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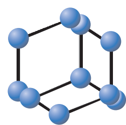

Feasibility of Using Adjunctive Optogenetic Technologies in Cardiomyocyte Phenotyping – from the Single Cell to the Whole Heart

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Abstract: In 1791, Galvani established that electricity activated excitable cells. In the two centuries that followed, electrode stimulation of neuronal, skeletal and cardiac muscle became the adjunctive method of choice in experimental, electrophysiological, and clinical arenas. This approach underpins breakthrough technologies like implantable cardiac pacemakers that we currently take for granted. However, the contact dependence, and field stimulation that electrical depolarization delivers brings inherent limitations to the scope and experimental scale that can be achieved. Many of these were not exposed until reliable *in vitro* stem-cell derived experimental materials, with genotypes of interest, were produced in the numbers needed for multi-well screening platforms (for toxicity or efficacy studies) or the 2D or 3D tissue surrogates required to study propagation of depolarization within multicellular constructs that mimic clinically relevant arrhythmia in the heart or brain. Here the limitations of classical electrode stimulation are discussed. We describe how these are overcome by optogenetic tools which put electrically excitable cells under the control of light. We discuss how this enables studies in cardiac material from the single cell to the whole heart scale. We review the current commercial platforms that incorporate optogenetic stimulation strategies, and summarize the global literature to date on cardiac applications of optogenetics. We show that the advantages of optogenetic stimulation relevant to iPS-CM based screening include independence from contact, elimination of electrical stimulation artefacts in field potential measuring approaches such as the multi-electrode array, and the ability to print re-entrant patterns of depolarization at will on 2D cardiomyocyte monolayers.

Keywords: Optogenetics, channelrhodopsin, halorhodopsin, archearhodospin, iPS-cardiomyocyte, cardiac, drug screening, re-entrant arrhythmia.

1. INTRODUCTION

Historically, investigating how cardiomyocytes turn on and off in response to small molecules or disease-causing mutation required animal explant material studied by a combination of patch clamp electrophysiology and electrode stimulation. Although this provides quantitative data with very high temporal resolution (>kHz) these approaches require contact with the cell, and with the dish which compromise throughput and preclude spatial resolution. The human heart beats over a modest physiological range (0.5-4Hz), and clinically relevant perturbations exert effects measured in tens of milliseconds. Therefore it can be argued that the primacy afforded to temporal resolution (which is at least two orders of magnitude more than is required) in this classical approach, at the expense of spatial resolution and scale, is ultimately disadvantageous. Alternative approaches using light-based stimulation of cardiac material in combination with optical or electrode based phenotyping platforms appear to offer solutions to some of the problems classical methodologies were not able to solve.

This review describes the development and application of optogenetic control strategies in cardiac biology. We focus on the three technical challenges that have been overcome to enable this. Firstly, the equipment infrastructure needed to pattern and control light with sub-millisecond, and sub-micrometer precision. Secondly, the gene products that make excitable cells light sensitive. And thirdly, the strategies for gene delivery in the context of various experimental space-scales. A graphical overview is presented in Fig. (**1**).

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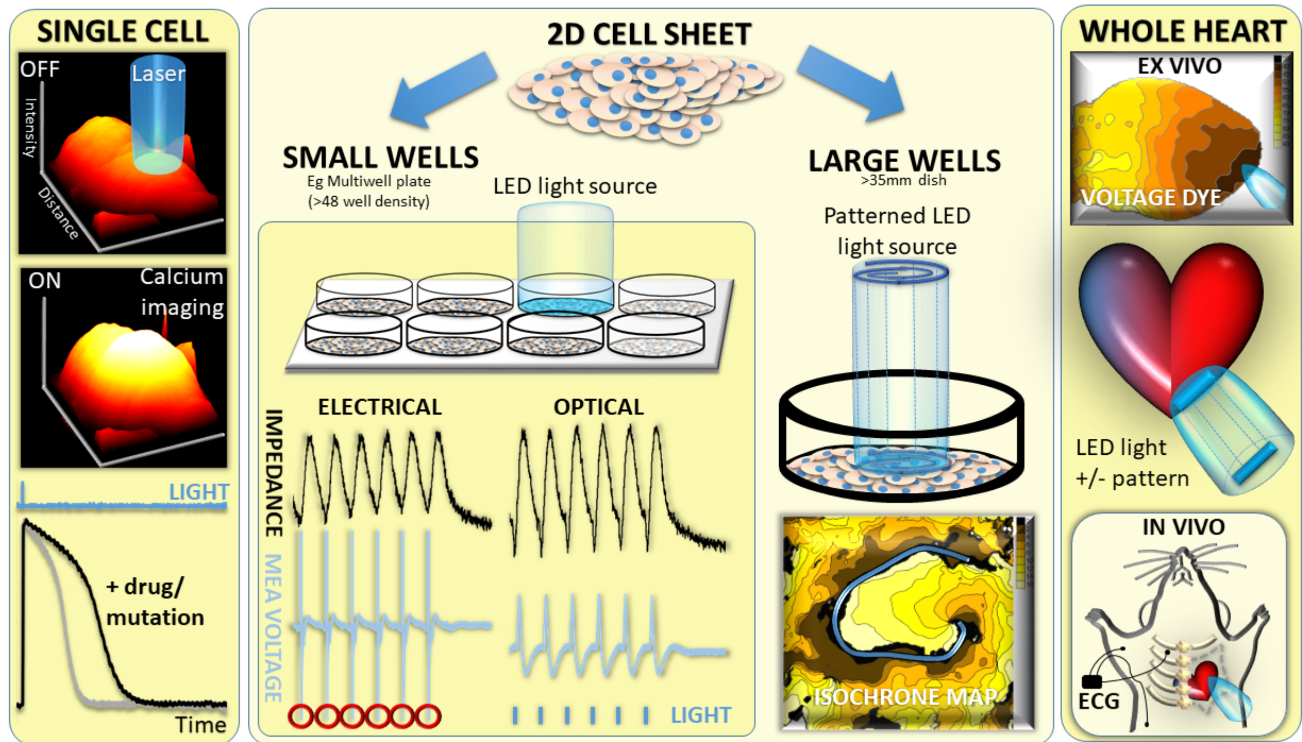

Fig. (1). An overview of depolarising optogenetic control, and compatible phenotyping readouts across relevant cardiac space scales. Selective single cell stimulation can be achieved in adult or iPS derived cardiomyocytes using laser light spatially restricted to subcellular volumes by an acousto-optic tunable filter, and combined with simultaneous optical measures of cell activation in order to reveal effect of small molecules or disease causing mutation [36]. In 2D cell sheets, small well formats (*e.g.* >10^4 cells/well) can utilise LED based all-or-none optogenetic stimulation to replace electrical pacing in existing commercial platforms, example data is shown from the CardioECR platform (ACEA Biosciences) where simultaneous measures of contractility by impedance are combined with an integrated MEA field potential under electrical (left) and then optical (right) stimulation. Note the lack of the pacemaker artefact (red circles) in the optically stimulated sample [*Daniels lab unpublished*]. By contrast, at larger cell numbers (10^6/well) and space scales (>2cm) re-entry waves can be printed, and controlled, with patterned LED light sources and observed by dye free motion analysis [18]. Finally, whole organ optical stimulation in the ex-vivo Langendorff perfused [32] or *in vivo* [53] rodent hearts can be used to study selective sympathetic nerve stimulation, optical pacing or defibrillation with voltage dye, or surface electrocardiogram readouts.

To help the reader choose appropriate combinations of contemporary technologies for their particular application, we summarize all commercial platforms incorporating an optical control capability, and all cardiomyocyte-based applications of optical stimulation reported to date. We show how these overcome barriers of traditional electrode stimulation that have led to contact independent screening of single cells; freedom from electrical stimulation artefacts in combined MEA/impedance platforms; production of complex (and clinically relevant) patterns of depolarization in 2D monolayer constructs in addition to application at the whole heart scale *in vitro* or *in vivo*.

1.1. Electrode Based Recording and Stimulation

Patch clamp and sharp electrode-based electrophysiology techniques precisely measure and control membrane potential and can be used to deduce individual ionic current contributions to the action potential morphology. While patch clamp is effectively the gold-standard technique for pharmacology and drug screening, it has two limitations. First, patch clamp is restricted to single cell preparations, as maintaining voltage clamp conditions over large areas is impossible. A consequence of this limitation is that patch recordings offer a limited window on cardiac physiology at the tissue level. Second, conventional single electrode techniques are intrinsically low throughput. The in- experienced user must manually impale each cell and perform measurements in series. High throughput patch systems can perform these experiments in parallel [1] but are mostly suited for measurements in cell-lines, are costly, and were generally considered to be less accurate than single cell measurements [1, 2] (but show recent improvements in this regard [3]).

Extracellular electrodes can indirectly measure action potential morphology (*e.g.* action potential duration) and are also useful for pacing protocols where the exact timing of evoked action potentials is an important control parameter, such as measurements of tissue repolarization dispersion and action potential and conduction velocity restitution [4, 5]. Extracellular electrodes can be fashioned from non-reactive metal wires, carbon electrodes, or electrodes patterned using photolithography techniques on glass surfaces. Single extracellular electrodes must be manually positioned using micromanipulators adding cost and complexity. Alternatively, electrodes can generate large electric fields that simultane-

ously stimulate all cells in a wide area, but mismatches of stimulation field size and observation area are not ideal as electricity is harmful to biological material. In contrast, photolithographically patterned electrodes enable higher throughput cardiac cell-culture studies as they can be fashioned into multi-electrode arrays (MEAs) which can record and pace many wells in parallel [4-6]. The main drawbacks with MEA based approaches are the cost of the dishes, which have limited re-use potential as the patterned electrodes are degraded by the salt solutions required for cell culture; the requirement for large numbers of cells to generate a measurable field potential; and the finite scales that can be achieved imposed by the wiring required for stimulating and recording electrodes.

Finally, electrical stimulation is not perfect. In addition to contact with the dish, or the cell (which constrains scale) stimulation is not homogeneous. Areas of depolarization and hyperpolarisation are produced within the stimulation field. The voltage a cell is exposed to depends on the distance, and orientation, with respect to the electrode. Voltage cannot be patterned to create clinically relevant entities. Faradaic reactions produced at the electrode produce radicals that are directly harmful to cells. At the whole animal scale, electrical pacing requires electrode contact with the heart, with associated problems in lead fragility, migration, and infection. Furthermore, power consumption, particularly during defibrillation, is both high, and from the patient perspective intensely painful. Alternative methods to depolarise cells, free from the inherent limitations of electrode stimulation have been sought and developed. The most versatile platform to date – optogenetics - allows photons to replace electrons in the initiation of an action potential.

2. OPTOGENETICS

Cardiac optogenetics is a catch-all term for techniques that allow cardiac cells to be controlled by light through a genetically encoded component. Optogenetics was pioneered in 2002 initially in neural tissue [7], and became increasingly popular once improved light-sensitive microbial opsin-channels (called channelrhodopsins (ChR2)) were produced [8]. Cardiac applications were first introduced in 2010 [9, 10] and are now rapidly being adopted by the experimental community as an alternative method for pacing and cardioverting tissue with light [11, 12]. This approach enables optical pacing, and overcomes many of the inherent limitations of direct electrical stimulation.

2.1. Optical Pacing

Optical pacing methods have several advantages derived from the exquisite ability to control light, and the ability of the relatively harmless photon to travel through transparent material, and even to penetrate tissue. This makes it possible to precisely target small areas within a field, with subcellular spatial precision if needed. Since the enabling component is genetically encoded, techniques to restrict optical activation to a subset of cells within mixed populations based on promoter activation is now straightforward. Light intensity is easily controlled, therefore fine control over the membrane potential by varying the illumination strength is possible. Patterns of optical stimulation are simple to create and project onto biological specimens. In addition, optical methods are easily implemented and are relatively inexpensive. This is particularly true when combined with optical readouts of cell physiology within microscopy platforms as the light path that optogenetics requires is invariably present.

Optical actuation methods have their own limitations which need to be considered. First, while ChR2s are generally well tolerated in cardiac cells, additional ion channels if leaky may alter the physiology of the cells beyond adding light sensitivity. Assessing the behaviour of the preparation with and without ChrR2s in dark conditions to determine whether opsins have detrimental effects on their preparation is advised. Second, intense light can cause cardiac cells to fire in the absence of ChR2s. Here, the mechanism is controversial but may include local temperature gradients causing calcium release [13-15]. While this is not a concern in most optogenetic experiments, researchers should ensure that wavelengths and optical power do not approach those used in these ChR2-free studies. Third, ChR2 transgene expression within a sample is variable, particularly with transient gene delivery methods. Since photocurrent depends on channel number, cells may display heterogeneous responses to the same light intensity. While this effect can be largely ignored in multicellular cardiac preparations as light sensitivity is effectively averaged over many cells, this becomes increasingly relevant as cell number reduces. Although transgenic approaches may facilitate more homogeneous transgene expression, they tend to do so at the expense of lower protein levels compared to transient methods, which therefore makes cells harder to capture with optical stimulation. This, and a number of other technical considerations for successful optogenetic application in single cells have recently been reviewed [12]. Finally, in the drug screening context it is possible that some of the "*hits*" may be exerting their effects through the optogenetic component. The first step of target deconvolution therefore requires electrode stimulation to identify that group of compounds.

3. OPTOGENETIC COMPATIBLE CELL PHENOTYPING

Stimulating the cardiomyocyte without being able to study the response is meaningless, compatible detection methods must be employed at the same time as light triggered depolarization. While patch and sharp electrode methods give precise measurement of membrane voltage, these methods usually result in damage to the cell, preventing repeated measurement, and constraining scale. While MEA systems facilitate higher throughput applications, these electrodes measure extracellular potentials, providing indirect readouts of membrane voltage and no information on internal ion concentration. Optical detection offers several advantages over electrode-based techniques. For example, MEA's have much lower spatial resolution than most optical based systems as the number of electrodes is far less than the number of pixels that can be acquired in the same area. Optical techniques are far less labour and equipment intensive, lending them to genuinely high throughput studies.

Images of cell activity can be obtained using exogenously applied dyes or proteins which are sensitive to membrane voltage, cell calcium or other ion species, or directly measure

cardiac contraction using dark field, phase or other light refraction-based techniques. It is beyond the scope of this article to review the indicator literature, but a recent review is available [12], and a few caveats are worth highlighting. First, while optical probes show relative changes in membrane voltage or ion concentration, obtaining absolute measurement of these parameters involves ratiometric techniques as well as extensive calibration [16], which is not the case for sharp or patch electrode methods. Optical measurements may also be adversely affected by tissue motion. While this can be reduced using pharmacological agents to uncouple motion from ionic activity, off-target effects may result from their use. Conversely, dye free imaging methods depend on tissue motion and are useful where emergent mesoscopic tissue properties are measured (*i.e.* wave speed and pattern formation as opposed to membrane voltage and ionic currents) [17, 18], but here care must be taken as voltage and calcium transients can occur with little or no tissue motion, and in three dimensional tissues (including thick monolayer preparations plated on soft substrates) the motion transients do not necessarily map directly to changes in membrane voltage [19, 20].

Finally, scattering effects, which occur when photons move through living tissue and their substrates, impose fundamental limits on the spatial resolution optical based detection and stimulation systems can achieve. Bishop *et al*. [21] reported that photon scattering effects blurs the location and time course of optically captured action potentials. This is especially problematic in connected sheets of cardiac cells, as each optically detected action potential averages data over several hundred micrometres, irrespective of detector pixel size. A similar effect will limit the spatial resolution of an optical stimulus: researchers should assume that several hundred cells are potentially excited by an idealized illumination point source. This specific limitation can be overcome within certain confocal microscopy configurations if single cell, or subcellular, stimulation is required. Therefore, although optical methods deliver the simplicity, scale, duration, and spatial resolution required at low cost, some applications which required kHz resolution may still rely on contact-based approaches. Photon scattering becomes particularly limiting for stimulation and observation in thick samples.

4. HARDWARE COMPONENTS

Development of optogenetic capabilities across the single cell to whole organ space scale has required parallel development of equipment infrastructure to exploit the advantageous properties light sensitive ion channels confer. Optogenetic high throughput imaging systems must have specialized hardware for light delivery, light detection, drug delivery and data storage, and may include robotics for automation. Several systems are commercially available, and are summarized in Table **1** [22-30]. The sophistication of optical stimulation in current platforms is limited to whole well actuation at present. Specific details about the versatility of optical control are lacking in product literature. The relevant parameters to consider are the light wavelength (which must match the opsin excitation spectrum), combined with the ability to control pulse duration, light intensity, and pulse frequency which underpin the pacing stimulus. If parallel phenotyping occurs, spill-over of stimulating light from one well to another can be problematic.

4.1. Light Delivery

Outside the "point-and-shoot" requirements of high-content commercial platforms there has been sustained effort to exploit additional capabilities derived from optical pacing. In contrast to electrode stimulation, excitation light can be delivered globally (evenly across the entire imaging field) or localized, either in a spot or as a pattern. Most systems either use a laser or light emitting diode (LED) as the photon source. Laser based systems can deliver higher intensity light to samples, but in most cases LED based systems are sufficiently bright. Both LEDs and lasers can be easily positioned in a standard optical system to illuminate the entire field of view by imaging light on the back focal plane of the imaging objective. Laser systems may include a galvonometer based or acousto-optical deflector (AOD) [31] based beam steering system in order to position light on the sample, or rapidly scan the sample for patterned illumination. LED based systems can deliver patterns using projector optics, which can be either liquid crystal or micromirror array based. While laser steering is conceptually simpler than using projection systems, the latter are generally less expensive and easier to maintain as the technology is found in consumer electronics [18, 32]. In addition, while lasers can be used in conjunction with projector based systems, the use of coherent light in micromirror based projectors can give rise to fringes as light is refracted on the mirror edges.

LED projection and laser AOD based systems have sub-millisecond temporal resolution sufficient for use in protocols requiring precise delivery of light energy. The stimulation frequency and duration of projection is determined by the refresh rate (typically 30 or 60 Hz for high-street devices) however this is not an intrinsic limitation of this platform and systems with 30KHz update frequencies are available. In addition, video rate projection systems can be used for most experiments as the LED light source can be controlled independently of the micromirror array, giving temporal microsecond resolution (*e.g*. required when obtaining a restitution curve).

4.2. Detection

The detection subsystem will largely depend on the type of signal required. Experiments that require precise measurement of action potential duration or membrane voltage upstroke velocity require measurements at kilohertz rates or faster. In some cases, this can be measured with a point detector (a photodiode or photomultiplier tube), but often contact based electrodes are used. By contrast, calcium transient duration, beat rate, conduction velocity and pattern formation (*e.g*. presence of re-entrant patterns of excitation) require imaging systems that capture data at lower temporal frequency but with higher spatial resolution. Many camera systems can deliver the minimum video rate (>20Hz) temporal resolution required from fluorescent samples, but measuring activation patterns and velocity from fluorescent signals requires faster and more sensitive, and therefore more expensive, cameras. Finally, dye-free measurements of conduction velocity or pattern formation can use very inexpensive cameras as light levels are not limiting, even at high frame rates.

Table 1. Commercially available high throughput cardiac screening platforms that include optogenetic stimulation.

HTS Platform	Type	Assays	Wells	Sensors	Actuation	Robotics	Refs.
cellOPTIQ Clyde Biosciences	Service academic	Ca, Vm, contractility	48	PMT (10Khz)	LED	Inverted microscope, Automated stage,	[22]
OptoPatch QState Biosciences	Service	Ca, Vm	NA	Camera based	LED	Custom	[23, 24]
OptoDYCE	Service academic	Ca, Vm Conduction velocity	96, 396	Camera	LED	Inverted Microscope Automated stage	[25]
FLIPR	Commercial Device	Ca (per well)	up to 1536	Camera	LED	Automated stage, fluid handling, plate handling (third party)	[26]
FDSS7000EX Hamamatsu	Commercial Device	FRET, Vm, Ca	up to 1536	Camera & PMT	Arc Lamp	Automated stage, fluid handling, plate handling	[27]
Axion Biosystems	Commercial Device	Extracellular potential (EEP)	48	MEA	LED	N/A	[28]
Nanion Cardioexcite 96	Commercial Device	EEP, contraction	96	MEA Impedance	LED	N/A	[29]
Vala Kinetic Image cytometer (KIC)	Commercial Device	Ca, video	Up to 1536	Camera based	LED	Automated stage, fluid handling	[30]

4.3. Environmental Control

Cardiac tissue phenotype depends on maintaining physiological conditions, as temperature and pH impact cardiac currents and metabolism. In most cases, the tissue is maintained close to 37°C, with adequate oxygenation and in a buffered medium. High throughput systems either depend on using a stage top incubation system, an enclosure that covers most of the microscope, use systems that fit within a standard incubator, or build systems that combine optics and environmental control in one unit. The optogenetic tools themselves also demonstrate thermal sensitivity, working better at body, rather than room temperature [12].

4.4. Sample Positioning and Drug Delivery

High throughput systems include some method of positioning the sample in the optical path of the detection and light delivery systems. This contrasts with multi-electrode (MEA) based systems, where detection electrodes are positioned in each sample (typically a multi-well plate). Simple high throughput systems consist of a motorized stage which positions a single multi-well plate in the optical path, allowing many samples to be measured sequentially. In these systems, a technician must swap multi-well plates in order to measure additional samples. More complex systems incorporate additional automated components to either move samples from an incubator to the imaging system or use in-incubator systems that can scan several plates on a platform, either by moving the samples or by moving components of the imaging path. The Hamamatsu FDSS/μCell system described above is notable because the optics within it allow all wells in a 96 well plate to be recorded simultaneously rather than sequentially. We are not aware that the theoretical capability to achieve this in higher well density formats has been demonstrated for cyclical cardiomyocyte activation.

Robotics is also used in automating administration of pharmacological agents to samples, especially in larger systems. In general, pharmacological agents are delivered *via* pipetting robot, although microfluidic approaches also have potential here. While precise delivery of pharmacological agents is an important component in any high throughput drug screening setup, optogenetics does not offer specific advantages in this area. Indeed, as cell number and well size decrease, optogenetic approaches start to lose some of their advantages – for example conduction velocity cannot be measured, patterned stimulation cannot be achieved or propagated, stimulating light can spill over into adjacent wells as it is scattered by the culture vessel.

4.5. Data Handling and Analysis

High throughput imaging systems can generate terabytes of raw data that must be stored before analysis. While data throughput and storage issues were rate limiting in the past, most modern computers, coupled to commercially available solid-state disk arrays can handle data produced from high throughput imaging systems. Data analysis, however, is still highly non-trivial and requires specialized software, which often must be developed or optimized for each application on a case-by-case basis. As large data libraries are established there would appear to be significant potential for advanced mathematical or computational modelling efforts to augment the data extraction process.

5. OPTOGENETIC TOOLS AND THEIR DELIVERY

Having discussed the technical aspects of equipment infrastructure, which in essence can be reduced to a stimulating light source in combination with appropriate detector apparatus, it is important to consider the optical interface that allows a light signal to be converted to an electrical one inside cells. The typical depolarizing or hyperpolarizing opto-

genetic toolset are derived from proteins found in green algae that enable them to swim towards light (Channelrhodopsin**,** ChR) or light dependent archaebacterial ion pumps that facilitate survival in high salt or at extremes of pH (Halorhodopsins, or Archaerhodopsin) [12]. The photosensitive mechanism in all of these tools derives from the common requirement for the vitamin A derivative retinal, and its photon induced *all-trans* to *13-cis* isomerisation, in order to open a gated ion pore. Retinal may be limiting in conventional tissue culture environments and supplementation is recommended for some applications [33].

As is typical for repurposed gene products from the natural world applied artificially to the cardiomyocyte; the baseline performance of the initial material can be enhanced by random, or directed mutation strategies and complemented by gene discovery efforts in other candidate organisms. The main limitations of the early channelrhodopsin tools were physical size, GC codon usage, which reduced expression levels, limited ion conductance 20-50 fS (typically x1000 less than voltage gated sodium channels), low retinal affinity, and slow kinetics. Collectively these compromise the photocurrent that can be generated. Although it is beyond the scope of this article to describe the efforts to improve ChR functionality, which can be accessed here [34], the most commonly used variant in cardiac applications remains the early described H134R ChR2 mutant. Since the physiological range of the cardiomyocyte does not exceed 6Hz it is possible that the relatively slow inactivating variants may be advantageous in cardiac applications as the triggered photocurrent depends on both the number and duration of channel opening among other things. Therefore, keeping pace with developments in optogenetic tools may be less relevant in slower samples like the human heart, compared to the 100Hz requirements of neurobiology for example.

By contrast to depolarizing channelrhodopsins, hyperpolarizing tools pump protons out (archaerhodopsin) or chloride ions in (halorhodopsins) in response to light. A number of variants are now described [35]. In general terms these have made less progress in cardiac application than the depolarizing equivalents. Since both hyperpolarizing and depolarizing optical control tools are genetically encoded, a natural obstacle to cardiomyocyte application is transgene expression, particularly in primary cardiomyocytes which have a limited viability. Since successful application of optogenetics depends on production of a photocurrent large enough to trigger depolarization, expression levels can be critical determinants of experimental outcome. A number of viral [18, 25, 29, 36-53] and non-viral methods [9, 10, 25, 29, 31, 32, 48, 54-63] have been successfully applied to the cardiomyocyte. For *in vivo* cardiac application tissue tropism with adeno-associated virus (AAV) types 1 or 6 might be preferable to type 9 used *in vitro* [64]. Table **2** summarises the gradual progress made in cardiac optogenetic application. It should be noted that no comparative study of optical actuators (*e.g*. ChR2 H134R *vs*. a more recent ChR2 such as CheTAtc) has been reported in cardiomyocytes to date. Many studies only consider applications in single cell types, only rarely demonstrating compatibility across adult ventricular, and more immature cell substrates like embryonic or induced pluripotent stem-cell cardiomyocytes (hES- or iPS-CM) *e.g*. [36] or space scales [38, 39]. Limited comparisons are available for the mechanism of gene delivery (spark cell *vs*. adenovirus) *e.g*. [25]. Finally, toxicity or efficacy studies using small molecules are relatively recent additions to this landscape, paralleling the conversion from proof of concept work in animal explant material to human stem-cell derivatives from commercial sources.

Although traditionally optogenetic control has been enabled by genetically encoded components it has been difficult to narrow the absorbance spectrum, increase the photocurrent, and reduce the photon number required for optical control to a level unlikely to cause phototoxic effects. Day-to-day challenges related to gene expression persist. These difficulties have prompted some to explore alternative strategies that still start with a photon, rather than an electron, as the trigger for depolarization but rely on different photophysical phenomena to generate a current. The simplest example of this apparently requires nothing more than IR irradiation [13-15, 65] (1862nm light, 3-4 ms pulse $^{-1}$, ~10 Jcm $^{-2}$ pulse $^{-1}$) which is thought to trigger depolarisation by a photothermal effect. Adaptations to the material interface cells are grown on can include photosensitive coatings like graphene that generate local current in response to incident light [66]. Cardiac application of these alternative strategies are limited but they may have advantages as they do not require gene transfer, time for protein expression, or membrane localisation. Moreover, their production should follow simple materials principles which may help iron out variability inherent in biological systems.

6. CONSIDERATIONS FOR IPSC CARDIOMYOCYTE SUBSTRATES

The unifying theme for this collection of articles is the added value that induced pluripotent stem cell (iPSc) derivatives may deliver in the drug discovery process. Although it is clear from Table **2** that pioneering work in optical control of cardiac tissues has relied on neonatal rat ventricular or atrial myocytes (NRVM or NRAM) or adult whole heart to establish feasibility, the potential cross-over to use human iPSc derivatives as a renewable *in vitro* cell model of disease [67] in place of these cell types has been demonstrated [25, 36, 29, 42, 44, 55].

The iPSc approach is particularly relevant for cells like cardiomyocytes or neurons that are inaccessible and terminally differentiated, as they cannot be expanded in culture and typically have a short life *in vitro* following isolation. Hundreds of patient genomes are now represented in iPS repositories established internationally [68]. The last decade has witnessed parallel advances in protocols for cell production, and also genome editing which complement the iPS approach. Collectively these have fostered the environment needed for the simultaneous emergence of cell providers (*e.g*. Cellular Dynamics International, Ncardia, Axol Bioscience) which are reliably able to manufacture material on a scale and quantity needed for the industrial application. It falls outside the scope of this article to cover how these cell types are produced, but it is worth noting that among excitable cells, neurons (cortical excitatory, motor neurons, interneuron, GABA, Dopaminergic, …), astrocytes, microglia, cardiomyocyte (atrial, nodal, ventricular) cardiac fibroblasts, endocardial, vascular smooth muscle (large and small vessel),

Table 2. Applications of cardiac optogenetic control across all reported space scales in chronological order with the most recent study first.

	Control Tool	Delivery Method	Cell Type	Supplier	Assay Performed	Purpose	Refs.
Single cell	CheTAtc	Adenovirus	vGPCM/hiPS-CM	Axol	Ca transient analysis (genetic R-GECO)	Toxicity	[36]
	Arch3 & GtACR1	Lentivirus	NRVM	Rat	Patched AP	Tool validation	[37]
	ChR2 H134R	AAV9	vMCM	Mouse	Singularised vMCM stimulation, patch	Proof of concept	[38]
	ChR2 L132C	Lentivirus	NRAM	Rat	Patched AP	Proof of concept	[39]
	ChR2 H134R	Transgenic (β-actin)	mES-CM	Mouse	White light, Ca (dye)	Proof of concept	[10]
2D sheet	ChR2 L132C	Lentivirus	NRAM	Rat	Voltage dye, spiral wave control	Efficacy	[40]
	ChR H134R	AAV 2.1 or mRNA	iPS-CM Cor4U	Ncardia	MEA field potential duration	Toxicity	[29]
	ChR2 L132C	Lentivirus	NRAM	Rat	Voltage dye, spiral wave termination	Efficacy	[41]
	OptoPatch	Lentivirus	iPS-CM Cor4U	Ncardia	Patched AP, Ca and Voltage (CaViar)	Toxicity	[42]
	ChR2 H134R	Adenovirus	iCELL	CDI	Ca and Voltage (dye)	Toxicity	[25]
	ChR2 H134R	Spark cell	iCELL	CDI	Ca and voltage (dye)	Toxicity	[25]
	ChR2 H134R	Adenovirus	Neonatal cardiac fibroblast	Rat	Voltage (dye)	Proof of concept	[43]
	OptoPatch	Lentivirus	iCell	CDI	Ca and Voltage (genetic)	Toxicity	[44]
	ChR2 H134R	Transfection	NRVM	Rat	Collagen matrix movement	Proof of concept	[54]
	Arch3	Cell (HEK293T)	NRVM & ESC-CM	Rat, H9.2 clone	MEA	Proof of concept	[55]
	ChR2 H134R	Adenovirus	NRVM	Rat	Ca (dye)	Proof of concept	[45]
	ChR2 H134R	Adenovirus	NRVM	Rat	White light contraction	Proof of concept	[18]
	ChR2 H134R	Adenovirus	NVRM	Rat	Calcium (dye)	Proof of concept	[46]
	ChR2	Lentivirus	NVRM	Rat	Voltage (dye)	Concept & toxicity	[47]
	eNpHR3.0	Lentivirus	NVRM	Rat	Voltage (dye)	Concept & toxicity	[47]
	ArchT	Cell (NIH3T3)	NVRM & ESC-CM	Rat	MEA	Proof of concept	[48]
	ChR2	Cell (NIH 3T3)	NVRM & Esc-CM	Rat	MEA	Proof of concept	[48]
	ChR2 L132C	Lentivirus	NRAM	Rat	Voltage (dye)	Spiral wave termination	[39]
	ChR2 H134R	Lentivirus	hES-CM	H9 clone	White light beat rate	Proof of concept	[49]
	ChR2 H134R	Cell (293T)	Adult & NVRM	Dog & Rat	Ca (dye) white light contraction	Proof of concept	[56]
Engineered Heart Tissue (EHT) or Organoid NONE TO DATE							
Whole heart	hChR2 H134R	Transgenic	whole heart	Mouse	Voltage dye, AV conduction, re-entrant circuit, intraventicular activation	Proof of concept	[32]
	hChR2 H134R	Transgenic (TOH)	whole heart	Mouse	Sympathetic control of heart rate	Proof of mecha-nism	[57]
	ArchT	AAV2/9	stellate ganglion	Dog	Heart rate, blood pressure, ischaemia induced ventricular fibrillation	Therapeutic	[50]
	ChR2 XXL	Transgenic	whole heart	Fruitfly	-	Proof of mechanism	[58]
	ChR2	Transgenic (M)	whole heart	Mouse	AV node conduction	Proof of mecha-nism	[59]

(Table 2) Contd....

	Control Tool	Delivery Method	Cell Type	Supplier	Assay Performed	Purpose	Refs.
Whole heart	hChR2 H134R	Lentivirus	cardiac slice	Rat	Voltage dye, re-entrant circuit ECG, Ca and Voltage dye	Efficacy	[51]
	hChR2 H134R	Transgenic (PNMT)	whole heart	Mouse	ECG, Ca and Voltage dye	Proof of mechanism	[60]
	ReaChR	AAV 9	whole heart	Rat	-	Proof of concept	[52]
	ChR2 H134R	Transgenic (α-Myhc)	whole heart	Mouse	Termination of electrically induced VT	Proof of concept	[31]
	ChR2 H134R	Transgenic (α-Myhc)	whole heart	Mouse	ECG, pacing and defibrillation Voltage dye	Proof of concept	[61]
	ChR2 H134R	Transgenic (α-Myhc)	whole heart	Mouse	-	Proof of concept	[54]
	ChR2 XXL	Transgenic	whole heart	Fruitfly	Heart rate	Proof of concept	[62]
	ChR2 H134R	Transgenic (α-Myhc & Purkinje fibre)	whole heart	Mouse	Voltage (dye), ECG, Ectopy, post MI VT	Proof of concept	[63]
	ChR2	AAV9	whole heart	Rat	Voltage (dye), electrode recording	Proof of concept	[53]
	ChR2 H134R	AAV9	whole heart	Mouse	*In vivo* pacing	Proof of concept	[38]
	ChR2 H134R	Transgenic	whole heart	Zebrafish	*in vivo* pacing and heart block	Proof of concept	[9]
	NpHR	Transgenic	whole heart	Zebrafish	*in vivo* pacing and heart block	Proof of concept	[9]
	ChR2 H134R	Transgenic (β-actin)	whole heart	Mouse	ECG	Proof of concept	[10]

For transgenic applications the promoter used is provided in brackets. Optopatch is the combination of an archaerhodopsin engineered as to work as a voltage indicator (QuasAr2) and a Channelrhodopsin, CheRiff, derived from *Scherffelia dubia*. CaViar is a genetically encoded calcium and voltage imaging tool based on Arch(D95N) and GCaMP5. Key: vGPCM = guinea pig ventricular cardiomyocyte; hiPS-CM = human induced pluripotent cardiomyocyte; NRVM = neonatal rat ventricular cardiomyocyte; NRAM = neonatal rat atrial cardiomyocyte; vMCM = ventricular mouse cardiomyocyte; AP = action potential; Ca = calcium; MEA = multi-electrode array; M = macrophage.

and pancreatic (alpha and beta) cells can be made on academic and sometimes commercial scales. In principle optogenetic applications to cell stimulation, as single cells (to investigate fundamental aspects of depolarisation) or as tissue constructs (to explore the propagation of depolarization within multicellular constructs that may be surrogates for clinically relevant entities like epilepsy, atrial fibrillation, or ventricular fibrillation) could be applied to any of these models.

While provision of a new human experimental platform for the heart was clearly welcome, a couple of observations are worth reflecting on. Firstly, the problem that loosely might be defined as maturation. A general finding for all iPS derivatives is that they approximate to the primary cell type of interest rather than completely recapitulate it. For example, in the case of the iPS cardiomyocyte, foetal rather than post-natal isoforms of contractile proteins are expressed [69]. If the gene of interest is not expressed, or expressed as an isoform not found in the adult the utility of the model is questionable. Even if expressed, the amount of protein might be lower than in the adult which compromises validity of certain functional assays. The net result of the collective features of immaturity result in a long lived (months) spontaneously beating, small, weak, mononuclear cell [70] reflecting a developmental stage only rarely affected by many of the disease relevant processes of adulthood that are of interest to pharma and academia. Producing cells with a more mature phenotype might be both a blessing, and a curse. In the case of the cardiomyocyte the gains would be more robust ionic currents, and larger contractile forces generated by proteins found in the adult heart. However, the loss of spontaneous contractility would compromise any phenotyping platform that did not have the ability to trigger depolarisation by electrical or optical stimulation. Moreover, a significant limitation of the adult cardiomyocyte is the limited viability in culture (days) which would make long term assays of cardiomyocyte form and function impossible.

A number of contemporary strategies have been developed to try and improve the raw iPS-CM product. These include chemical maturation [71, 72], 2D patterning for single cells [73] or cell aggregates [74], 3D culture environments either as organoids [75] or Engineered Heart Tissue (EHT) [76, 77]. Interestingly inclusion of an electrical stimulation protocol to the EHT model can further improve certain parameters of interest [78, 79] including formation of recognisable T-tubule architecture, increased mitochondrial density, and switch to fatty acid oxidation for energy production. It is not clear if similar approaches applied to a 2D cell sheet will produce similar changes, or whether optical stimulation could replace electrical stimulation in the extended training protocols described in EHT's that lead to improvements in the final cardiomyocyte product.

Considering that although the size of the normal heart is dominated by the cardiomyocyte population, it is worth recalling that by cell number they are only 30% of the total. This has fostered interest in incorporation of additional cell types into 2D and 3D experimental constructs, particularly

cardiac fibroblasts, epicardial cells and the autonomic neurons that have previously been used to augment conduction velocity in cultured neonatal myocyte models [18]. The hope is that these will engineer out variability seen in single cells and contribute to maturation of the cardiomyocyte population which more faithfully reflects the postnatal heart. They should do this without introducing problems attributable to asymmetric, or uneven secondary cell distribution within aggregates. Potentially inclusion of secondary cell-types may reduce signal and increase experimental noise from cardiomyocytes in certain experimental applications. For example, fibroblasts will absorb chemical dyes (increasing background noise) but will not generate a dynamic voltage or calcium transient in response to a depolarizing stimulus (reducing signal). An additional concern is the cost and complexity of these experiments. EHT's are typically grown and stimulated for weeks before detectable improvements become apparent, this might limit their utilisation within screening pipelines to the end of the drug discovery process even within large commercial organisations.

As the size and complexity of constructs increases experimental possibilities expand from single cell measurements of action potential duration, calcium transient visualisation and force toward more complex macroscopic 2D phenomenon such as conduction velocity, or re-entry. These experiments typically demand millions of cells as determining velocity requires measures of distance (provided by the cell mass) over time (a property of the camera). In addition, smaller cell areas are unable to sustain mesoscopic re-entry phenomena. At even greater scales, it may be possible to incorporate iPS material into models mimicking ventricular architecture [80] for structural models of heart disease. Since the cost and time to produce material at this scale is significant, the versatility of optical control to impart regional, and reversible stimulation or hyperpolarization without traumatic injury to the construct may be a considerable advantage in maximising experimental opportunities while minimising the associated costs.

7. EXPANDED EXAMPLES

Optogenetic stimulation simply represents a way to depolarize a cell or tissue with light, therefore it can be used to provide experimental consistency at fixed stimulation rates, used to generate restitution curves at variable rates, or employed to produce patterned stimulation of defined shape, speed and size at tissue scale. As shown in Fig. (**1**), application of the technique has been demonstrated from the single cell, to the whole heart in a variety of different experimental contexts (summarised in Table **2**). It can be argued that the added value of optical stimulation becomes increasingly apparent in cardiovascular research as space scales increase into ranges where macroscopic re-entrant arrhythmia can be generated and propagated as there is currently no other method capable of producing this experimental substrate. Additionally, there is a large unmet clinical need for effective antiarrhythmic agents where this approach appears relevant. In this section we consider a few examples that illustrate how optical pacing (section 1.2.1) has been combined with cardiomyocyte phenotyping (section 1.3) and in particular the additive value it brings.

7.1. Single Cell Scale

Potentially this represents the most cost effective scale to work at, but pre-supposes that the experimental model works well when singularised. Although this may be true for adult ventricular cardiomyocytes it is a less valid assumption for the current single iPS-CM [81]. The principle concern at this scale is the variable spontaneous depolarization frequency of the iPS-CM model. Since beat frequency affects the action potential duration, this introduces noise into the interpretation of a cellular phenotype (calcium transient, or action potential duration). By Nyquist sampling theory, higher rates of spontaneous activity (where action potential shortens) reduce apparent differences between control and disease/drug treated cells. Since pacing captures only at rates faster than the intrinsic beat rate, optogenetic stimulation of single cells automatically becomes restricted to a subpopulation within the dish, but can be combined with patched action potential [37-39], or Ca^{2+} dye [10] based phenotyping. A small group of potent ion channel modulators have been studied using optical control with genetically encoded calcium indicators [36] in the iPS-CM model (left panel of Fig. **1**). Early and delayed after depolarizations, in addition to changes to calcium transient duration or intensity were detected. These drug induced changes have also been reported in a number of iPS-CM channelopathy patient models, reviewed [82]. Therefore, in theory it may be possible to run the experiment in reverse and use it as a screening platform for agents ameliorating disease phenotype. However, in practice the level of input required from the operator for single cell selection, and stimulation brings many of the limitations of patching to bear and throughput is therefore reduced. In addition, variability in the single cell response to genetically inscribed optical pacing creates experimental inefficiencies.

7.2. 2D Multi-well Plate

The use of a cell syncytium averages out the requirements for ChR2 transgene expression, and homogenises the observed response to stimulation, so optical control becomes easier to deliver in this setting. There is also experimental data suggesting that iPS-CM's behave differently in groups rather than in isolation [83]. At this scale it is only possible to stimulate the whole well, but phenotypic readouts can include MEA field potential, dye measurements of membrane voltage or calcium transient, or white light measures of contractility. In the context of the MEA approach one of the main limitations of electrical stimulation is the detection of the pacing stimulus by the MEA electrode. This is often two orders of magnitude larger than the field potential generated by the cells, and therefore separating one signal from the other can be challenging. The MEA does not detect photons, so optical stimulation does not have this limitation, an example is shown for simultaneous impedance and MEA field potential measurement stimulated at 1Hz from our group in the centre left panel of Fig. (**1**). Applications in this context depend on the phenotyping method used, and for example if an all optical approach is used can include small molecule toxicity evaluation across a range of pacing frequencies for prolonged (>40hr) periods of time [44] or with sufficient bandwidth to be considered genuinely high throughput [25]. The added value of optical stimulation in this format at present may be limited, especially as beat frequency (typically

0.5-1Hz) is one of the key quality control parameters used by the major cell manufacturers. However, if discovery or toxicity pipelines are to be future proofed for the emergence of matured iPS-CM's (which will have suppression of the intrinsic beat rate) considering investing in this capability may be advantageous in the near future. In the high content setting, although there may not be much difference in the transient duration across the 0.1-1.5 Hz spectrum there would be significant experimental implications for dwell time, which would have cumulative impacts at scale. Optical stimulation can be used to manage this problem within any high density plate format as there is no need for electrodes within the dish. Therefore, this platform may facilitate high content assessments for toxicology, or disease specific drug discovery, particularly for disease phenotypes that can be captured and quantified by MEA, impedance, or voltage/calcium imaging.

7.3. 2D Mesoscale

Prior formats are poor surrogates of tissue properties such as conduction velocity, or re-entrant arrhythmia. At space scales above 2cm (approximately 1 million iPS-CMs) these phenomena become possible to measure, and control optically [18, 39, 40, 41]. An example of a spiral wave printed onto a 2D cell sheet of defined helicity and period is shown in the centre right panel of Fig. (**1**). Although many of the diseases subjected to an iPS modelling approach demonstrate abnormalities suggestive of overt arrhythmogenesis as single cells, it is a general observation that events in patient populations occur much less frequently than the dish based models may suggest. For example, prevalence of any manifestation of disease in Long QT is generally <50% by age 40 [84] by which time approximately 4 billion cardiomyocytes will have completed more than 1 trillion activations. Therefore, it can be argued that just as hERG inhibition may represent a safety signal in a single cell assay, we lack experimental tools to predict with any degree of certainty if single cell events will ultimately translate into tissue level events in clinical populations. Therefore, from a disease screening perspective the reductionist approach of looking for things that are apparently efficacious in single cell, or small 2D constructs may not be directly relevant to larger space scales where there appears to be an additional level of inertia in order prevent overt catastrophe. We are not aware of any applications to date that have investigated this by using a combination of iPS-CM disease models at the mesoscale with optically triggered re-entry but theoretically this appears worth investigating.

7.4. Animal Models

The fact that ChR2 can be incorporated into the genome without an obvious consequence to animal behaviour or cardiac function argues that its expression alone may be relatively harmless. This suggests that unexpected experimental outcomes are perhaps more related to how the optical control tool is being activated or how the material is being studied rather than the additional presence of this transgene. Applications at the whole heart scale either *ex vivo* or *in vivo* (right panel of Fig. **1**) are possible, and can be combined with surface electrodes [10, 50, 60, 61] pacing electrodes (*e.g.* to trigger ventricular tachycardia [31]) or imaging based mapping of tissue level activation [32, 51, 53, 60, 61]. Applications have tended to focus on dissecting out the role of the autonomic nervous system, as expression of the optical control tool can be restricted to certain cell types, or investigation the potential for optical pacing or defibrillation as an alternative to the current electrode based methods. Since a number of mouse models of inherited cardiac conditions have been developed it should be possible to introduce transgenic optical control in order to move away from proof-of-concept studies towards disease relevant applications. This may be better achieved in the *ex vivo* Langendorff perfusion system, which has no intrinsic rate of contractility, as the *in vivo* situation (500-600bpm) has only a very small window for optical capture which will probably only be possible to access *via* ECG gating of the light pulse.

CONCLUSION

Optogenetic approaches to cell stimulation have clear advantages at both ends of the space scale. In particular, at the single cell scale a principle limitation of field stimulation is a lack of single cell specificity such that the entire well is stimulated, even though only one cell is observed. Established practice for singularised ventricular cells typically requires a complete change in cells and media after 15 minutes of electrical pacing even though by that point only a handful of cells will have been studied, this is a particularly wasteful approach for IPS derivatives which are costly to make, maintain and singularise. The contact dependence of electrical stimulation also builds cost and complexity into multi-well dish formats and typically sets an upper limit on scale defined by the electrodes and the connections needed to the stimulus generator. Electrodes are typically not transparent and therefore occupy part of the field of view in imaging experiments. Optogenetic controls have no wires, have subcellular stimulation capability and can therefore avoid these issues [36]. That is not to say single cell optical control is straight forward, a number of technical considerations apply [12] which in our experience appear harder to overcome in single cells, compared to 2D cell sheets.

Even at space scales large enough to sustain and propagate re-entrant arrhythmia electrical stimulation is unable to create the pathological entities that have been recorded in patients such as the rotors and spiral waves in atrial fibrillation [39]. Therefore, these have only been studied as they form randomly. This is inefficient, and therefore impossible to apply in a screening pipeline as the arrhythmia would not be present when observed, and even if it were observed it would not be standardised either for a particular arrhythmia size, shape, or speed. Since optogenetic control comes from light, although typically stimulation required for a toxicology assay is little more complex than "point and shoot", adopting shape based light stimulations to print stereotyped patterns onto 2D [18], or 3D shapes [23] repeatedly is possible. Since re-entrant arrhythmia (AF, VT) is a scourge of modern medicine, one argument for the current lack of effective therapies is the absence of appropriate models for drug development and testing; the optogenetic strategy appears to offer a solution to this problem.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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