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Optogenetic investigation of cardiac arrhythmia mechanisms

Feola, I.

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Chapter 2

OPTOGENETIC ENGINEERING OF ATRIAL CARDIOMYOCYTES

Iolanda Feola, MSc; Alexander Teplenin, MSc;
Antoine A.F. de Vries, PhD; Daniël A. Pijnappels, PhD.

Laboratory of Experimental Cardiology, Department of Cardiology, Heart Lung Center Leiden; Leiden University Medical Center, the Netherlands.

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SUMMARY

Optogenetics is emerging in the cardiology field as a new strategy to explore biological functions through the use of light-sensitive proteins and dedicated light sources. For example, this technology allows modification of the electrophysiological properties of cardiac muscle cells with superb spatiotemporal resolution and quantitative control. In this paper, the optogenetic modification of atrial cardiomyocytes (aCMCs) from 2-day-old Wistar rats using lentiviral vector (LV) technology and the subsequent activation of the light-sensitive proteins (*i.e.* ion channels) through light-emitting diodes (LEDs) are described.

Keywords: Optogenetics, Atrial Cardiomyocytes, Lentiviral vectors, Optical mapping, Light-emitting diode (LED)

INTRODUCTION

Optogenetics is a new technology to control cellular function by a combination of genetic engineering and light application. Optogenetics has revolutionized neuroscience by offering the possibility to control at high spatial and temporal resolution various biological processes both *in vitro* and *in vivo*.[1] Despite these unique features, optogenetics has not yet been extensively explored in cardiac research. Nevertheless, several scientific contributions have shown how light-gated ion channels can modulate the transmembrane potential of cardiomyocytes giving rise to excitatory or inhibitory responses, controlled in time, space and magnitude.[2,3] Channelrhodopsins are excitatory proteins that upon illumination can evoke an action potential (AP) in excitable cells due to the passive inward flow of positively charged ions.[4] When instead light induces protons or potassium ions to leave the cell or chloride ions to enter the cell, cellular excitation is inhibited.[5-9] Due to their specific properties, light-activated ion transporters may be very helpful to gain additional insight into the mechanisms underlying cardiac arrhythmias, which may inspire the exploration of new strategies for treating electrical disturbances in the heart. Besides via light-gated ion transporters, optogenetics offers many other possibilities to control cellular behavior, including transcription as well as intracellular and receptor signaling and could thereby further improve our understanding of cardiomyocyte biology and heart function.[10-12]

The following section describes how aCMCs, isolated from the hearts of 2-day-old Wistar rats, can be successfully optogenetically engineered by forced expression of a depolarizing optogenetic tool, *i.e.*, Ca^{2+}-permeable channelrhodopsin (CatCh) following LV-mediated transgene delivery.[13] In addition, details are provided on how to study the functional consequences of LED-mediated CatCh activation by optical voltage mapping. For example, exposure of CatCh-expressing aCMCs to 10-ms blue light pulses induces a photocurrent strong enough to evoke an action potential (AP) in these cells.[14]

MATERIALS

Reagents for isolating aCMCs

- Isoflurane.
- Fibronectin working solution (100 µg/ml): dilute the fibronectin stock solution 10 times with phosphate-buffered saline (PBS) and store at 4°C.
- Solution A: 0.02 g/l phenol red, 136 mM NaCl, 5 mM KCl, 0.2 mM $Na_2HPO_4.2H_2O$, 0.44 mM KH_2PO_4, 5.5 mM D-glucose and 20 mM HEPES-acid free. Adjust the pH to 7.4-7.5 by adding 5 M NaOH at 21°C. Sterilize by filtration through a 0.22 µm pore size cellulose acetate bottle top filter and store at 4°C.
- Solution B: 1 mM $CaCl_2.2H_2O$, 60 mM $MgCl_2.6H_2O$ in Solution A. Store at -20°C.
- Solution C: DNase I solution. Mix 500 units of deoxyribonuclease with 800 µl Solution A. Store at -20°C.

- Dissociation medium: mix 90,000 units of collagenase type I (Worthington Biochemical, Lakewood, NJ, USA) with 200 ml of Solution A, 2 ml of solution B and 200 µl of solution C. Sterilize by filtration through a 0.22 µm pore size cellulose acetate bottle top filter and store at -20°C.
- Growth medium: mix 450 ml of 1× Ham's F10 nutrient mix with 10 ml of heat-inactivated fetal bovine serum (HI-FBS), 10 ml of heat-inactivated horse serum (HI-HS) and 10 ml of penicillin-streptomycin stock solution (5000 U/ml). Store at 4°C.
- Solution D: 2.8 M sodium ascorbate, 107 mM pyruvic acid, 500 mM D-glucose and 5.7% (w/v) bovine serum albumin (BSA).
- CMC medium: add 15 ml of solution D to 250 ml of Dulbecco's modified Eagle's medium (DMEM)-low glucose. Next, add 25 ml of 10× Ham's F10, 192.5 ml of sterile water, 4 ml of 7.5% sodium-bicarbonate, 0.305 ml of 0.2 M L-glutamine, and 10 ml of penicillin-streptomycin solution. Store at 4°C.
- Sterile glass beads, 6.7-7.3 mm ø.
- Mitomycin-C solution: dissolve 2 mg Mitomycin-C powder (from *Streptomyces caespitosus*) in 4 ml PBS. Sterilize by filtration through a 0.22 µm pore size cellulose acetate syringe filter and store at 4°C.
- Round glass coverslips (15 mm ø).
- Primaria cell culture dishes (60 mm ø).
- Falcon 70 µm mesh size cell strainers.
- Costar 24 well clear TC-treated multiple well plates.

Reagents for producing self-inactivating LVs (SIN-LVs)

- 293T cells.[15]
- SIN-LV shuttle plasmid pLV.MHCK7.CatCh~eYFP.WHVPRE and derivatives thereof.[14]
- SIN-LV packaging/helper plasmids psPAX2 and pLP/VSV-G.
- DMEM-high glucose (HG) + 10% FBS.
- DMEM-HG + 5% FBS + 25 mM HEPES-NaOH (pH 7.4).
- TrypLE Express.
- 150 mM sterile NaCl solution.
- 1 mg/ml PEI solution (pH 7.4): weigh 45 mg of linear polyethylenimine (Mw 25,000), add 100 µl of 1M HCl and 40 ml of sterile water of 80°C. Shake vigorously to facilitate dissolution. Adjust the pH to 7.4 with 37% HCl. Increase the total volume to 45 ml with

sterile water. Sterilize by filtration through a 0.22 µm pore size cellulose acetate syringe filter and store in 1.8-ml aliquots at -80°C.

- 20% sucrose solution: weigh 100 g of sucrose and add 500 ml of 10× PBS. Increase the volume to 500 ml with sterile water. Use a magnetic stirrer to facilitate dissolution. Sterilize by filtration through a 0.22 µm pore size cellulose acetate bottle top filter and store in 50-ml aliquots at 4°C.
- Polyallomer ultracentrifuge tubes for SW28 or SW32 rotor.
- Millex sterile 0.45 µm pore size syringe filters (33 mm ø).
- BD Plastipak sterile 50-ml syringes with Luer Lock system.
- PBS-1% BSA: dissolve 1 g of BSA in 100 ml of PBS by gentle stirring with a magnetic stirrer. Sterilize by filtration through 0.22 µm pore size cellulose acetate syringe filters and store in 1.6-ml aliquots at 4°C.

Combination of LED and optical mapping system

- HEPES-buffered, phenol red-free DMEM/F12.
- Di-4-ANEPPS solution (1 mg/ml): dissolve 5 mg of di-4-ANEPPS powder in 5 ml sterile dimethyl sulfoxide (DMSO) and store in 500-µl aliquots at 4°C and protected from light.
- MiCAM ULTIMA-L imaging system (SciMedia, Costa Mesa, CA, USA).
- Excitation filter (530 nm pass, Semrock, Rochester, NY, USA).
- Dichroic mirror (520-560 nm reflect >90%, >600 nm pass >85%, Semrock).
- Emission filter (>590 nm pass, Semrock).
- 470-nm Rebel LED mounted on a 25 mm ø round CoolBase (Luxeonstar, Brantford, Ontario, Canada).
- Plano-convex lens (1 inch ø, 25.4 mm focal length) (Thorlabs, Munich, Germany).
- Stimulus generator STG2004 (Multichannel System, Reutlingen, Germany).
- Custom-made platinum electrode pair for bipolar point stimulation.
- Power meter PM100D (Thorlabs).
- Brain Vision Analyzer 1208 software (Brainvision, Tokyo, Japan).

METHODS

aCMC isolation and culture

Isolation of aCMCs

- Anaesthetize 2-day-old Wistar rats (n»50) via 4-5% isoflurane inhalation. Ensure adequate anaesthesia by checking the absence of reflexes.
- Rapidly excise the hearts and collect them in a plastic Petri dish containing ± 10 ml ice-cold Solution A.
- Separate the atria from the ventricles (*see* Note 1).
- Remove Solution A until the bottom of the Petri dish just stays covered with fluid. Chop the atrial tissues in small pieces and rinse the tissue with 4 ml of Solution A to get rid of erythrocytes (*see* Note 2).
- Prepare 12 ml of an ice-cold 1:1 mixture of dissociation medium and Solution A (Solution E). Remove solution A as much as possible with a 1000-µl pipetman and add 7 ml of Solution E to the Petri dish with atrial tissue pieces.
- Transfer the atrial tissue pieces in Solution E to a sterile 50-ml Erlenmeyer flask with screw cap containing sterile glass beats.
- Incubate under gentle agitation at 37°C for 35 min.
- Transfer the fully digested material (5 ml) to a sterile 15-ml polypropylene screw cap tube on ice and leave the undigested material (2 ml) in the Erlenmeyer flask.
- Add 4 ml of fresh Solution E to the Erlenmeyer flask and incubate once again under agitation at 37°C for 35 min.
- Transfer the content of the Erlenmeyer flask to the 15-ml tube with the remainder of the atrial digest and pellet the cells by centrifugation for 10 min at 150×*g*. Suspend the cell pellet in 12 ml of prewarmed growth medium. Equally divide the cell suspension over 4 Primaria cell culture dishes and incubate at 37°C in a humidified 95% air/5% CO_2 atmosphere (culture conditions) for 120 min to allow preferential attachment of non-cardiomyocytes (mainly cardiac fibroblasts).
- Pass the growth medium with the non-adhered cells (mainly cardiomyocytes) through a nylon cell strainer with a mesh size of 70 µm to remove undigested tissue fragments and cell aggregates.
- Count and seed the cells on fibronectin-coated glass coverslips in 24-well cell culture plates. To obtain confluent aCMC monolayers seed 8×10^5 cells/well.

Preparation of fibronectin-coated glass coverslips

- Place a sterile glass coverslips on the bottom of each well of a 24-well cell culture plate.

- Add 300 µl of fibronectin solution to each well.
- Incubate for 1 h under culture conditions.
- Collect the fibronectin solution (can be used 2 more times) and leave the coverslips to dry in the flow hood for about 60 min.

Antiproliferative treatment with mitomycin-C

- The day after isolation dilute the mitomycin-C solution 50 times in growth medium and add 300 µl/well of a 24-well cell culture plate.[16]
- Incubate for 2 h under culture conditions.
- Rinse the cells twice with PBS.
- Add 1 ml of CMC medium + 5% HI-HS of 37°C/well and maintain the cells under culture conditions.
- Refresh the culture medium once a day.

Production of vesicular stomatitis virus G protein (VSV-G)-pseudotyped SIN-LVs

Seeding of 293T cells in 175-cm^2 cell culture flasks

- Use 293T cells of low passage number for LV production. Check the 293T cells using an inverted phase-contrast microscope. When the cultures are ± 80% confluent, remove the medium and rinse each 175-cm^2 cell culture flask once with 10 ml PBS.
- Add 1.5 ml of TrypLE Express/flask and after 1 min incubation gently rock the flask to facilitate cell detachment. Once the cells have detached from the plastic support collect them in DMEM-HG + 10% FBS.
- Generate a single cell suspension by forcefully pipetting the cell suspension up and down 3-5 times to disrupt remaining cell clusters without destroying the cells (can be checked microscopically).
- Suspend 7×10^7 viable 293T cells in 85 ml DMEM-HG + 10% FBS and add 20 ml of the cell suspension to each 175-cm^2 cell culture flask (*i.e.*, 1.65×10^7 cells/flask). Make sure the cells become evenly spread over the plastic support (*see* Note 3).

Transfection of 293T cells

- The day after, before starting with the transfection make sure that the cells are ± 60-70% confluent, evenly spread over the surface of the culture flasks and viable (*see* Note 4).
- Prepare the DNA/NaCl solution by diluting 154 µg of plasmid DNA (molar ratio SIN-LV shuttle plasmid:psPAX2:pLP/VSV-G is 8:5:5) in 4.4 ml of 150 mM NaCl in a sterile 50-ml polypropylene screw cap tube (*see* Note 5). Add the DNA to the NaCl solution instead of

the other way around and mix by gentle vortexing. The cell type(s) in which and the level at which the transgene is expressed will be determined by the promoter that drives its expression (Figure 1).

- Prepare the PEI/NaCl solution by adding 504 µl of 1 mg/ml PEI solution to 4296 µl of 150 mM NaCl in a sterile 50-ml polypropylene screw cap tube. Mix the PEI with the NaCl solution by gentle vortexing.
- Add 4.4 ml of the PEI/NaCl solution to the DNA/NaCl solution in a dropwise fashion. Gently rock/swirl the tubes during the addition of the PEI solution. Homogenize the content of the tubes by vortexing for 10 s (*see* Note 6).
- Incubate the tubes for 15 min at RT to allow the formation of DNA/PEI complexes, and then add 2 ml of this transfection mixture to each flask. Move the flasks to completely mix the transfection mixture and culture medium.

Replacement of the transfection medium by DMEM-HG + 5% FBS + 25 mM HEPES-NaOH (pH 7.4)

- Replace the transfection medium in each of the 175-cm^2 cell culture flasks by 15 ml fresh DMEM-HG + 5% FBS + 25 mM HEPES-NaOH (pH 7.4) (*see* Note 7).

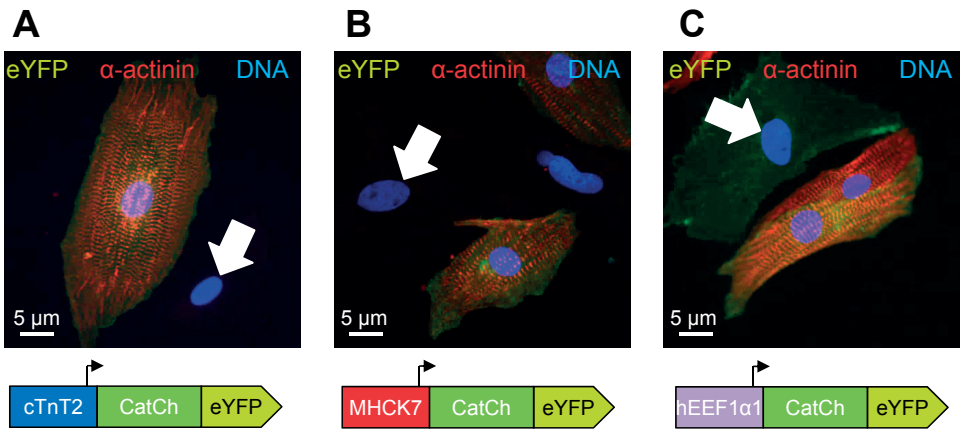

Figure 1. Immunocytological confirmation of aCMC-specific transgene expression. aCMC cultures were incubated with CatCh~eYFP-encoding SIN-LVs in which transgene expression was driven by (A) the striated muscle-specific MHCK7 promoter,[20] (B) the cardiomyocyte-specific chicken Tnnt2 promoter[21] or (C) the ubiquitous human EEF1A1 promoter. The MHCK7 and Tnnt2 promoter give rise to transgene expression in α-actinin$^+$ cells (*i.e.*, cardiomyocytes) only, while the promoter of the housekeeping gene EEF1A1 directs transgene expression in α-actinin$^+$ cells as well as α-actinin$^-$ cells (*i.e.*, cardiac fibroblasts [white arrows]). The blue fluorescence corresponds to cell nuclei stained with Hoechst 33342.

Harvesting of the culture supernatants and concentration/purification of the SIN-LV particles

- Before harvesting the LV particle-containing culture supernatants, ± 36-48 h after transfection, check the transfection efficiency of the 293T cells using an inverted fluorescence microscope (Figure 2).
- Transfer the culture medium of each pair of two 175-cm^2 cell culture flasks to a sterile 50-ml polypropylene screw cap tube.
- Centrifuge the tubes for 10 min at 3,000×g and RT in a tabletop centrifuge.
- Push the cleared culture media through 0.45 µm pore size polyethersulfone syringe filters and collect the filtrate in autoclaved 38.5-ml polyallomer ultracentrifuge tubes.
- Carefully move a 5-ml stripette containing 7 ml of 20% sucrose in PBS through the cell culture medium to the bottom of the ultracentrifuge tube and slowly release 5 ml of the sucrose solution to underlay the culture medium with a sucrose cushion.
- After taring with DMEM-HG + 5% FBS + 25 mM HEPES-NaOH (pH 7.4) spin the ultracentrifuge tubes for 2 h with slow acceleration and without braking at 15,000 revolutions/min and 4°C.
- Aspirate the supernatant and place the ultracentrifuge tube upside down on a piece of sterile filter paper to absorb the remaining supernatant.
- Add 400 µl of ice-cold PBS-1% BSA to the LV particle-containing pellet in each ultracentrifuge tube.
- Place each ultracentrifuge tube in upright position in a 50-ml polypropylene screw cap tube and incubate overnight at 4°C in the cold room while shaking gently and with the cap closed.

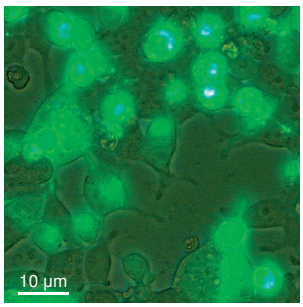

Figure 2. Fluoromicrograph of 293T cells 36 h after co-transfection with psPAX2, pLP/VSV-G and pLV.MHCK7.CatCh~eYFP.WHVPRE. LV yield strongly depends on the transfection efficiency, which should be >90%. The rounded shape of the cells is largely due to the cytotoxic effects associated with the accumulation and aggregation (brightly fluorescent perinuclear dots) of the CatCh~eYFP fusion protein in the LV producer cells.

2

Aliquoting of the concentrated SIN-LV suspensions

- Collect the SIN-LV suspensions in one of the ultracentrifuge tubes.
- Wash each ultracentrifuge tube with 100 µl of ice-cold PBS-1% BSA and transfer the wash solution to the collection tube.
- Divide the supernatant on ice in 50-100 µl aliquots using precooled 0.5-ml microtubes for storage at -80°C (*see* Note 8).

Transduction of aCMCs

Transduction

- Four days after aCMC isolation thaw the SIN-LV stock on ice and add the desired amount of SIN-LV particles to prewarmed CMC medium + 5% HI-HS.
- Gently mix to ensure homogenous distribution of SIN-LV particles and replace the culture medium with 400 µl of inoculum/well of a 24-well cell culture plate (*see* Note 9).

Medium refreshment

- ± 24 h after transduction aspirate the inoculum, wash the cells once with PBS and add 1 ml of prewarmed CMC medium + 5% HI-HS/well of a 24-well cell culture plate.

Light stimulation and optical voltage mapping

Preparation of the optical mapping system

- Place the plano-convex lens on top of the 470-nm LED.
- Align the LED assembly with the center of the MiCAM ULTIMA-L camera.
- Connect the lens-LED complex to the stimulus generator.

Preparation of aCMC cultures

- Check the aCMC monolayers for structural homogeneity, ± 72 h after transduction, using an inverted phase-contrast microscope (*see* Note 10) (Figure 3).
- Check the aCMC monolayers for homogeneity of transgene expression by visualizing the enhanced yellow fluorescent protein tag fused to the channelrhodopsin with the aid of an inverted fluorescence microscope (*see* Note 11) (Figure 4).
- Prepare an 8 µM di-4-ANEPPS solution in prewarmed DMEM/F12.
- Replace the CMC medium by 500 µl of the potentiometric dye solution and incubate for 10 min under culture conditions.

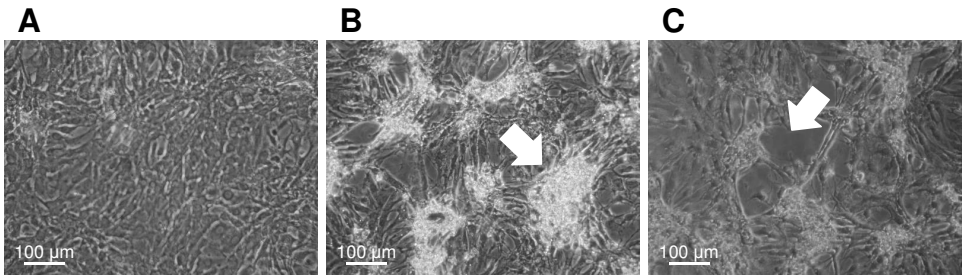

Figure 3. Morphology of aCMC monolayers at day 9 after isolation. A, Non-interrupted, homogenous aCMC monolayer well suited form optical mapping studies. B, Inhomogenous aCMC monolayer containing star-shaped cell aggregates (white arrow). C, Non-continuous aCMC monolayer due to the presence of acellular areas (white arrow). Cultures (B) and (C) do not allow reliable acquisition of whole culture optical mapping data.

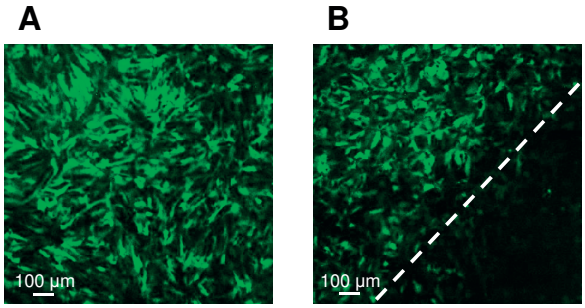

Figure 4. Fluoromicrograph of confluent aCMC cultures 3 days after transduction with LV.MHCK7.CatCh~eYFP.WHVPRE. A, Homogeneously transduced aCMC culture. B, Heterogeneously transduced aCMC culture. Only cultures showing (near-)quantitative transduction and homogenous transgene expression should be used for optical mapping experiments.

- Aspirate the di-4-ANEPPS solution and add 500 µl DMEM/F12.
- Place the cells under the MiCAM ULTIMA-L camera.

Optical voltage mapping

- Focus the MiCAM ULTIMA-L camera and check for functional homogeneity by electrical point stimulation at a frequency of 1 Hz using 10-ms rectangular pulses of 8 V (*see* Note 12).
- While recording pace the cultures at a frequency of 1 Hz using 10-ms blue light pulses at the maximum current tolerated by the LED (*i.e.*, 700 mA) (Figure 5).

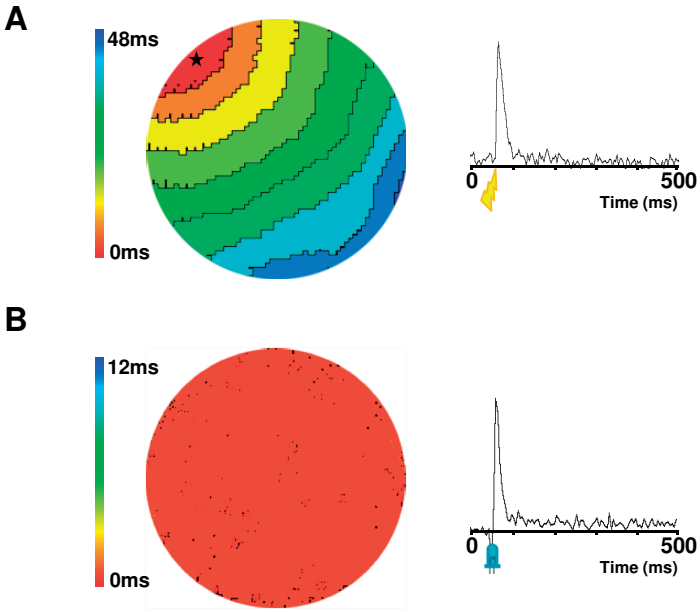

Figure 5. Activation maps (left) and optical signal traces (right) of CatCh-expressing aCMC monolayers following (A) electrical or (B) optical induction of APs. The activation map in (A) shows uniform convex AP propagation initiated at the bipolar pacing electrode (star). The activation map in (B) shows synchronous induction of APs in the entire monolayer following 10-ms exposure to 470-nm LED light (0.08 mW/mm^2 irradiance).

NOTES

1. Selective removal of the atria can be accomplished by placing an opened scissor tightly around the ventricles and gently moving the scissor upwards until the atria will fall on top of the blades of the scissor and can be cut off.

2. Standardization of the chopping procedure is complicated due to subtle differences in the technique used by individual researchers. Experience has taught us that both insufficient as well as excessive cutting strongly reduces cardiomyocyte yields.

3. Differences in 293T cell density within each culture flask or between culture flasks should be avoided by frequent homogenization of the cell suspension during the seeding procedure and by ensuring that the shelves of the CO_2 incubator are perfectly horizontal (check with a spirit level).

4. For LV production, only use 293T cells of low passage number (*i.e.*, <60). Furthermore, don't use 293T cells that have been seeded at too low a density, "over-trypsinized" or allowed to grow overconfluent.

5. The quality of the packing/helper construct and of the LV shuttle plasmid strongly influences LV yields. To achieve a high transfection efficiency, the plasmid DNA

preparations should ≥95% supercoiled as checked by agarose gel electrophoresis, have a low endotoxin content and contain minimal amounts of (in)organic contaminants.

6. The PEI/NaCl solution should be added to the DNA/NaCl solution instead of the other way around to ensure a high transfection efficiency.[17]
7. 293T cells should be transfected at the right cell density (*i.e.*, subconfluency). If the cell cultures are already ± 60-70% confluent in the morning of the day following seeding, the 293T cells should be transfected at that moment and refreshment of the transfection medium should be done in the late afternoon (*i.e.*, after 6-8 h).
8. VSV-G-pseudotyped LV stocks can be repeatedly thawed and frozen without significant loss of functional titer provided that they are kept on ice/at 4°C during inoculum preparation.[18]
9. The transduction efficiency of target cells by LVs is not only depending on the vector dose and infection period but also on the inoculum volume/vector concentration. To achieve the highest possible transduction efficiency, the inoculum volume should be kept as small as possible.[19] However, the target cells should still be covered with sufficient culture medium to keep them healthy.
10. The aCMC monolayers should not show acellular areas and/or star-shaped aggregated cell clusters.
11. The aCMC monolayers should show homogenous transgene expression.
12. The aCMC monolayers should not show spontaneous activity and areas of conduction slowing or block. Moreover, the monolayers should not show areas of significant optical signal prolongation.

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