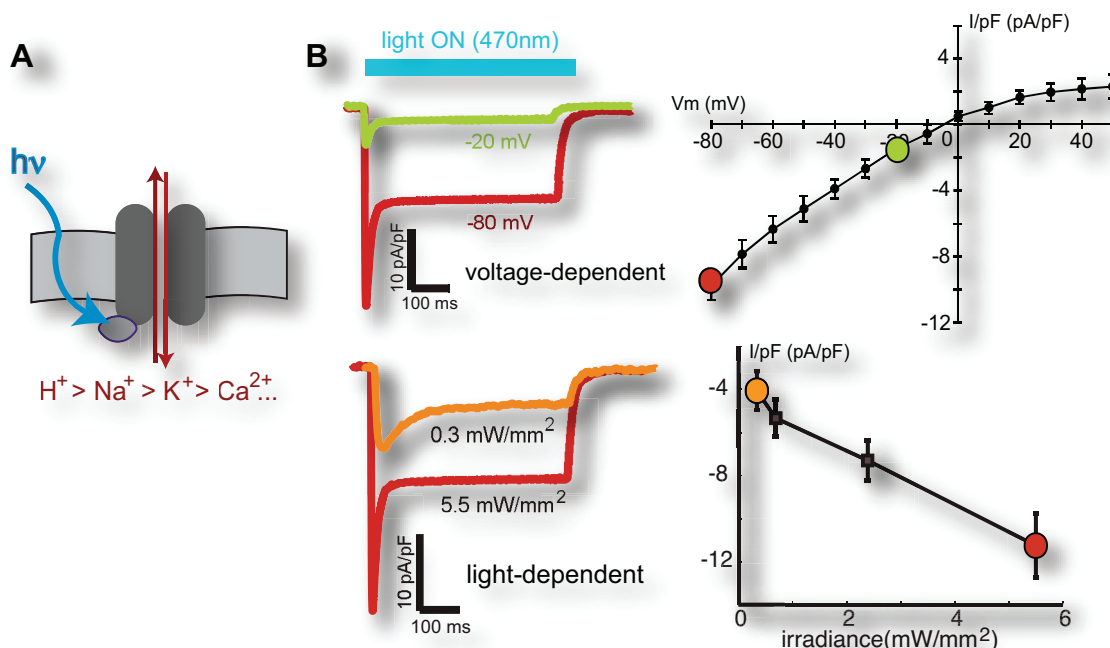

Fig. 1. Biophysical properties of channelrhodopsin2 (ChR2). *A*: ChR2 is a light- and voltage-dependent ion channel; all-*trans*-retinal, intracellularly available and covalently bound to ChR2, acts like a chromophore (sensing photons) to facilitate ChR2 opening and the transport of cations with differential preference from H^+ to Ca^{2+}. *B*: ChR2 produces voltage- and light-dependent current. The resultant current is predominantly inward/excitatory with a fast peak and sustained component; ChR2 exhibits strong inward rectification with a reversal potential around 0 mV [current-voltage relationship for the sustained component shown from Jia et al. (64)]; shown is also a current-irradiance relationship and selected traces for ChR2 current under different voltage clamps (*top*) and irradiance levels (*bottom*). V_m, membrane potential.

0.25–2.42 pS (82), depending on the method of estimation. For comparison, single Na^+-channel conductance in muscle cells can be orders of magnitude higher than the ChR2 conductance, as high as 18 pS (115). Zimmermann et al. (145) used freeze-fracture electron microscopy and particle counting as well as whole cell conductance and capacitance measurements to estimate ChR2 density of expression (typical case) and found about 2,000 channels/μm^2. Taken together with certain probability of being opened, this allows for whole cell current estimates. As discussed below, one of the first ChR2 single amino-acid mutants (H134R) was generated to increase conductance by two- to threefold with minimum sacrifice of kinetics (46, 96).

Conceptual and quantitative understanding of the function of ChR2 is aided by recent mathematical models, proposed by Hegemann and colleagues (53) and modified by others (49, 101, 121). A four-state model is currently favored, with two open states (a high-conductance and a low-conductance, light-adapted one) and two closed states. The photon absorption and isomerization of retinal is a near-instantaneous process (130), so that ChR2 conformational changes, after light sensing, determine its photocurrent kinetics. Current models reflect reasonably well light dependence but oversimplify voltage dependence.

The Optogenetics Toolbox

Different microbial opsins, developed and adopted for use in mammalian cells, compose the "optogenetics toolbox" (Fig. 2A). These include both proteins that generate depolarizing/excitatory currents, e.g., channelrhodopsins, and proteins that produce inhibitory/hyperpolarizing currents, such as the chloride pump HR from *Natronomonas pharaonis* adopted for mammalian use (eNpHR) (45) and some BR-like proton pumps, e.g., AR-3 from *Halorubrum sodomense* (30) and the shorter wavelength-activated pump from the fungus *Leptosphaeria maculans* (Mac) (30).

AR is the most potent inhibitory opsin to date. When compared with HR and Mac, it offers larger photocurrent and faster recovery from inactivation. Similar to HR and Mac, AR provides an outward current, with a highly negative reversal potential and only about 20% drop in current when going from 0 to −120 mV. By its action, despite being an efficient proton pump, AR changes the H^+ concentration (pH) only mildly, i.e., the intracellular pH can increase by about 0.15 for 1 min of continuous illumination (30). The single channel conductance of AR is unknown but by whole cell current is comparable in amplitude to the ChR2 current. For example, about 1-nA current was induced by strong illumination in neurons (30) for unknown expression levels.

Having both excitatory and inhibitory optogenetics tools of comparable performance truly opens the possibility for optical control of membrane potential, i.e., shaping the action potentials and/or the frequency response of the system. A method for optimized tandem expression of excitatory and inhibitory opsins has been recently developed (73). Genetic engineering is currently used to expand and optimize available opsins in three important aspects: light sensitivity, speed, and spectral response (Fig. 2B). These efforts, mainly carried out in the laboratories of Deisseroth, Bamberg, Hegemann, Tsien, Boyden, and several others, have resulted in various ChR2 mutants
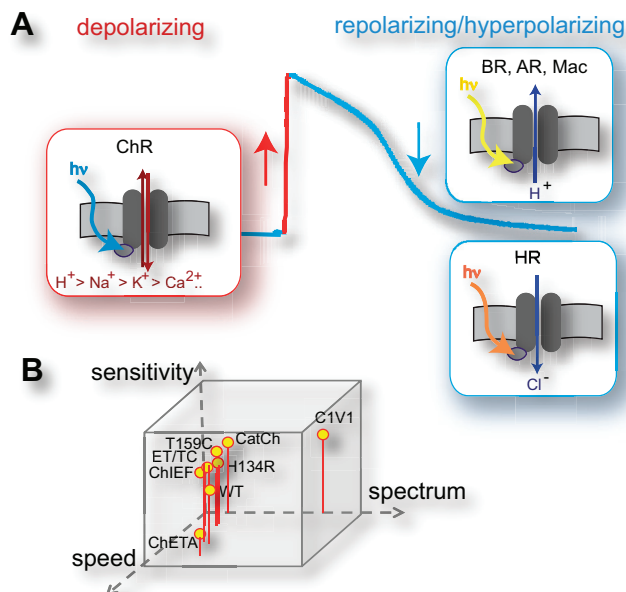

Fig. 2. The optogenetics toolbox. *A*: available optogenetic tools for manipulation of membrane potential include excitatory/depolarizing ion channels, e.g., channelrhodopsins and derivative mutants and inhibitory/hyperpolarizng pumps from 2 major classes: proton pumps like bacteriorhodopsin (BR), archaerhodopsin (AR), and *Leptosphaeria maculans* (Mac) and chloride pumps like halorhodopsin (HR). *B*: genetic engineering is used to expand and optimize available opsins in 3 main aspects: light sensitivity, speed, and spectral response; some examples are shown with their relative position in this parameter space, including direct ChR2 mutants with single amino-acid substitutions (H134R, T159C, ET/TC, the Ca^{2+} permeable CatCh, and the speed-optimized ChETA), hybrids of ChR1 and ChR2 (ChIEF), and hybrids of ChR1 and VChR1 (C1V1) for red-shifted variants. WT, wild-type.

with single amino-acid substitutions, including the higher-conductance H134R (96), T159C (14), and ET/TC (14); the Ca^{2+} permeable CatCh (72) and the speed-optimized ChETA (50); hybrids of ChR1 and ChR2 (ChIEF) (82); and hybrids of ChR1 and VChR1 (C1V1) for red-shifted variants (142), among others. Additionally, ChR2 variants were molecularly engineered to prolong open state and obtain stable switch properties (15). Figure 2B attempts to qualitatively place some of these modified ChR2 derivatives in the parameter space of sensitivity, speed, and spectral response. In most cases, optimization of one aspect, e.g., conductance, comes with a trade-off in another aspect, e.g., speed. Table 1 summarizes important parameters for these available voltage actuators. A more extensive quantitative comparison of existing genetically engineered opsins can be found in several excellent reviews (14, 89, 136). Other efforts to improve the optogenetics toolbox include reduction of toxicity, better membrane targeting, better cell specificity, and general optimization of expression (25, 47).

Optogenetics in Neuroscience

Neuroscience applications have successfully used the specificity offered by optogenetics to dissect neural circuits and connectivity, linking specific neuron populations to behavior and disease presentation. These studies include applications to better understand learning (58), olfactory processing in vivo (8), depression (3), narcolepsy (2), sleep disorders (26), fear (65), and addiction (84). Optogenetics was used to validate

Table 1. *Voltage and biochemical actuators*

| | | | **Voltage Actuators Based on Microbial (Class I) Opsins** | | | | | | |

Excitatory/depolarizing ChR-based actuators

Opsin/mutation	Actuation mechanism	Spectrum λ_{abs}, nm	Biophysical properties compared with wild-type ChR2						Reference
			I_p, nA	I_{ss}/I_p	τ_{ON}, ms	τ_{OFF}, ms	τ_{INACT}/τ_{DES}, ms	τ_{REC} (S1–S2), s	
ChR2	Cation ion channel, $H^+>Na^+>K^+>Ca^{2+}$	470	~1*	~0.25*	~5*	~10*	~50*	~5*	(27)
ChR2-H134R	Cation ion channel, $H^+>Na^+>K^+>Ca^{2+}$	470	↑	↑↑	~	~	~	~	(96)
ChR2-T159C	Cation ion channel, $H^+>Na^+>K^+>Ca^{2+}$	470	↑	~	~	↑	~	~	(14)
ChR2-ET/TC (ChETA$_{TC}$)	Cation ion channel, $H^+>Na^+>K^+>Ca^{2+}$	470	↑	↑	~	~	~	↓↓	(14)
CatCh (ChR2-L132C)	Ion channel, ↑Ca^{2+} conductance	474	↑	↑↑↑↑	↑	↑↑	↑↑	—	(72)
ChETA (ChR2-E123A)	Cation ion channel, $H^+>Na^+>K^+>Ca^{2+}$	470	↓	↑↑↑	~	↓	↓↓	↓↓	(50)
ChIEF (ChR1 + ChR2)	Cation ion channel, $H^+>Na^+>K^+>Ca^{2+}$	450	↑	↑↑↑↑	↑↑	↑↑	↑↑	—	(82)
C1V1 (ChR1 + VChR1)	Cation ion channel, $H^+>Na^+>K^+>Ca^{2+}$	540	↑	↑↑	↑	↑↑↑	↑↑	↑	(137, 142)
Step-function opsins, C128S/D156A	Cation ion channel, $H^+>Na^+>K^+>Ca^{2+}$	470 (on) 535 (off)	↑	↑↑↑↑	↑	↑	Minimal inactivation	—	(11, 15)

Inhibitory/hyperpolarizing opsin actuators

Opsin		λ_{abs}	Biophysical properties				Reference
			I_p, pA/pF; nA (mW/mm^2)	Activation, ms	Deactivation, ms	τ_{REC} (S1–S2), s	
HR (eNpHR)	Cl^- pump	590	4; 0.15 (<10)	—	τ_{OFF} <10	>50	(30, 47)
Arch/ArchT	H^+ pump	566	12; 0.35 (<10)	<5 (onset); ~300 (to plateau)	τ_{OFF} <10, and ~400 (to full elimination of the effect)	<30	(30)
Mac	H^+ pump	540	9; 0.25 (<10)	—	τ_{OFF} ~50	—	(30)

| | | **Biochemical Actuators Based On Class II Opsins** | | | |

Construct		λ_{abs}	τ_{ON}, s	τ_{OFF}, s	Reference
Opto-β_2-AR	↑ G_s protein signaling	500	~2	~3	(4)
Opto-α_1-AR	↑ G_q protein signaling	453	~0.1	~0.5	(4)
bPAC (cAMP)	↑ cAMP	455	~23	~12	(118)

For voltage actuators, channelrhodopsin (ChR)-based excitatory and inhibitory voltage actuators are listed along with some quantitative parameters compared with values for wild-type ChR2. Listed are the mechanism of actuation, the peak absorption wavelength (λ_{abs}), peak current (I_p), ratio of sustained current-to-peak current (I_{ss}/I_p), time constant of activation (τ_{ON}), time constant of deactivation (τ_{OFF}), time constant of inactivation/desensitization (τ_{INACT}/τ_{DES}), and time constant of recovery from inactivation upon consecutive stimuli (τ_{REC}). *Actual values vary with irradiance, voltage, temperature, cell type, etc. Some values for inhibitory opsins are not known. For biochemical actuators, 3 examples are given based on class II opsins along with their mechanism of action and characteristics. See main text for definitions of other abbreviations.

blood oxygen level-dependent MRI signals via direct links to local electric events (79). More translational studies tackled questions related to epilepsy and termination of seizures (123), Parkinson's disease, and deep brain stimulation to counteract it (7, 44, 77), countering visual degeneration in retinitis pigmentosa (21), resuming respiratory control (43), better optical nerve stimulation of skeletal muscle (83), and optimized stem cell differentiation (119, 131). This is a small subset of the wide spectrum of studies conducted thus far; recent more comprehensive reviews provide further information (41, 136).

Beyond Neuroscience and Toward Cardiac Applications

Overview of early work in cardiac optogenetics. Starting in 2010, only a couple of publications have appeared to date that extend optogenetics utility to cardiac muscle. Arrenberg et al. (9) used a zebra fish model to express both excitatory (ChR2) and inhibitory (HR) opsins. Employing structured illumination, they demonstrated the use of the optogenetics to spatially map the exact pacemaking region in zebra fish during development. They also demonstrated reversible range of rhythm disorders triggered optically. Bruegmann et al. (20) published the first mammalian application of optogenetics in heart. They combined viral expression of a ChR2 variant with a cytosine-adenine-guanine promoter into mouse embryonic stem cells with targeted differentiation and purification of embryonic stem cells-derived cardiomyocytes for in vitro demonstration of optical pacing. Furthermore, they generated transgenic mice with cardiac ChR2 expression, in which normal rhythm was perturbed in vivo by light pulses and focal arrhythmias were induced by long pulses. Simultaneously and independently, our group

demonstrated a nonviral optogenetic stimulation (63, 64). Considering the heart's dense and well-coupled environment, we used cell delivery to demonstrate optical pacing in vitro. We termed this strategy a tandem cell unit (TCU) approach, where dedicated (nonexcitable) donor cells expressing light-sensitive ion channels (ChR2) and capable of electrical coupling with cardiomyocytes, can effectively inscribe light sensitivity to cardiac tissue. Our study also demonstrated the first integration of high-speed/high-resolution optical imaging with optogenetics-based actuation for a fully optical interrogation of excitable tissue and quantitative comparison of wave propagation upon optical versus electrical stimulation. Two more in vitro studies were published approximately at the same time, one using a cardiac cell line HL-1 in which ChR2 was expressed by electroporation (56) and the second using lentiviral delivery of ChR2 into human embryonic stem cells, followed by cardiomyocyte differentiation (1). Both studies applied microelectrode arrays to confirm electrical response upon optical stimulation. Abilez et al. (1) combined their experiments with computational modeling of the function of ChR2 into cardiac tissue.

Cardiac electrophysiology and optogenetics. When compared with neuronal electrophysiology, cardiac action potentials are longer and more complex, reflecting the close integration of electrical and mechanical function, with a prominent role for Ca^{2+} as intermediary. Physiologically relevant frequencies of electrical response are about an order of magnitude lower than for neurons. Myocytes are big and tightly coupled via gap junctional proteins, forming a syncytium. Excitation waves, under normal conditions, follow well-known paths, as reflected in the highly regular ECG. Albeit cell-type variation is lower than in the brain, the heart still features cell subpopulations with distinct electrophysiological profiles, e.g., atrial and ventricular myocytes as well as conduction system cells, including sinoatrial pacemaking cells, atrioventricular nodal cells, and Purkinje cells. Finer subdivisions exist within these classes, e.g., transmural distinction of ventricular myocytes or subpopulations of cells within the sinoatrial node.

In consideration of these characteristics, it is likely that optimization of optogenetics tools and their specific application follow different routes compared with neuroscience. For example, higher-speed opsins are not as relevant for the heart; higher conductance opsins are quite relevant considering the high electrotonic load. While in neuroscience the ideal excitatory pulses are very brief and information is encoded mostly by the frequency of the pulses, for cardiac applications it may be interesting to explore longer, lower intensity pulses. The importance of the waveform of the stimulating pulse both for pacing and defibrillation has been long recognized in the cardiac literature (42, 66, 106, 107, 111, 127). More specifically, energy minimization has been pursued via waveform optimization (106), and it is known that a typical rectangular monophasic pulse, for example, does not provide the most efficient stimulus (66). It can be speculated that the conceptually different optogenetic mode of stimulation (Fig. 3B) may be inherently optimal. This is due to the stimulus (the actual induced photocurrent) being shaped based on instant real-time feedback about the membrane voltage; i.e., unlike electrical pulse stimulation, the ChR2 current pulse effectively will terminate when a certain voltage is reached because of the channel's voltage sensing and inward rectification. Therefore,
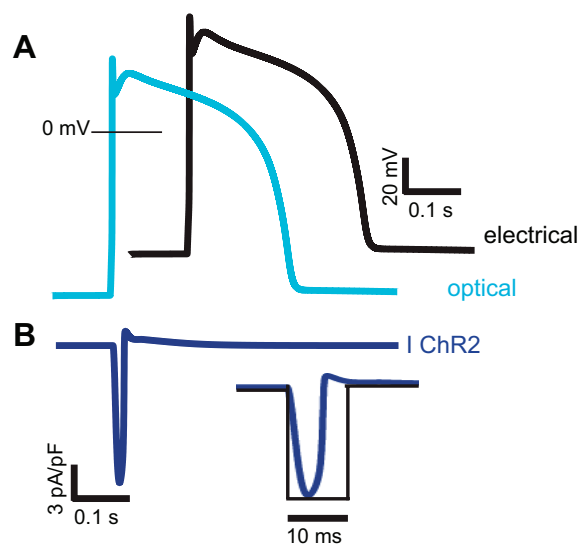

Fig. 3. Optogenetic stimulation of cardiac cells. *A*: human ventricular myocytes, stimulated electrically (5 ms, 10 pA/pF) and optically (10 ms, 5 mW/mm^2), produce very similar action potentials. *B*: the underlying ChR2 current during a cardiac action potential is fast inward current that gets reversed by the change of voltage and becomes briefly outward for positive voltages. *Inset*: optogenetic stimulation is inherently waveform optimized (compared with electrical rectangular pulses) because of the built-in feedback control by voltage, i.e., the inward current injection self-terminates once the membrane has been depolarized. The results are from computer simulations with the ten Tusscher myocyte model, integrated with a ChR2 model, modified from Nikolic et al. (101).

it may be more energy efficient, especially at longer low-irradiance pulses. It is harder to speculate about the performance of optogenetic pulses for cardioversion and defibrillation; computer modeling and in vitro experiments can provide more insight how the prestimulus state may affect the outcome. Figure 3 illustrates the response of a ventricular myocyte to comparable electrical and optical stimulation using computer simulations (unpublished data) with the ten Tusscher ventricular cell model (122), including a version of ChR2 modified from earlier papers (101, 121).

Inscribing light sensitivity in cardiac tissue. Common techniques for genetic modifications, including for optogenetics, are shown schematically in Fig. 4A. Transgenic mice present an attractive experimental model and can be generated with specifically targeted cell types. Various strains of transgenic ChR2-expressing mice were developed by Feng and colleagues for in vivo neuroscience research (128), and many of these are currently available through the Jackson Laboratory. To date, no commercially available mice with cardiac expression of optogenetic tools have been produced. Recently, Witten et al. (134) have developed a more elegant general strategy for cell type-specific expression of opsins in rats. They used recombinase-driver rat cell lines that can drive the gene expression in specific cell types with Cre recombinase under the control of relatively large regulatory regions (>200 kb). This approach allows for faster generation of experimental rat models of interest than transgenic animals and will likely be used in cardiac applications as well. Such Cre driver lines confer another level of selectivity when combined with viral vector delivery, in addition to promoter-determined selectivity. For cardiac applications, one can envision interest in targeting the
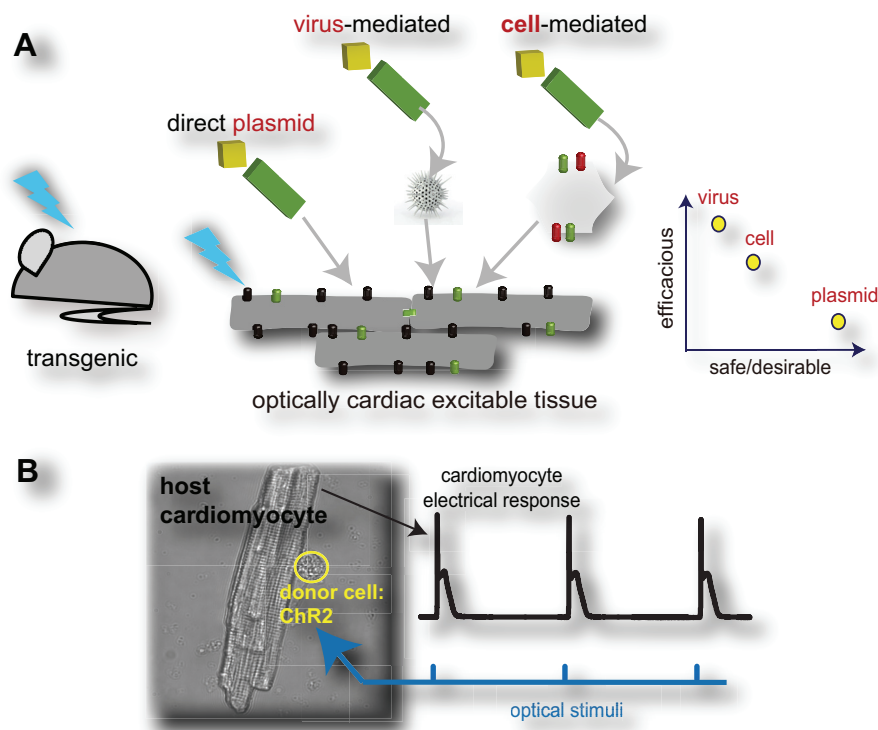
Fig. 4. Inscribing light sensitivity in cardiac tissue. *A*: the most common approaches to optogenetic transduction include the generation of a transgenic animal or different ways of gene delivery: direct plasmid transfection, virally mediated, or cell mediated; the relative efficacy and safety of these approaches is depicted. *B*: the cell delivery approach works for well-coupled cells like in the myocardium where a tandem cell unit can be formed between a nontransduced myocyte and a nonexcitable donor cell; shown are a canine adult ventricular cell and a donor ChR2 HEK cell where optical stimuli (at 0.2 Hz) drive action potentials in the myocyte [modified with permission from Jia et al. (64)].

conduction system as a whole or regions of it, i.e., just the sinoatrial node or just the Purkinje fibers. While specific gene expression (HCN4, Cx40) or locally enriched transcription factors, such as Contactin-2 (103), have been investigated for the conduction system, more work is needed to make relevant promoters available for cell-specific targeting, similar to the arsenal available for neuroscience.

In addition to transgenic animals, gene targeting in vitro or in vivo can be achieved by direct DNA delivery (by electroporation or other transfection methods), viral delivery, or cell delivery. Through concerted efforts to optimize the optogenetics toolbox, multiple laboratories use repositories like Addgene and make their constructs publicly available. Of the shown delivery methods (Fig. 4*A*), viral delivery using lentivirus or adeno-associated virus (AAV) is the most common in optogenetics applications (136) because of the high efficiency and potential specificity if a cell/tissue-specific promoter is used. Successful long-term expression with lentivirus and AAV has been demonstrated not only in mice (2) and rats (7) but also in primates (51). For cardiac use, optimization of viral delivery will involve a search for the most suitable AAV serotype and small promoters for cell-specific expression (the small payload of AAV of <4.7 kb limits promoter size). The cell delivery approach (Fig. 4*B*) is based on the TCU strategy and relies on coupling of the donor cells with the native myocytes with coupling strength beyond 2 nS (64). It is particularly relevant to cardiac applications and use of stem cell delivery. It does not address cell-specific labeling of native myocytes, but it allows for optimization of the donor cells for better opsin performance in vivo.

When applying optogenetics to cardiac tissue, it was unknown a priori if the required chromophore for ChR2 operation can be found in sufficient amounts. For example,

bulk tissue measurements with chromatography and UV techniques (HPLC/UV) (67, 68) indicate that cardiac muscle may contain lower amounts of endogenous retinoids compared with liver, kidney, adipose tissue, and the brain. Yet, the few cardiac applications discussed above did not use exogenous retinal, thus suggesting that some (possibly sufficient) amounts of retinal were available. It is interesting to note that the human embryonic kidney cells (HEK293) used in our TCU experiments (64) and in other optogenetics studies are known for their optimized retinoid machinery (19), whereas myocytes are most likely not. We also had some success with direct optical stimulation of ChR2 cardiomyocytes in cell culture without exogenous retinal addition (64).

Challenges for light access in the heart. In neuroscience applications, precise injections for optogenetic targeting of desired brain locations and implantation of stimulating or recording devices are done by widely available stereotactic systems. Furthermore, implantable devices evolved rapidly in brain research since 2007 (7) to the current fully integrated systems in freely moving animals, in some cases with wireless powering (6, 129, 133).

An analogous approach to the stereotactic system is not in place for cardiac gene or cell delivery. The challenge is to do this in a live beating heart, without the firm support and point of reference naturally offered by the skull for the brain. Optical fiber conduits for imaging purposes have been developed before for cardiac applications (22, 23, 57, 75); now this intramural optrode approach may be adopted for possible localized gene or cell delivery as well as optical stimulation and recording. Alternatively, for optical stimulation, surface-conforming solutions, where the device is moving with the contracting heart, may come from recent new developments in stretchable electronics and optoelectronics (70, 71), i.e., light-

emitting diode matrixes can be organized to conform and follow accessible surfaces, epicardial or endocardial, with minimally invasive procedures similar to the use of inflatable balloons. As dense and highly scattering medium, the heart may require further efforts in developing red-shifted optogenetics tools, similar to VChR1 and C1V1 (142). Two-photon excitation offers an alternative way of increasing wavelength. Recently, two studies (104, 108) confirmed effective two-photon excitation of ChR2, reporting high two-photon absorption cross section of about 260 GM at 920 nm; thus it may be possible to stimulate deeper tissue (>0.5 mm) by surface illumination even in the dense cardiac muscle. Nevertheless, accessibility of certain endocardial conduction system structures may provide some immediate opportunities for interesting research questions amenable to optogenetic solutions.

Energy for optical stimulation. The optical stimulus strength needed to trigger a response is typically measured in units of irradiance (in mW/mm^2). Strength-duration curves link minimum irradiance and pulse duration and capture the overall energy for optogenetic stimulation (Fig. 5A). The energy will be influenced by a multitude of factors, including expression levels and functionality of the opsins, the host cell electrophysiological milieu (balance of depolarizing and repolarizing currents), the cable properties of the tissue and electrotonic load for activation, the efficiency of light delivery/penetration, and so on. In Fig. 5A, strength-duration curves are assembled from published data for cardiac cell monolayers with the TCU approach (64) and for ventricular and atrial tissue in transgenic

Fig. 5. Energy for optical stimulation of cardiac tissue. *A*: strength-duration curves (irradiance and pulse duration needed to pass the threshold for stimulation) are assembled from published data for cardiac cell monolayers with the tandem cell unit approach and for ventricular and atrial tissue in transgenic mice; these are compared with the much higher values reported for stimulation in neural applications in vitro and in vivo: *1*) data from multiple papers, including Boyden et al. (17), Wang et al. (128), Cardin et al. (24); *2*) data from Bruegmann et al. (20); and *3*) data from Jia et al. (64). *B*: energy to stimulate depends on the spatial distribution of gene or cell delivery of ChR2: computational data [Boyle et al. (18)] show that lower energy is needed for direct gene delivery in myocytes if a localized area is transduced; while for a sparser distributed expression, inert cell delivery may be more efficient.

mice (20); these are compared with the much higher values reported for stimulation in neural applications in vitro and in vivo, including Boysen et al. (17), Cardin et al. (24), and Wang et al. (128). The results are surprising if we only consider cable properties. In such case, neural applications should have not required higher energies than cardiac. Furthermore, in Bruegmann et al. (20) atrial myocytes were found to express ChR2 at higher levels and produce bigger functional currents than ventricular myocytes. Yet, when they were tested at the tissue level, a puzzling result was the higher energy needed to excite atrial muscle than ventricular tissue, which theoretically should have presented bigger electrotonic load. Clearly, multiple factors are at play. Considering that gene targeting will rarely result in perfectly uniform expression, we also explored computationally how the energy of stimulation will depend on the spatial distribution of gene (viral) or cell delivery (Fig. 5B). Direct ChR2 expression in native myocytes (gene delivery) and indirect expression via nonexcitable cells (TCU approach of cell delivery) were simulated using a stochastic algorithm to yield different levels of localization and consolidation of the islands of ChR2-expressing cells. The results show that lower energy is needed for (viral) gene delivery in myocytes if a single localized area is transduced; while for a sparser distributed expression, inert cell delivery may be more efficient (18).

When it is considered for cardiac pacing, an interesting question arises if optogenetic activation can be more energy efficient than electrical stimulation. Our in vitro experiments hinted at such possibility (64). If one compares directly the charge delivered by ChR2 current and electrical current injected in a rectangular pulse, then the waveform in the two cases (Fig. 3) suggests that optical stimulation may yield benefits for longer pulses but the instant-upstroke electrical pulses are more efficient at short durations. Without a doubt, stimulus delivery to the site of interest will profoundly affect the overall energy.

Unique cardiac utility. It has to be emphasized that the power of optogenetics is in offering unique ways to better address basic science questions. Whether it can transcend into more translational therapeutic uses remains to be seen for neuroscience and for cardiac applications.

As a basic science tool in cardiac research, optical pacing can offer contactless stimulation with higher spatiotemporal resolution, cell selectivity, and new ability for parallelization. When compared with classical stimulation by an electric field, optogenetics achieves better spatiotemporal addressing (cellular and subcellular) because of a combination of factors, but most importantly: *1*) selective cell type expression by virtue of genetic targeting and *2*) superior light focusing and controlled delivery of desired and well-localized excitatory or inhibitory action unlike the typical complex polarization induced by an electric field, involving islands of opposite polarity (known as virtual electrodes) around the target spot. These features can be useful for a number of research questions. One set of problems deals with probing and confirmation of cell-to-cell coupling, e.g., cardiomyocyte-fibroblast coupling or coupling between donor (stem) cells and host cardiomyocytes in regenerative cardiomyoplasty. Currently, there is no direct and specific method to address these questions in vivo in the native tissue. Optogenetics may offer solutions via selective cell type-specific expression and optical stimulation, if light access prob-
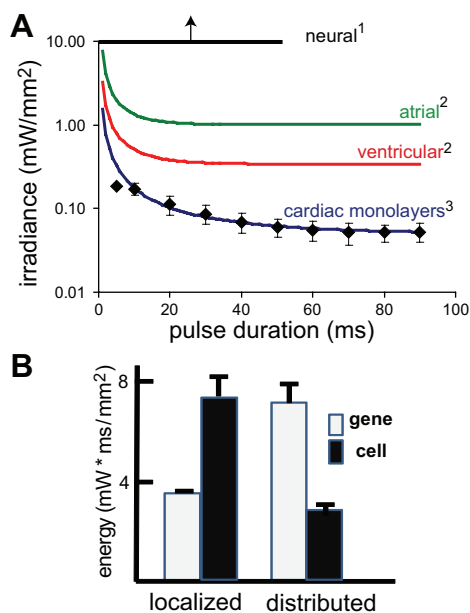
lems are resolved. A second related set of problems deals with initiation of focal arrhythmias. Suspected common sites, e.g., automaticity at endocardial Purkinje network locations (36, 110), can be systematically studied by cell-specific expression and perturbation by light to induce or suppress such activity. Critical contributions of different parts of the pacemaking and conduction system can be probed, especially by inhibitory opsins, as demonstrated in the zebra fish study (9). Such approaches may not only help understand arrhythmia induction but may offer new anti-arrhythmic strategies. A third set of problems suitable for in vitro investigation are related to mechanisms of reentrant arrhythmias and their termination. Precise dynamic optical probing can be used to address the exact nature of reentrant activation and the state of the reentrant core, spiral wave versus leading circle (61). The search for mechanisms of atrial fibrillation (60) or ventricular fibrillation (29), mother rotor, wandering wavelets, or other, can be better tackled by fine stimulation tools to establish vulnerability, and may have real impact on devising better defibrillation strategies.

Classical defibrillation works by synchronous depolarization of a critical mass of the myocardium (>95%) using strong electric shocks; lower energy alternatives are pursued by the proper timing of multiple electrical shocks to interact with and extinguish reentrant waves underlying an arrhythmic episode (85, 109). Because of penetration depth issues, optogenetic termination of arrhythmias (cardioversion and defibrillation) is likely to explore methods similar to low-energy electrical

cardioversion without the need to capture 95% tissue. In addition, when compared with electrical methods, optogenetics offers superior localization control of excitatory or inhibitory perturbations. Global hyperpolarization (or forced repolarization) is hard to achieve by electric fields; thus optical suppression offers a new tool. It is interesting to point out successful termination of epileptic seizure activity by light (using inhibitory HR) in hippocampal tissue slices in vitro (123), as epilepsy and cardiac arrhythmias share some mechanistic similarities. The concerns with in vivo optical cardioversion and defibrillation are not with the magnitude of the photocurrents to overwrite ongoing activity but with the need for spatially distributed light delivery to successfully affect tissue.

It is unclear whether optogenetics can prove a "disruptive technology" for in vivo pacing and defibrillation, considering the success of the current devices. However, if potential safety concerns are resolved and if critical benefits are demonstrated, e.g., substantial battery life extension or pain reduction for defibrillation, then perhaps optical devices will be a good alternative.

Optogenetics strengths may yield other translational solutions. The possibility to combine optical stimulation with optical readout is particularly attractive (Fig. 6). After some early reports of single site or low-resolution optical measurements in conjunction with optogenetic stimulation in brain (3, 141), we demonstrated the combined use of high-speed ultra-high-resolution optical mapping with optical stimulation (64) (Fig. 6B). Such an approach can be adopted for all-optical
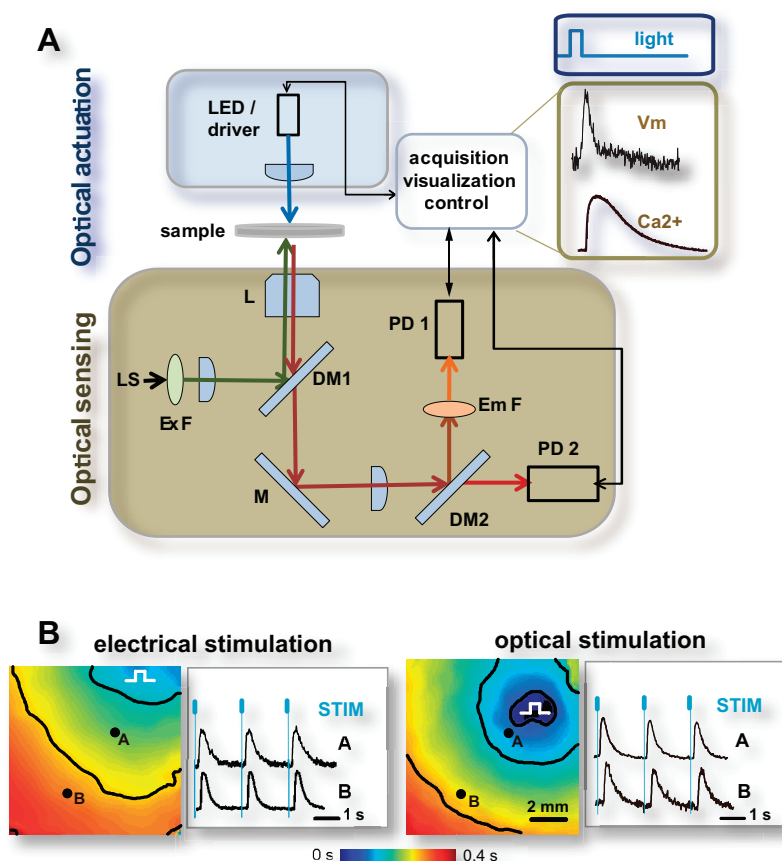

Fig. 6. All-optical actuation, sensing, and control of cardiac function. *A*: system for all-optical contactless cardiac electrophysiology, built around a microscope. Optical actuation is achieved through collimated light-emitting diode (LED)-produced light; optical sensing of voltage (V_m) and calcium (Ca^{2+}) is done by voltage-sensitive dye (di-8-ANEPPS) and calcium-sensitive dye (Rhod-4), respectively; shown are actual records (light pulse was 50 ms, 0.2 mW/mm^2). Computer controls the LED driver and the acquisition by the photodetectors (PD1, PD2), thus allowing for a closed-loop feedback control. L, objective lens; LS, light source for imaging; Ex F and Em F, excitation and emission filters, respectively; M, DM1, and DM2, full and dichroic mirrors, respectively. *B*: all-optical interrogation of cardiac function over time and space by combining high-resolution optical mapping with optogenetic actuation [used with permission from Jia et al. (64)]: waves of excitation in cardiac monolayers, triggered by electrical and optical pacing at 0.5 Hz, and captured by activation maps. Color represents time of activation; isochrones are shown in black at 0.15 s. Calcium transients (Rhod-4) in response to electrical or optical stimulation are shown from two locations, normalized fluorescence. Blue marks indicate time of stimulation (electrical pulses were 10 ms; and optical, 20 ms each). In *A* and *B*, cell delivery approach of ChR2 was used.

interrogation of cardiac electrophysiology (voltage or calcium) (Fig. 6A). Because of its completely contactless nature, it naturally lends itself to parallelization and scalability, as well as closed-loop feedback control.

The contact-requiring aspect of the classic electrophysiology techniques (often implemented manually) has been the main reason for the low-throughput nature of this method and has prevented its parallelization over the last 60 years. It is only in the last five years that modest success has been achieved at developing a new generation of automated planar clamp (74, 81, 114), not by removing the contact requirement but by achieving it using microfluidics and precise on-chip suction onto microfabricated holes. Thus developed "planar patch" currently allows for up to 48 channels of simultaneous clamping (93), a great improvement over the single-pipette manual operation. Nevertheless, these new developments remain well below (about 2 orders of magnitude below) the standard for high-throughput screening methods needed in drug discovery and in screening for possible side effects of already developed drugs (114). Therefore, one of the possible translational aspects of cardiac optogenetics is likely to come in realization of true high-throughput screening technology for drug testing.

More than optical control of voltage. The common use of microbial opsins, discussed here, relates to control of electrical activity, i.e., TM voltage. However, recent efforts have been made to extend optogenetics to include a broader range of tools for sensing and control of various physiological parameters (37), especially protein-protein interactions and cellular signaling. A recent example of a cardiac application used flavin-binding opsins to create light-induced fusion of calcium ion channels (Cav1.2) for better understanding of how oligomerization and channel clustering may affect the produced current in cardiomyocytes (35). Furthermore, in addition to the microbial class I opsins for direct control of voltage (Fig. 2), the optogenetics toolkit has been expanded to include derivatives of the vertebrate class II opsins, commonly referred to as OptoXR (4). These are G protein-coupled proteins that can interact with intracellular messengers, including cAMP, phosphatidylinositol 3-kinase, and 1,4,5-trisphosphate, and can provide precise optical interrogation of biochemical signaling. Even though such uses may tolerate lower temporal resolution, the selectivity and the spatial precision of manipulation offered by optogenetics tools are still desirable. Table 1 summarizes some of the available opsins for biochemical actuation.

Conclusions

Optogenetics has already proven to be an indispensable research approach in neuroscience. A vast number of useful light-sensitive actuation tools have been produced and made publicly available. We discussed how such tools can be adopted for cardiac applications and what challenges need to be addressed. Obviously, new developments are needed to accelerate cardiac use of optogenetics, especially in vivo. Only a handful of studies have been published to date, yet many current research questions on cardiac arrhythmias are amenable to optogenetic solutions, thus cardiac electrophysiology is likely to embrace and benefit greatly from this emerging technology.

DISCLOSURES

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

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