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Stimulating Cardiac Muscle by Light: Cardiac Optogenetics by Cell Delivery

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Abstract

Background—After the recent cloning of light-sensitive ion channels and their expression in mammalian cells, a new field, optogenetics, emerged in neuroscience, allowing for precise perturbations of neural circuits by light. However, functionality of optogenetic tools has not been fully explored outside neuroscience; and a non-viral, non-embryogenesis based strategy for optogenetics has not been shown before.

Methods and Results—We demonstrate the utility of optogenetics to cardiac muscle by a tandem cell unit (TCU) strategy, where non-excitable cells carry exogenous light-sensitive ion channels, and when electrically coupled to cardiomyocytes, produce optically-excitable heart tissue. A stable channelrhodopsin2 (ChR2) expressing cell line was developed, characterized and used as a cell delivery system. The TCU strategy was validated *in vitro* in cell pairs with adult canine myocytes (for a wide range of coupling strengths) and in cardiac syncytium with neonatal rat cardiomyocytes. For the first time, we combined optical excitation and optical imaging to capture light-triggered muscle contractions and high-resolution propagation maps of light-triggered electrical waves, found to be quantitatively indistinguishable from electrically-triggered waves.

Conclusions—Our results demonstrate feasibility to control excitation and contraction in cardiac muscle by light using the TCU approach. Optical pacing in this case uses less energy, offers superior spatiotemporal control, remote access and can serve not only as an elegant tool in arrhythmia research, but may form the basis for a new generation of light-driven cardiac pacemakers and muscle actuators. The TCU strategy is extendable to (non-viral) stem cell therapy and is directly relevant to *in vivo* applications.

Keywords

optogenetics; channelrhodopsin2; light-sensitive ion channels; cardiac; optical mapping

Introduction

The simplest known opto-electrical transducers in nature are a class of light-sensitive transmembrane proteins, best represented by bacteriorhodopsin, converting photons into

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transmembrane voltage via proton pumping. Since their discovery[1], the prokaryote rhodopsins have been viewed as potential bioelectronics components[2] due to offered ultra-fine spatiotemporal control by light. The latter is of equal interest in excitability control of eukaryotic cells. The cloning of Channelrhodopsin2 (ChR2) by Nagel, Hegemann and colleagues[3] expanded the field beyond microorganisms. These ion channels provide excitatory current with relatively fast kinetics and can effectively trigger electrical impulses (action potentials) in excitable cells. Since 2005[4, 5], numerous neuroscience applications *in vitro* and *in vivo* delineated a new research area, termed "optogenetics"[4, 6–12]– the precise interrogation, stimulation and control by light of excitable tissue, genetically altered to become light-sensitive.

Use of optogenetics in other excitable tissues, e.g. cardiac, skeletal, smooth muscle, has been virtually non-existent until very recently[13–15]. At the end of 2010, Bruegmann et al.[13] combined viral expression of a ChR2 variant with a CAG promoter into mouse embryonic stem cells (ESCs) with targeted differentiation and purification of ESC-derived cardiomyocytes for *in vitro* demonstration of optical pacing. They also generated transgenic mice with cardiac ChR2 expression, in which normal rhythm was perturbed by light pulses and focal arrhythmias were induced by long pulses. A transgenic zebra fish was used by Arrenberg et al.[14] to spatially map the exact cardiac pacemaking region by structured illumination. At the same time, our group succeeded in developing the first non-viral strategy to optogenetics that does not rely on embryogenesis and is applicable at the syncytial level – work presented in abstract form in 2010[15], and reported here.

Unlike the brain, cardiac tissue is composed of densely packed, highly coupled cardiomyocytes, integrating electrical and mechanical function. The heart's electromechanical function requires synchrony of excitation waves for efficient global contraction, achieved by cell-cell coupling via gap junction channels formed by Connexin43 (Cx43) in the ventricular portion of the heart. Here, we exploit the heart's high coupling aspect, to develop and validate a non-viral cell delivery system for expression of light-sensitive ion channels. Fig. 1 illustrates the concept of a "tandem cell unit" (TCU), formed by a host cardiomyocyte and a non-excitable donor cell, carrying exogenous ion channels, e.g. ChR2. Biophysically, for this unit to be functional (to fire an action potential upon light excitation), low-resistance coupling is needed for closing the local electric circuits. Our group has previously validated this concept for generation of a two-cell pacemaking unit using cardiomyocytes and stem cells expressing a pacemaking ion channel[16].

The TCU strategy, if proven successful, has potential safety advantages over viral delivery methods used in all prior optogenetics studies, and may be applicable for study and treatment of cardiac rhythm disorders. In this work, we demonstrate the utility of optogenetics in the development of a more robust and energy-efficient solution for cardiac stimulation/actuation by: 1) employing a non-viral, cell delivery strategy to create optically-excitable cardiac muscle, extendable to *in vivo* applications; 2) applying biophysical methods to validate the TCU strategy in cell pairs and in cardiac syncytium *in vitro*; 3) demonstrating an all-optical sensing and actuation in a cardiac syncytium by combining optical stimulation with high-resolution optical mapping for quantification of wave properties under optical vs. electrical stimulation.

Methods

Detailed description of the methods is available as Online Supplement.

As a proof-of-principle cell delivery system, we developed a stable HEK cell line expressing ChR2 and capable of Cx43-mediated coupling[16] to cardiomyocytes to generate optically-

excitable cardiac tissue. The ChR2 plasmid, developed by the Deisseroth's lab, was obtained from Addgene, Cambridge, MA (pcDNA3.1/hChR2(H134R)-EYFP), grown in replication-deficient bacteria, purified and sequenced to confirm the published map. Transfection of the HEK293 cells (ATCC, Manassas, VA) was done using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA), followed by 500μg/ml Geneticin (GIBCO Invitrogen) selection to achieve >98% expression.

We used whole-cell patch clamp to confirm ChR2-mediated inward current inducible by pulses of blue light (470/40nm) in the developed cell line. The TCU concept was validated using a dual-patch technique[16] in cell pairs of adult canine cardiomyocytes (CM), isolated as described before[16] and HEK-ChR2 cells, as well as in co-culture of neonatal rat CM[17–19] with ChR2-expressing HEK cells at initial plating ratio 100:1 or 45:1. Without explicit suppression of proliferation, within the 2–3 days to experiments, the effective ratio was several folds lower. These *in vitro* syncytia were used for tissue-level optical mapping of excitation and contraction. In some experiments, carbenoxolone (CBX), a gap-junction uncoupler, was applied as described to probe the role of cellular coupling in the TCU approach.

Electrical stimulation was provided through Platinum electrodes, driven by a computer-controlled stimulator. For most experiments, optical stimulation was through the dish bottom, by focused light from a blue LED (470nm, 1.35 cd, 20mA) or a fiber-optics coupled high-power blue LED (470nm, 1.6A), connected to the TTL output of a second computer-driven stimulator.

Optical tracking of excitation waves was done using Rhod-4, a calcium-sensitive probe, with excitation at 525/40nm and emission at 610/75nm to avoid interference of the stimulation light with the dye's excitation spectrum. A non-conventional distributed tangential illumination was used here to accommodate optical stimulation and for superior contrast. Emitted light was collected through high-NA optics and a fast intensified CMOS camera. Records of contractility were done with Hamamatsu ImagEM EMCCD camera on a confocal Olympus FluoView™ FV1000.

Data from multiple samples are presented, as indicated, using standard deviation, standard error of the mean or 95% confidence intervals. Statistical testing for functional data on propagation was done in Matlab using a standard two-way ANOVA, followed by a Tukey-Kramer correction for multiple comparisons; p-values are listed for all comparisons. Normality of the distributions was confirmed by Kolmogorov-Smirnov test in Matlab, except for data on coupling in cell pairs, where nonparamateric population statistics was applied (median and interquartile range, IQR). Curve fitting was done also in Matlab using the robust nonlinear least-squares fit method, with pre-specified equations for the desired curve types, e.g. a sigmoidal curve or exponential decay.

Results and Discussion

Development and characterization of a cell delivery system for non-viral optogenetics

To validate the TCU strategy for cardiac optogenetics, as a proof of principle, we developed a stable HEK cell line expressing a variant of ChR2. Fig. 2a–e illustrates the properties of such donor cell line. Preserved expression and functionality were established for the HEK −ChR2 cell line after multiple freeze-thaw cycles and multiple passages (passages 2 to 30 were used for functional experiments), Fig. 2a. Successful expression is possible in other non-excitable cell types, including mesenchymal stem cells, Supplementary Fig. 1, that may yield more clinically relevant cell delivery systems.

Confirmation of ChR2 functionality was done by whole-cell voltage clamp. Quantification of the steady-state light-sensitive ion current in single HEK−ChR2 cells (Fig. 2b–c) revealed that the channel is closed and non-contributing during dark periods regardless of transmembrane voltage, and has a mildly inwardly rectifying current-voltage (I–V) relationship when blue light is applied. Overall, significantly higher steady-state current densities were seen in our donor cells at all voltages in the I–V relationship, even at the low irradiance used here (0.24 mW/mm^2), compared to previously reported light-induced ChR2 current in HEK cells[20], C. elegans muscle cells[10] or in cardiomyocytes[13]. For example, for comparable irradiance levels, at holding potential of −40mV, about 20 times higher steady-state current densities were measured in our donor cells compared to ventricular myocytes from a transgenic mouse expressing ChR2[13]. These data confirm high expression levels and/or functionality of ChR2 in the developed cell delivery system, important for optimizing light stimulation parameters.

Recent comprehensive characterization of ChR2 current kinetics indicates fast activation (<5ms), deactivation (<10ms) and inactivation (<50ms)[20], thus making it suitable as excitatory (action potential – generating) current for cardiomyocytes during external optical pacing at relevant frequencies (5–12 Hz for rodents, 1–3 Hz for humans). Indeed, our kinetics characterization (Fig. 2d–e) estimates the activation and deactivation time constants for ChR2-mediated current in the ms range. Therefore suitable rates for cardiac pacing are attainable even without genetic modifications, as previously done for neural applications, where faster optogenetic tools in conjunction with much shorter action potentials allowed for pacing rates up to 200Hz[9].

In contrast to the robust expression of ChR2 in HEK cells, much lower yield was seen when directly transfecting cardiomyocytes with ChR2 using nucleofector electroporation. This prevented direct synthesis of a large-scale ventricular syncytium from ChR2-expressing myocytes. Nevertheless, individual ChR2-expressing neonatal rat ventricular myocytes were excitable and contracting when optically stimulated and quiescent otherwise (Supplementary Movie 1).

Validation of the TCU strategy for cardiac excitation

The TCU approach for cardiac optogenetics, i.e. inscribing light-sensitivity into cardiomyocytes and cardiac tissue without their direct genetic modifications, was validated in cell pairs of CM and HEK−ChR2 cells, as well as in a synthesized large-scale cardiac syncytium. ChR2-expressing donor cells were most often found to aggregate in small clusters among the neonatal CMs rather than disperse as single cells, Fig. 3a. The donor cells expressed a significant amount of Cx43, as confirmed by a Western blot (Fig. 3b). Interestingly, substantially more Cx43 protein was seen in the HEK−ChR2 cells compared to the parental cell line without ChR2 – an observation that warrants further investigation.

Functional response of TCUs to optical stimulation was first confirmed in cell pairs of adult canine CM and HEK−ChR2 cells using dual-clamp to estimate coupling (Fig 3c–d) and to record light-triggered action potentials in the cardiomyocytes, Fig. 3e–f. A histogram of measured coupling in spontaneously formed cell pairs (n=31) of canine CM and HEK−ChR2 over 48 hour period is shown, Fig 3c. A robust response was seen in a wide range of coupling values spanning an order of magnitude, starting as low as 1.5 nS. Interestingly, a similar low critical coupling value (1.5 – 2 nS), below which TCU functionality failed, was found previously in the generation of a two-cell pacemaking unit by a donor cell carrying HCN2 (a gene encoding for the pacemaking current I_f) and a cardiomyocyte[16]. Extreme uncoupling abolished the light-sensitivity of the myocytes in the TCUs – values below 1.5nS and pharmacological uncoupling with cabenoxolone provided further proof for gap

junctions' critical role in the TCU functionality for neonatal rat and for adult canine myocytes, Fig 3f and Supplementary Movie 2.

In a functional TCU pair, the cardiomyocytes generated normal action potentials upon stimulation by blue light (470nm, 0.13 mW/mm^2, 10ms pulses), Fig 3e, indistinguishable from electrically-triggered ones. The donor cell's membrane potential followed passively by a low-pass filtered version of an action potential, Fig. 3e. In a spatially-extended (several centimetres) two-dimensional cardiac syncytium of randomly mixed neonatal rat CMs and HEK−ChR2 (45:1 initial plating ratio), robust synchronous contractions were registered upon stimulation by blue light 2–3 days after plating (Supplementary Movie 3).

Wave properties of cardiac syncytium in response to optical vs. electrical stimulation

Synthesized optically-excitable cardiac tissue was then subjected to further functional testing. Synchronized wave propagation is essential for the heart's normal functionality and efficient mechanical contraction; lethal arrhythmias occur when the generation or propagation of these excitation waves is altered (failure to initiate, abnormal propagation velocity and/or path). Accordingly, we have developed an ultra-high resolution optical mapping system[18, 19] to dissect cardiac wave propagation during external pacing or arrhythmic activity over a centimeter-scale (>2cm) with subcellular resolution (22μm/pix) at 200 fps using fast voltage and calcium-sensitive dyes[18]. This optical mapping system was made compatible with simultaneous optical excitation, Fig. 4a, so that excitation light for the fluorescence measurements did not induce ChR2 excitation and ChR2 excitation did not interfere with the measurements. While mapping was done here with Rhod-4, a calcium-sensitive fluorescent dye, suitable voltage-sensitive probes with similar spectral properties can also be used, e.g. di-4 or di-8-ANEPPS[18]. In normal pacing conditions, cardiac calcium transients are an excellent surrogate for action potentials, and calcium dyes outperform voltage-sensitive dyes in signal-to-noise ratio.

Optical mapping of propagating waves triggered by localized electrical and optical stimulation in the same sample, revealed similar conduction velocities and calcium transient morphologies, thus confirming equivalent triggering abilities for both modes of stimulation, Fig. 4b–e and Supplementary Movie 4. Pure cardiomyocyte cultures and co-cultures of cardiomyocytes and HEK cells without ChR2 served as controls. At the mixing ratios used here, the presence of HEK cells, with or without ChR2, did not alter the recorded calcium transients (Fig. 4c–d), p=0.36 with ChR2, p=0.44 without ChR2. However, the mixing ratio was a significant factor ($p<0.0001$) in modulating CV, as revealed by a two-way ANOVA, i.e about 30% drop in CV was seen at initial plating ratios of 45:1 (CM:HEK) while a ratio of 100:1 lead to a smaller (non-significant, p=0.06) reduction, Fig. 4e. The presence of ChR2 did not contribute as a significant factor in CV modulation (p=0.16), even though a slight trend to a decrease was seen. Further titration (higher mixing ratios) and/or localized spatial distribution are likely to minimize these effects. Electrical and optical pacing in light-sensitive samples (CM:HEK+ChR2) resulted in identical wave propagation properties. The controls (CM only and CM+HEK without ChR2) were quiescent and never produced excitation in response to light triggers.

The response of the syncytium to optical excitation was captured by constructing a strength-duration curve, describing minimum irradiance over a range of pulse duration values for a point excitation of the 2D syncytium (2mm fiber-optic coupled controllable LED), Fig 4f. The fitted curve revealed a particularly low minimal irradiance levels (average rheobase[21] for excitation of about 0.05mW/mm^2) – at least an order of magnitude lower than previously shown values for optical stimulation of ventricular or atrial tissue[13]. Within the tested diameter for light delivery, macroscopic excitability remained uniform across the tissue.

However, we anticipate that donor cell clustering (Fig. 3a) needs to be addressed in order to achieve maximal spatial resolution of excitation, down to the single cell level.

Considering the electromechanical nature of cardiomyocytes, we also show direct light-triggered muscle contraction, confirming intact excitation-contraction coupling in single myocytes or hybrid cardiac tissue (Fig. 4g and Supplementary Movies 1–3). This demonstration of mechanical response triggered by light-sensitive ion channels suggests possible development of light-driven actuators with efficient energy transfer and illustrates the feasibility for direct optogenetic control in other muscles.

Energy needs in cardiac optogenetics

Previous studies in neuroscience have reported optical energies used to excite single neurons or brain tissue[4, 6, 22] in a wide range of high values (approximately 8 to 75 mW/mm^2). The well-coupled spatially-extended cardiac tissue was expected to present higher load for optical stimulation, thus possibly requiring even higher irradiance values. Yet, surprisingly, in Bruegmann et al.'s study[13], significantly lower light levels (0.5 to 7 mW/mm^2) were sufficient to optically stimulate cardiac tissue *in vitro* or *in vivo* for a wide range of pulse durations.

In our TCU approach, during optical pacing in the cell pairs or in the two-dimensional cardiac syncytium, we measured irradiance at 470nm as low as 0.006 mW/mm^2. The strength-duration curve (Fig 4f) further corroborated low irradiance needed across pulse durations. Interestingly, this is much lower than reported for neuroscience applications and about 1–2 orders of magnitude lower than previously reported values for cardiac excitation in cells and tissue[13] for comparable pulse durations. The significantly lower optical energy needed in our study can be explained, at least partially, by the superior light sensitivity of the donor cells as seen in the higher ChR2 current densities, Fig 2c. Further differences when compared to optical excitation of ventricular and atrial tissue may stem from dimensionality (2D here vs. 3D[13]).

It is important to note, that in contrast to a previous study[13], no (pro-arrhythmic) re-excitations were observed during longer stimulation pulses (up to 1s) at our low illumination intensities, close to the rheobase of the strength-duration curve. This low light intensity is an important factor in minimizing heat-related effects, phototoxicity and in considering future implantable devices.

An interesting question concerns comparison of energy needed for electrical vs. optical pacing. In our system, energy needed for supra-threshold stimulation of two-dimensional cardiac syncytium can be estimated as follows: For typical electrical pacing (5V, 0.2A, 0.01s pulses), we obtain 10mJ. For optical stimulation with an LED (5V, 0.02A, 0.01 – 0.05s pulses), we obtain 1 to 5mJ. Considering the possibility for optimization of light focusing, as well as the active development of more efficient light-emitting diodes (more lumens per watt), it is likely that optical stimulation may be more energy efficient than its electrical counterpart. As pacemaking (unlike cardioversion) is a topologically simple problem, i.e. a spatially-localized (light-sensitive) cell ensemble can be used, the TCU strategy may prove particularly valuable in pursuing potential clinical applications, given the above energy considerations. However, the challenges of achieving efficient light delivery to a densely packed tissue, such as the myocardium, should not be underestimated, and ultimately *in vivo* testing is needed.

Potential benefits of the TCU strategy and *in vivo* considerations

When comparing the TCU strategy to the direct expression of ChR2 in native myocytes (mostly by viral methods), several important differences are worth mentioning, see also

Supplementary Fig 2: (1) the donor (D) cells are non-excitable and typically do not have major repolarizing currents, i.e. the ChR2 inward current is their main current, unlike native CMs; (2) the D-cells have higher membrane impedance at rest due to smaller/negligible inward rectifier, I_{K1}, and typically have a more depolarized resting potential; (3) compared to CM-CM coupling, the D-CM coupling is typically somewhat reduced. We ask the question: What factors in the TCU approach affect the ease of excitation and how is the TCU response to light different than the response of a homogeneous myocardial tissue with direct expression of ChR2?

For a spatially-extended tissue, when the cell pair (TCU) is connected to some "load" of excitable cells (CMs), the system can be abstracted to a Source-Neighbor-Load (S-N-L) triad for easier analysis. A simplified equivalent circuit of such a triad is presented in Supplementary Fig 2d and analysis is provided in detail in the Online Supplement.

The main results of this analysis (Supplementary Fig 2d) are that the ease of optical excitation will improve with: (1) higher membrane impedance for the source/donor cell (as seen for a D vs. CM source cell), since that makes it closer to an ideal current source, and a higher excitation current is available for the neighboring CMs for the same light-induced current in the source; (2) lower membrane impedance of the immediate CM neighbors, determined by their K+ conductance; (3) better S-N coupling but reduced coupling in the load and reduced overall impedance of the load. It is important to note, that for spatially dispersed donor cells, as here, the S-N coupling in the S-N-L triad plays a dual role, i.e. it affects both the S-N charge transfer and the equivalent "load" presented by the tissue. Since the two have opposite effects on the ease to excite, there may be an optimal D-CM coupling for excitation via the TCU strategy.

Having a dedicated donor cell may also provide additional benefits by allowing donor cell optimization independent of the properties of the target tissue, i.e. maximizing the light sensitivity by achieving high current densities in the donor with proper cellular environment for the function of the ChR2 channels, for example.

The presented cell delivery method can be viewed as an easy and accessible research tool for basic studies *in vitro*. Its feasibility for potential *in vivo* cardiac pacing is supported by prior relevant studies, including one using MSCs with a depolarizing current, I_f, in a canine heart[23], where the cells' survival and functionality was demonstrated for at least several weeks. In that study, about 0.7mil cells were delivered, 40% of them carrying the HCN2 gene, encoding for an I_f-like current, expressed at comparable densities to our ChR2 current[24]. The cell number needed for localized excitation can be further inferred from considerations of tissue properties, i.e. estimates of "liminal length" (the minimum size of tissue capable of exciting the rest)[25, 26], which in some cases can be derived from the strength-duration curve. Recent estimates by computer simulations of a related problem – localized generation of early or delayed afterdepolarizations (EADs/DADs)[27] - yielded about 0.7 mil cells in a cluster required for maximum load (worst case scenario), i.e. 3D highly coupled ventricular myocardium. With decreased coupling and other load reductions, this cell number also decreased. Since we did not perform experiments with a confined cell region in 2D, it is hard to directly relate to these estimates. Nevertheless, these studies support the general feasibility of *in vivo* cell delivery for optical pacing by the TCU approach, provided that an appropriate light delivery solution is found. Optimizing the light sensitivity of the donor cells partially alleviates these challenges. Compared to pacing, cardioversion and defibrillation are topologically more complicated problems due to their inherent spatial component and requirement for spatially distributed light-sensitivity and light delivery. Stability and long-term functionality of ChR2 *in vivo* have not been studied

rigorously, but preliminary data from ChR2 use in primate brains over several months are promising in terms of persistence and lack of toxicity[28].

Conclusions

In summary, our study highlights the utility of optogenetics for cardiac applications by using a strategy inspired by the specific properties of cardiac tissue, i.e. high cell-cell coupling. The optogenetic approach offers high spatiotemporal resolution for precise interrogation and control of excitation, seemingly without interfering with essential cardiac tissue properties. Therefore, it presents a new versatile actuation tool in cardiac research for dissection of arrhythmias. Furthermore, cardiac optogenetics based on the TCU strategy, presented here, may evolve in a more translational direction and lead to a new generation of optical pacemakers and potentially cardioverter/defibrillators. The feasibility of this is supported by several critical features of the method presented here: 1) desirable pacing rates achievable with the current kinetics of ChR2; 2) finer control of excitation and repolarization in shaping cardiac action potentials, and potentially in terminating arrhythmias is possible by a combination of light-sensitive ion channels providing outward current[7, 8] and ChR2; 3) the cell delivery platform demonstrated here may offer a safer alternative to viral delivery for *in vivo* applications; 4) optical fibers are inherently more biocompatible than metal electrode leads for *in vivo* pacing; 5) preliminary energy estimates point to potential fold improvements in energy consumption with optical vs. electrical pacing – important for extending battery life in implantable devices.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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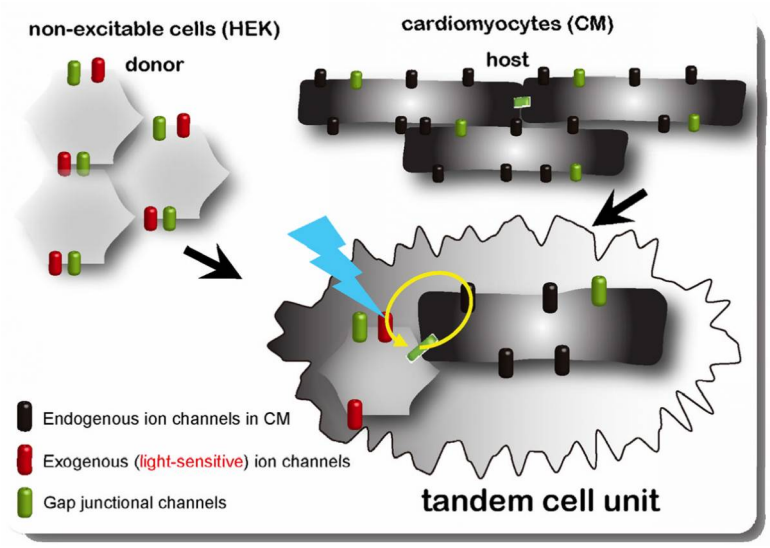

Figure 1.
The functional "tandem cell unit" (TCU) concept of donor-host cells. Non-excitable cells (e.g. HEK cells here) are transfected to express a light-sensitive ion channel (ChR2). When coupled via gap junctions to excitable cardiomyocytes (CM) they form an optically controllable functional TCU, i.e. the CM will generate an action potential upon light-triggered opening of the depolarizing ChR2 in the HEK cell.

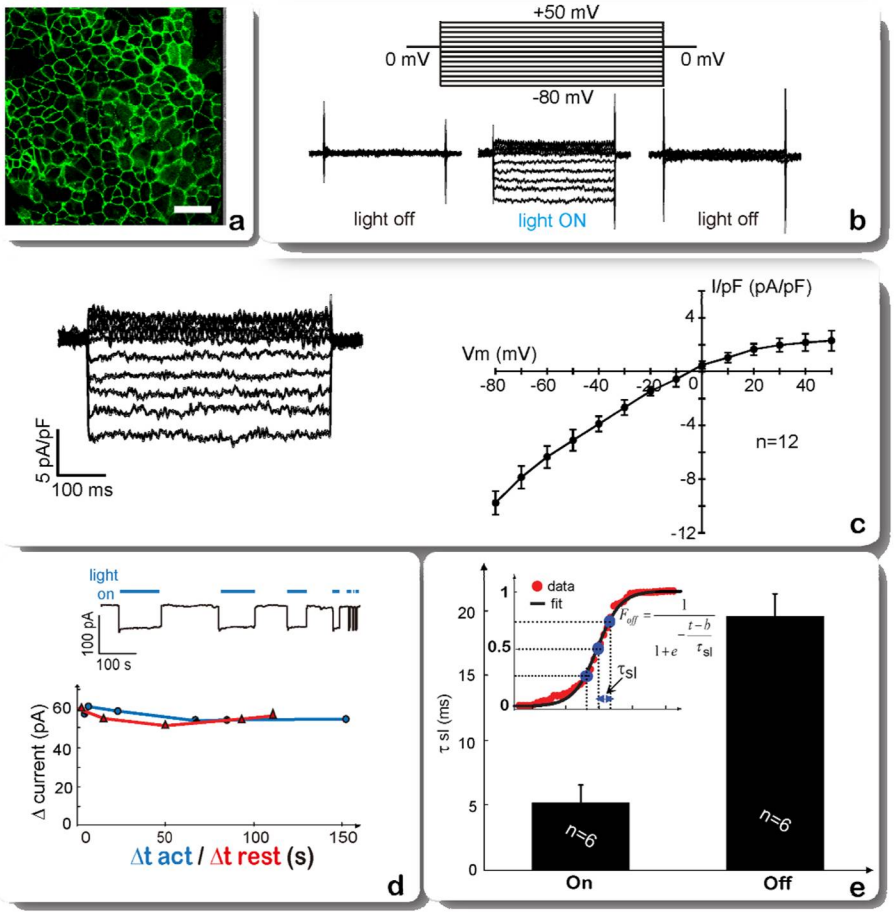

Figure 2.

Development and functional characterization of a cell delivery system for ChR2. **a:** Stable HEK−ChR2 cell line – shown is EYFP-reported ChR2 expression in the 10th passage after transfection and purification; scale bar is 50μm. **b**: Voltage-clamp test protocol and example traces for quantification of the steady-state ChR2 current in single HEK−ChR2 cells with 500ms voltage pulses in the range (−80 to +50mV) with and without excitation light for ChR2 on (470nm, 0.24 mW/mm^2). **c**: Example curves for the light-sensitive component after subtraction of current in dark, and the resultant average current-voltage (I–V) relationship for n=12 cells, cell capacitance 43.3±7.5pF, data are presented as mean±SD. **d**: Magnitude of the light-triggered current does not depend on the duration of rest (Δt rest) or activation (Δt act), thus indicating relatively fast deactivation in the examined range. Holding potential is −80mV. **e**: Kinetics of activation (on) and deactivation (off), quantified by a τ_{sl} parameter in the sigmoid curve fits to the light-controlled current transitions (see inset); bar graphs represent mean±SEM.

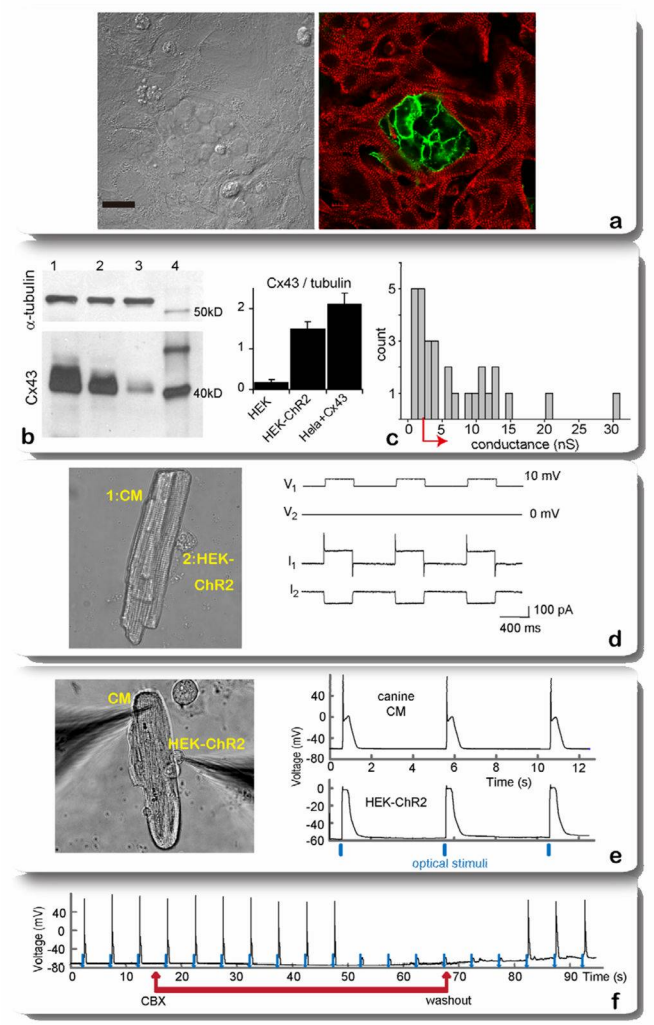

Figure 3.

Implementation and validation of the TCU concept for neonatal rat CM and adult canine CM coupled to HEK+ChR2 cells. **a**: Phase and fluorescence images of neonatal rat CM and HEK−ChR2 co-culture. Immunostaining in red forα-actinin (CMs), green is EYFP-ChR2-expressing HEK cells, typically forming small clusters as shown. Scale bar is 20μm. **b**: Western blot for Cx43 andα-tubulin (at 55kD) in the cell delivery system (HEK−ChR2), column 2; column 1 shows a positive control of stably transfected HeLa-Cx43 cells; column 3 shows parental HEK cells without ChR2; column 4 shows the ladder – MagicMark™ bands in kDa; Normalized (Cx43/tubulin) expression is provided for four gels (mean ±95%CI). **c**: Histogram of measured coupling conductances in TCUs of canine CMs and HEK−ChR2 cells, n=31, median value of 4nS and IQR (2 – 11nS); red arrow indicates coupling levels allowing optical excitability of the TCUs. **d**: Dual whole-cell voltage clamp of a TCU - adult canine ventricular CM (1) and HEK−ChR2 cell (2). Voltage steps (V1=10mV, 0.4s), applied to the canine CM (cell 1), induced junctional currents (I2) in this cell pair (estimated g.j. conductance of 11nS). **e**: Action potentials in a cell pair (canine CM and HEK−ChR2 cell, phase image on the left) in response to optical pacing (0.13 mW/mm^2, 10ms pulses). Due to coupling, the HEK cell exhibits a low-pass filtered version of the CM-generated action potentials. **f**: Action potentials in a cell pair (canine CM and HEK−ChR2

cell) in response to continuous optical pacing before, during and after washout of uncoupler carbenoxolone (CBX).

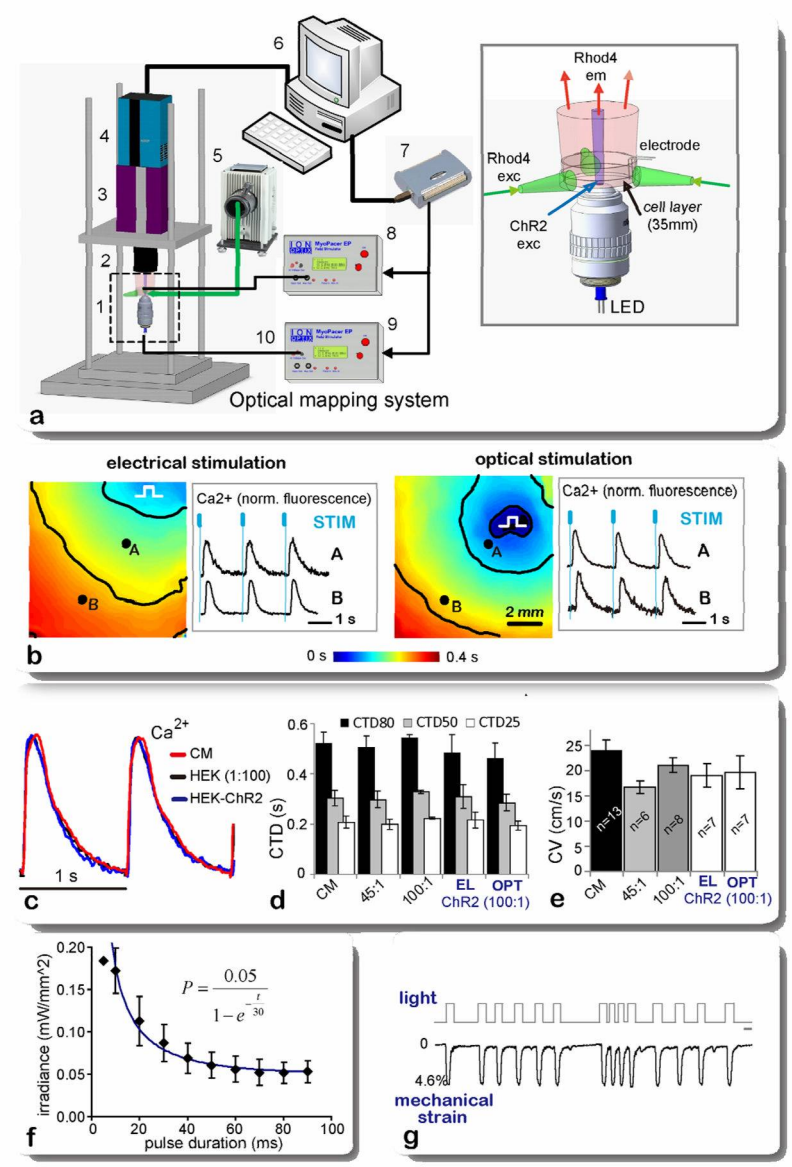

Figure 4.

Optical control of cardiac tissue function over space-time: light-triggered excitation waves and light-triggered contractions. **a**: Experimental setup for ultra-high resolution high-speed optical imaging and optical control of cardiac excitation: 1) experimental chamber with tangential light illumination for calcium imaging, focused LED illumination on a moveable stage for ChR2 excitation (see inset on the right); 2) high-NA optics for high-resolution macroscopic imaging; 3) Gen III MCP intensifier; 4) pco 1200hs CMOS camera; 5) light source, excitation filter and optical light guides for tangential excitation; 6) computer system and software for data acquisition and control of electrical and optical stimulation; 7) interface for stimulation control; 8) controllable stimulator for electrical pacing (analog output); 9) controllable stimulator for optical pacing (TTL output). 10) LED for ChR2 excitation, driven by the TTL stimulator output. **b**: Activation maps in a cardiac monolayer by electrical and optical pacing at 0.5Hz. Color represents time of activation; isochrones are shown in black at 0.15s. Calcium transient traces in response to electrical or optical stimulation are shown from 2 locations (A and B), normalized fluorescence. Blue marks

indicate time of stimulation (electrical pulses were 10ms, optical −20ms each). **c**: Normalized Ca^{2+} transients from CM monolayer (red), CM:HEK (black) and CM:HEK +ChR2 co-culture 100:1 (blue) at 1Hz pacing. **d**: Quantification of calcium transient duration (CTD) – CTD25, CTD50 and CTD80 for pure CM monolayer, 45:1 and 100:1 CM:HEK, as well as 100:1 CM:(HEK+ChR2) co-culture under electrical and optical pacing at 1Hz. **e**: Comparison of conduction velocity (CV) among the same 5 groups as in (**d**); for **d** and **e** optical pacing was at irradiance of 0.01– 0.04mW/mm^2, 50ms pulses; data are shown as mean±95%CI; listed number of samples applies to both; **f**: Strength-duration curve (along with the equation for the fitted curve) obtained for optical pacing in co-cultures (100:1 CM:HEK ratio) at 30°C, n=8, mean±SEM. **g**: Example contractility recording from optically-driven CM+HEK+ChR2 – displacement normalized to cell length. Scale bar is 1s.