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Optogenetic Control of Cardiomyocytes via Viral Delivery

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Abstract

Optogenetics is an emerging technology for the manipulation and control of excitable tissues, such as the brain and heart. As this technique requires the genetic modification of cells in order to inscribe light sensitivity, for cardiac applications, here we describe the process through which neonatal rat ventricular myocytes are virally infected in vitro with channelrhodopsin-2 (ChR2). We also describe in detail the procedure for quantitatively determining the optimal viral dosage, including instructions for patterning gene expression in multicellular cardiomyocyte preparations (cardiac syncytia) to simulate potential in vivo transgene distributions. Finally, we address optical actuation of ChR2-transduced cells and means to measure their functional response to light.

Keywords

Optogenetics; Adenovirus; Channelrhodopsin; Cardiomyocytes; Optical mapping; Multiplicity of infection

1 Introduction

Optogenetic control of cardiomyocytes is achieved through the genetic insertion of light-sensitive ion channels and/or pumps of microbial origin (opsins) to render cells controllable by light. This technique can be used for contactless manipulation of activity in excitable tissues such as the brain and heart. In neuroscience, optogenetics has become the tool of choice to dissect neural circuits and brain connectivity in health and disease through cell-specific targeting since its first reported application in 2005 [1]. Since then, a myriad of studies have linked specific neural populations to behavior (for review *see* refs. 2–5) and addressed their contribution to such conditions as depression [6], anxiety [7], addiction [8, 9], and sleep disorders [10]. Pioneering novel optical therapies have stemmed from this technique in the treatment of diseases, such as epilepsy [11, 12], Parkinson's [13–15], and retinal degeneration [16]. Only more recently (since 2010) has optogenetics been applied in the heart (for review *see* ref. 17). In vitro cardiac applications have thus far explored the utility of expressing channelrhodopsin-2 (ChR2), an excitatory opsin, through viral means in a mouse embryonic stem cell line followed by differentiation to cardiomyocytes [18, 19] as well as through a tandem cell unit approach where nonexcitable cells, expressing ChR2, are coupled to cardiomyocytes, thus inscribing light sensitivity to the cardiac syncytium [20].

The microbial opsins at the core of optogenetics can produce either depolarizing (excitatory) currents or hyperpolarizing (inhibitory) currents in mammalian cells. Excitatory opsins, such

as channelrhodopsin (ChR), can provide fast kinetic currents of sufficient amplitude to trigger action potentials, whereas inhibitory opsins, such as halorhodopsin (HR) and archaerhodopsin (AR) can suppress activity via fast onset of hyperpolarization [21]. When an opsin is activated by a photon of the appropriate wavelength light, the chromophore all-*trans*-retinal, which is covalently bound to the channel, is isomerized to 13-*cis*-retinal resulting in the opening of the channel. Depending on the channel, cations (ChR, AR) or anions (HR) flow with or are pumped against the electrochemical gradient across the cell membrane to change transmembrane potential. Mutant opsins are also available which are designed to improve upon light sensitivity, speed, and spectral response [22, 23]. In the case of cardiac optogenetics, the most commonly used mutant excitatory opsin is ChR2(H134R). ChR2(H134R) was one of the first single amino acid mutants generated and results in a 2–3× increase in channel conductance with minimal effect on kinetics [24].

Optogenetics opens the possibility for an all-optical investigation of neural and cardiac electrophysiology when optical mapping (using voltage- and calcium-sensitive dyes) is combined with these new optical actuation tools. Our 2011 study was the first to combine optical stimulation with high-speed, high-resolution optical mapping [20]. The integration of these techniques allowed the quantitative comparison of wave propagation and conduction properties of both optical and electrical stimulation. As will be discussed in this chapter, care must be taken when choosing opsins and optical sensors, such as calcium- and voltage-sensitive dyes, for a combined experiment, as excitation and emission spectra may overlap making data interpretation difficult.

This chapter describes the process for direct virally mediated opsin expression in cardiomyocytes, including methods for spatial gene patterning in cardiac syncytium. We specifically outline step-by-step procedures for in vitro infection of neonatal rat ventricular cardiomyocytes (NRVMs) with an adenovirus containing the transgene for ChR2(H134R) fused to an eYFP reporter protein with a ubiquitous CMV promoter. This technique is also applicable to (and has been tested in) other cardiac cell types (including adult cells) with appropriately designed optogenetic viruses and optimization of the viral infection protocol as described here.

2 Materials

2.1 Isolating and Preparing Cells for Adenoviral Infection

1. NRVM culture media: M199 media (Invitrogen, Grand Island, NY, USA) supplemented with 10 % fetal bovine serum (FBS), 12 μM L-glutamine, 0.02 μg/mL glucose, 0.05 μg/mL penicillin–streptomycin, and 10 mM HEPES. Store at 4 ºC. Warm to 37 ºC before use.
2. NRVMs prepared according to Jia et al. [20].

2.2 Determining the Optimal Multiplicity of Infection

1. Custom-designed Ad-ChR2(H134R)-eYFP or other virus containing an appropriate light-sensitive opsin. Viral titer is 9×10^{11} viral particles (VP)/mL (*see* Note 1).
2. Phosphate-buffered saline (PBS).

3. NRVM culture media: M199 media (Invitrogen, Grand Island, NY, USA) supplemented with 10 % FBS, 12 μM l-glutamine, 0.02 μg/mL glucose, 0.05 μg/mL penicillin–streptomycin, and 10 mM HEPES. Store at 4 ºC. Warm to 37 ºC before use.

4. NRVM infection media: M199 media (Invitrogen, Grand Island, NY, USA) supplemented with 2 % FBS (*see* Note 2), 12 μM l-glutamine, 0.02 μg/mL glucose, 0.05 μg/mL penicillin–streptomycin, and 10 mM HEPES. Store at 4 ºC. Warm to 37 ºC before use.

5. Fibronectin (BD Biosciences, San Jose, CA, USA): Prepare a stock solution (5 mg/mL) by adding 1 mL distilled water to the vial, aliquot, and store at −20 ºC. For coating cell culture dishes, dilute stock solution in PBS for a final working concentration of 50 μg/mL. Unused fibronectin can be stored at 4 ºC for up to 1 week.

6. Glass-bottomed dishes (14 or 20 mm) (In Vitro Scientific, Sunnyvale, CA, USA).

7. Tyrode's solution: Prepare a 5× concentrated Tyrode's solution by dissolving the following amounts of compounds in 1 L of distilled water: 1.02 g $MgCl_2$, 2.01 g KCl, 39.45 g NaCl, 0.2 g NaH_2PO_4, and 5.96 g HEPES. This concentrated solution can be stored at 4 ºC for several months. To make a working Tyrode's solution mix 400 mL distilled water with 0.46 g d-glucose, 750 μL $CaCl_2$ (for 1.5 mM Ca^{2+} in final volume), and 100 mL of 5× Tyrode's stock. The concentrations (in mM) are as follows: NaCl, 135; $MgCl_2$, 1, glucose, 5; HEPES, 5; KCl, 5.4; $CaCl_2$, 1.5; NaH_2PO_4, 0.33. pH should be adjusted to 7.4 with NaOH.

8. Propidium Iodide (Life Technologies, Grand Island, NY, USA): Stain is packaged as a 1 mg/mL solution. For a final working concentration of 2 μg/mL, dilute 2 μL of stock solution in 1 mL of non-phosphate-containing solution, such as Tyrode's.

2.3 Patterned Transgene Expression

1. Sylgard® 184 Silicone Elastomer Kit (Fisher Scientific, Pittsburgh, PA, USA).

2.4 Optogenetic Actuation and Feedback

1. Quest Rhod-4, AM (AAT Bioquest, Sunnyvale, CA, USA): Prepare a stock solution of 0.5 mM which can be stored at −20 ºC until experimental use. The solvent contains 20 % Pluronic (F127) and 80 % dimethyl sulfoxide (DMSO). Dilute to 10 μM in room-temperature Tyrode's solution for cardio-myocyte staining.

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[1]Depending on the viral titer, a dilution may be required prior to infection. In our case, Ad-ChR2(H134R) (original titer: 9×10^{11} VP/mL) is diluted in 1 mL of PBS for a final working concentration of 9×10^{8} VP/mL.

[2]High levels of protein in the culture media (with 10 % FBS) can interfere with the binding of the adenovirus (or any viral vector) to their cellular receptors. Therefore, a lower serum infection media (with 2 % FBS) is used during infection as a trade-off with no-serum media which cardiomyocytes do not tolerate well.

3 Methods

3.1 Isolating and Preparing Cells for Adenoviral Infection

Cells specific to this protocol (NRVM) are isolated using a previously published technique [20]. In short, the ventricular apex is excised from 2- to 3-day-old Sprague-Dawley rats and enzymatically digested overnight with trypsin at 4 ºC and then with collage-nase at 37 ºC the following morning. Cardiac fibroblasts are removed from the cell suspension by a two-stage pre-plating procedure. At the end of the isolation procedure, cardiomyocytes are counted and resuspended in NRVM culture media (*see* Subheading 2 for recipe) to a concentration of 1.125×10^6 cells/mL.

3.2 Determining the Optimal Multiplicity of Infection

This section describes the viral infection of cardiomyocytes with opsin genes using a suspension approach with proper dosing for maximum efficiency while maintaining cell viability. Carry out all procedures under a Biosafety Level 2 (BSL-2) cabinet unless otherwise specified (*see* Note 3).

1. Prepare a cell suspension of NRVM at a concentration of 1.125×10^6 cells/mL in NRVM culture media (containing 10 % FBS, *see* Subheading 2 for recipe).
2. Remove adenovirus from storage at −20 ºC and place on ice (*see* Note 4).
3. Separate the total volume of cell suspension into three (or more) conicals for multiplicity of infection (MOI) testing (*see* Note 5). For this protocol, we will describe MOI testing at 0 (control), 25, and 100.
4. Calculate the amount of viral particles needed for infection at the specified MOI (*see* Note 6):

$$\text{Viral particles (VP)} = (\text{total number of cells}) \times (\text{MOI})$$

   For example, to infect 2.25×10^6 cells (2 mL cell suspension at 1.125×10^6 cells/mL) at MOI 25, 56.25×10^6 VP are required.
5. Calculate and measure out the volume of virus needed for infection:

$$\text{Viral volume } (\mu\text{L}) = \frac{\text{Viral particles (VP)}}{\text{Viral titer (VP}/\mu\text{L)}}$$

[3]All work with adenoviruses should be completed under the NIH guidelines for working with recombinant DNA. Infections are performed under a certified BSL-2 hood, and personal protection equipment (gloves, lab coat) are worn at all times while handling infectious materials. In addition, for decontamination, all pipets, dishes, and conicals are rinsed with 10 % bleach and autoclaved.
[4]Adenoviruses should not undergo multiple freeze/thaw cycles as they could negatively affect performance. Ad-ChR2(H134R) is stored at −20 ºC in a 25 % glycerol solution (2× storage solution: 10 mM Tris pH, 100 mL NaCl, 1 mM $MgCl_2$, 0.1 % BSA, and 50 % glycerol).
[5]The amount of virus needed for efficient infection of cells (i.e., successful, widespread expression of the transgene and low cytotoxicity) will differ depending on the cell type. For NRVMs, MOIs ranging from 25 to 100 were tested.
[6]VP represent the total amount of particles in a volume irrespective of their function. Transducing units (TU), plaque-forming units (PFU), or infectious units (IFU) represent the number of functional viral particles in a given volume. For most viral preparations the VP:PFU ratio ranges from 20:1 to 50:1.

For example, to infect a 2 mL suspension of NRVM (2.25×10^6 total cells), a total viral volume of 62.5 μL is required.

6. Mix the viral volume (μL) calculated in **step 5** with infection media (containing 2 % FBS, *see* Note 2 and Subheading 2 for recipe), so that the volume of virus and infection media maintain the original cell concentration of 1.125×10^6 cells/mL (*see* Note 7).

7. Spin down the cells at 720 × *g* for 4 min.

8. Aspirate the culture media from the conical taking care not to disrupt the cell pellet, and resuspend the cells in the infection media and viral particle mixture from **step 6**.

9. Incubate the conicals at 37 ºC, 5 % CO_2, for 2 h with agitation every 15–20 min during this time (*see* Note 8).

10. Spin down the cells at 720 × *g* for 4 min.

11. Aspirate the infection media from the conical, again taking care not to disrupt the cell pellet, and resuspend the cells in fresh NRVM culture media maintaining the original cell concentration of 1.125×10^6 cells/mL.

12. Plate the cells in a monolayer at a density of 350–470 k cells/ cm^2 on fibronectin-coated (50 μg/mL, *see* Note 9) glass- bottomed dishes.

13. Image transgene expression based on fluorescence of the reporter molecule, in this case eYFP (*see* Fig. 1).

    Since optimization of the MOI requires maximizing expression efficiency and minimizing cytotoxic effects, propidium iodide (PI) staining can be used to quantify cell death. PI is a membrane-impermeant DNA stain, excluded from viable cells and commonly used to detect dead cells in a given population.

14. Prepare a 2 μg/mL solution of PI diluted in a Tyrode's solution (*see* Subheading 2 for recipe). This fluorescent stain is light sensitive, and therefore the application must be completed in the dark.

15. Add 0.5 mL of the 2 μg/mL solution to the glass-bottomed dish containing the cells of interest.

16. Incubate the cells with PI for 2 min.

17. Remove the dye solution and wash with fresh Tyrode's solution.

[7] In this protocol, infection is completed with the cells in suspension. Provided the cells (like NRVM) tolerate remaining in suspension for a sufficient period of time, infection in this manner allows for better access of the viral particles to the cell surface (membrane) and (in our hands) results in healthier cells post-infection. NRVMs tolerate this protocol well; however it may not be suitable for all cell types.

[8] Make sure to shake hard enough to disrupt the pellet of cells and resuspend them sufficiently in order to fully expose them to the virus for infection.

[9] Neonatal cells show preference for fibronectin-coated surfaces, whereas adult cells tend to show preference for laminin-coated surfaces [26].

18. Image cell viability based on the fluorescence of PI using appropriate excitation and emission filter sets (*see* Fig. 2). When PI is bound to nucleic acids, the maximum excitation and emission are 535 nm and 617 nm, respectively.

19. Based on the overall transgene (ChR2(H134R)-eYFP) expression and cell viability, determine the optimal MOI to be used in future experiments (*see* Note 10). For NRVM infected with our custom-designed Ad-ChR2(H134R)-eYFP, the optimal MOI was found to be 25 based on maximal expression and minimal cell death.

3.3 Patterned Transgene Expression

The ability to infect NRVM cells in suspension, as opposed to in dish infection after cell plating, allows for patterned optogenetic transgene expression, as described in this section. To date, we have patterned islands of ChR2(H134R)-eYFP-expressing cells using silicone elastomer stencils, in addition to graded mixtures of infected and uninfected cells mimicking potential in vivo transgene expression patterns.

1. Thoroughly mix silicone elastomer in the ratio of 10 parts base to 1 part curing agent (total weight of this mixture is 2.2 g).

2. Pour elastomer mixture into a 60 mm petri dish. Allow the elastomer to spread out and cover the entire bottom of the dish. This volume will yield a 200–250 μm thick layer of silicone.

3. Bake in an oven at 50–60 ºC for 2 h. Alternatively, the elastomer can be cured at room temperature for 24 h.

4. In the meantime, coat glass-bottomed dishes with 50 μg/mL of fibronectin (*see* Note 9). Cover dishes and leave in the incubator (37 ºC, 5 % CO_2) for 1–2 h.

5. When the elastomer is finished setting, cut out stencils, such as that illustrated in Fig. 3, in the shape of the desired pattern. The stencil illustrated here will create an island of light-sensitive cardiomyocytes surrounded by normal, uninfected cardiomyocytes or vice versa.

6. When the glass-bottomed dishes have been coated with fibronectin, remove the solution and carefully place the stencils on the glass-bottomed dish in the desired location (*see* Note 11).

7. Plate a droplet of infected cells (350–470 k cells/cm^2) in the stencil cutouts and let sit for 30–40 min (*see* Note 12). This will be sufficient time to allow the infected NRVM to begin adhering to the dish. The droplet volume is determined in such a way that the open stencil area should be covered at a target density of 350–470 k cells/cm^2.

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[10] Viral performance may vary between batches. Therefore, a repeat optimization protocol may be necessary.

[11] When placing stencils on the fibronectin-coated glass- bottomed dishes, make sure that the dish is dry enough so that the stencil sticks. If not, the cell droplet will spread under the stencil when cells are plated.

[12] Ensure that the droplet is of a large enough volume so as not to dry up during this first step of the cell plating procedure. In our experience, a 10 μL droplet is of a sufficient volume and size when placed within a 3 mm diameter stencil.

8. Carefully, remove the stencil and plate uninfected cells at the same plating density in a monolayer on the glass-bottomed dish.

9. To plate uniform, graded mixtures of infected and uninfected cells, mix various concentrations, such as 35 % infected cells and 65 % uninfected cells, that have been maintained in suspension during infection, and plate these mixtures at a density of 350–470 k cells/cm^2.

10. Image transgene expression based on fluorescence of the reporter molecule (*see* Fig. 4 for examples of transgene distributions).

3.4 Optogenetic Actuation and Feedback

This section describes means of functional testing of the cardiomyocyte samples transduced with the opsin of interest. It demonstrates a combined optical actuation and optical sensing. The macroscopic (large field of view) optical mapping described in this section is done using a custom-developed ultrahigh-resolution system which has been previously described in detail [20]. Other charge-coupled diode (CCD)-, photodiode array (PDA)-, and complementary metal-oxide-semiconductor (CMOS)-based optical mapping systems can also be adapted to this protocol (for review *see* ref. 25). Alternatively, the optical sensing can be done with a microscope-integrated photodetector [17]. Here we focus on the choice of optical sensors (i.e., calcium- and voltage-sensitive dyes) and how to trigger optical excitation in NRVM monolayers.

All measurements described here are recorded at room temperature and in the dark so as not to photobleach the fluorescent dyes and/or activate the opsins.

1. Remove a dish containing NRVM monolayer containing cells expressing ChR2(H134R) from the incubator (37 ºC, 5 % CO_2).

2. Remove culture media from the dish, and incubate cells with 10 μM Quest Rhod-4AM (*see* Subheading 2 for recipe), a robust calcium-sensitive dye (*see* Note 13), diluted in room- temperature Tyrode's solution for 20 min.

3. Remove dye solution, and wash the cells with a 20-min incubation in fresh room-temperature Tyrode's solution.

4. Place a freshly stained glass-bottomed dish on the optical mapping setup (*see* Note 14).

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[13]The calcium-sensitive dye—Quest Rhod 4 AM—was chosen in this instance because of the compatibility of its excitation and emission spectra with ChR2 (*see* Table 1 for spectral characteristics). In choosing a dye for optical mapping, it is also critical to account for the reporter gene of the opsin (in this case, eYFP), as it is not ideal to excite it in a way that emits too much light during optical mapping, potentially masking the optical response of the monolayer. As also highlighted in Table 1, since di-8-ANEPPS has the same spectral characteristics as Rhod 4, this voltage-sensitive dye may be used in place of the calcium-sensitive dye in the experiments described in this protocol. It should be noted, however, that di-8-ANEPPS has been reported to have a lower fractional change in fluorescence at the cellular level [25] and, consequently, optics may need to be adjusted in order to collect enough light for an analyzable optical signal.

[14]Our custom-developed macroscopic optical mapping system [27], as shown in Fig. 5a, includes a CMOS camera (pco, Germany), capable of recording at 200 frames per second over 1,280 × 1,024 pixels; a Gen III fast-response intensifier (Video Scope International, Dulles, VA); light collection optics (Navitar Platinum lens, 50 mm, f/1.0) and emission filter (610/75 nm); excitation light source (525 nm, Oriel); and an adjustable imaging stage. Excitation light for the calcium- or the voltage-sensitive dye is delivered using tangential illumination (i.e., at a 90° angle with respect to the imaging axis) by a QTH lamp with branching liquid light guides. Excitation light for the opsins is provided through the bottom of the dish (*see* Notes 16 and 17).

5. Record electrical activation of the monolayer by pacing with custom-built platinum electrodes connected to a Myopacer Cell Stimulator or equivalent (*see* Fig. 5b for example of electrical pacing output and Note 15).

6. Record optical activation of the monolayer using light pulses with a wavelength specific to the opsin integrated into the virus (i.e., 470 nm for ChR2(H134R)) (*see* Fig. 5b for example of optical pacing output and Notes 16–[18]). According to a strength–duration curve, the pulse duration can be decreased if higher optical power is used. For example, for our samples, 10-ms optical pulses of 0.01 mW/mm^2 are sufficient to trigger activity in NRVM uniformly expressing ChR2(H134R) (*see* Note 19).

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[15]Propagation data, in the cases of both electrical and optical stimulation, are collected using the commercially available software CamWare (pco, Germany). Raw data is then processed and analyzed in custom-developed Matlab software to quantify propagation, conduction velocity, and transient morphology, among other measures. In addition, all data are typically spatially filtered (Bartlett filter; kernel size: 10 pixels) as well as temporally filtered (Savitzky–Golay filter; order: 2, width: 11).

[16]To date, we have used two types of illumination systems with which to optically stimulate light-sensitive monolayers: LED and laser based. The choice of illumination system will, in part, depend on the power requirements of the excitable syncytium and the use of any fiber optics downstream of the light source, which can decrease power at its delivery point.

[18]The energy for optical stimulation is measured and reported in terms of irradiance (mW/mm^2). Irradiance is measured at the site of light delivery using an optical power meter with an appropriately sized sensor area (0.785 cm^2). All reported irradiance measurements are taken at the end of each experiment as slight, unintentional alterations in the position of the sample and/or optical fiber can significantly alter the resultant power measurement. In addition, if optically illuminating from the bottom of the glass-bottomed dish (*see* Fig. 5a), it is also important to measure the power through a glass-bottomed dish as it could attenuate light. Threshold irradiance levels required to trigger propagating action potentials in test samples are directly related to the expression levels and functionality of the virally introduced opsins.

[19]The availability of retinal is necessary and essential for the proper light-sensing abilities of ChR2 and other opsins. As a chromophore, retinal covalently binds to opsins to form photosensitive receptors. Without retinal, no functional opsin channels can be assembled. In our experience with NRVM, we have not had to add exogenous retinal, as NRVMs contain suf-ficient retinal for opsin function. It has, however, been reported that the addition of exogenous retinal can increase optical responsiveness [30].

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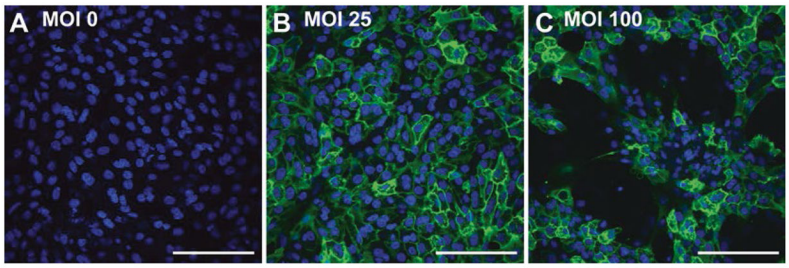

Fig. 1.

ChR2 expression in cardiomyocytes: Representative fluorescent images of NRVMs infected with Ad-ChR2(H134R)-eYFP at (**a**) MOI 0, control; (**b**) MOI 25; and (**c**) MOI 100. *Blue stain* is DAPI. Scale bar is 100 μm

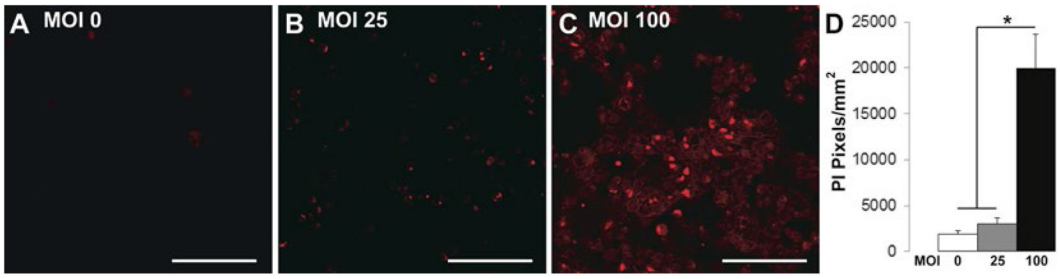

Fig. 2.

Cell viability upon viral dosing: Representative fluorescent images of NRVMs infected with Ad-ChR2(H134R)-eYFP and stained with PI at (**a**) MOI 0, control; (**b**) MOI 25; and (**c**) MOI 100. Scale bar is 100 μm. (**d**) Quantification of PI staining presented as average number of pixels with PI stain above threshold normalized to area (mm^2)

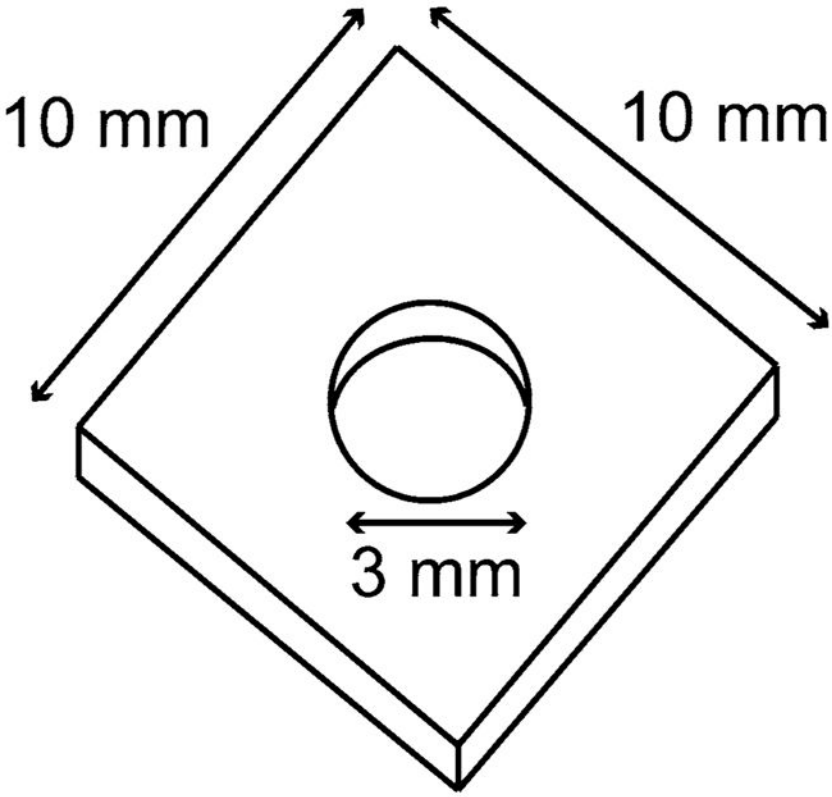

Fig. 3.

Patterning stencils: Example schematic of silicone elastomer stencil used to pattern an island of ChR2-expressing NRVMs surrounded by normal, uninfected NRVMs. Not drawn to scale

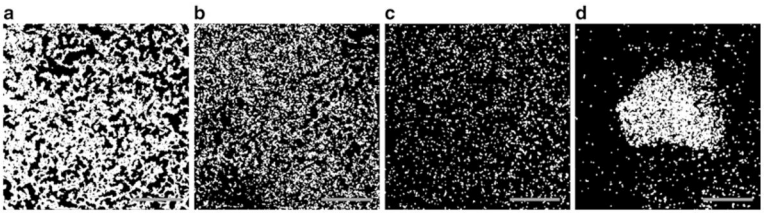

Fig. 4.

Spatial patterning of ChR2 expression: Representative binarized distributions of ChR2-expressing NRVMs (eYFP, *white*) in four configurations: (**a**) global expression of ChR2; (**b**) mixture of 35 % ChR2-expressing NRVMs and 65 % non-infected NRVMs; (**c**) mixture of 10 % ChR2-expressing NRVMs and 90 % non-infected NRVMs; (**d**) island of ChR2-expressing NRVMs

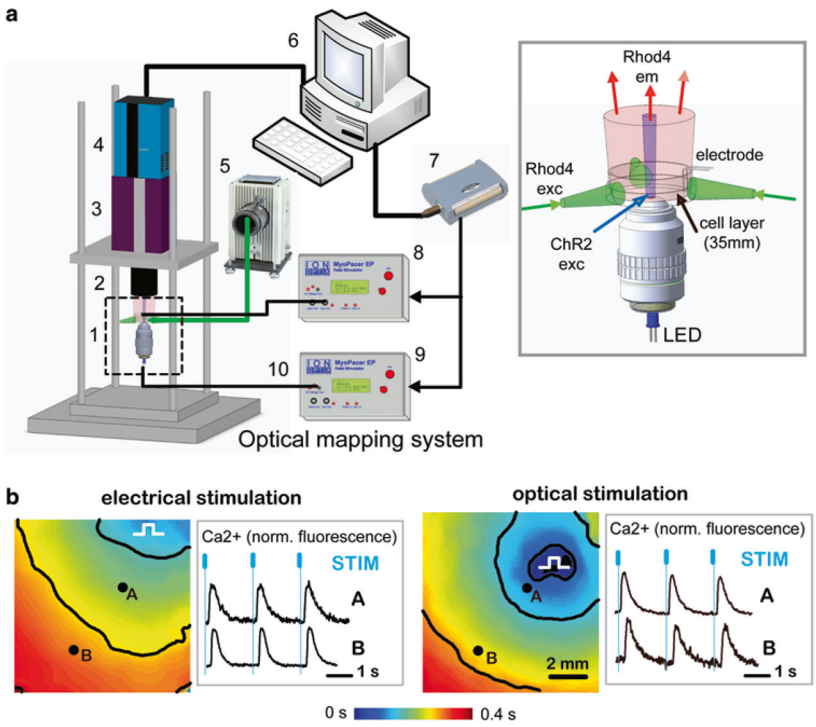

Fig. 5.

Combining optical actuation with optical imaging: (**a**) Experimental setup for high-resolution, high-speed optical imaging and optical control of cardiac syncytia. (**b**) Activation maps in cardiac syncytia by electrical and optical pacing at 0.5 Hz. Normalized calcium transients (acquired with Rhod 4 staining) are shown from two locations—A and B

Table 1

Excitation spectra for opsin, reporter protein, and imaging dyes

	Peak excitation (nm)	Peak emission (nm)
Opsin		
ChR2	470±20	n/a
Reporter protein		
eYFP	515	530
Imaging dyes		
Ca^{2+} (Rhod-4)	525±40	585±40
V_m (di-8-ANEPPS)	525±40	610±75