Video Article

Live Cell Imaging of Primary Rat Neonatal Cardiomyocytes Following Adenoviral and Lentiviral Transduction Using Confocal Spinning Disk Microscopy

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Abstract

Primary rat neonatal cardiomyocytes are useful in basic *in vitro* cardiovascular research because they can be easily isolated in large numbers in a single procedure. Due to advances in microscope technology it is relatively easy to capture live cell images for the purpose of investigating cellular events in real time with minimal concern regarding phototoxicity to the cells. This protocol describes how to take live cell timelapse images of primary rat neonatal cardiomyocytes using a confocal spinning disk microscope following lentiviral and adenoviral transduction to modulate properties of the cell. The application of two different types of viruses makes it easier to achieve an appropriate transduction rate and expression levels for two different genes. Well focused live cell images can be obtained using the microscope's autofocus system, which maintains stable focus for long time periods. Applying this method, the functions of exogenously engineered proteins expressed in cultured primary cells can be analyzed. Additionally, this system can be used to examine the functions of genes through the use of siRNAs as well as of chemical modulators.

Video Link

The video component of this article can be found at https://www.jove.com/video/51666/

Introduction

Primary rat neonatal cardiomyocytes have long been used for investigating cardiomyocyte function *in vitro*[1]. They are easy to isolate from rat pups by several well established methods[2-4]. The most common method employs collagenase or trypsin to digest connective tissue of the heart prior to cell isolation. Researchers have also developed methods for isolating cardiomyocytes from adult rodents[5-8] as well as neonatal mice[9,10].

This protocol describes a method for isolating cardiomyocytes from neonatal rat pups, employing a two-step enzyme digestion procedure. Trypsin is first used O/N at 4 °C, and then purified collagenase at 37 °C. Incubation of the heart tissue with trypsin O/N at 4 °C reduces the steps necessary to harvest cells compared to methods using sequential incubations in a warm enzyme solution[2]. In addition, by using purified collagenase rather than crude enzymes, lot-to-lot variability can be eliminated, thus providing enhanced reproducibility.

Functional studies of a particular protein often employ a protein expression system using an adenovirus[11-13] and/or a lentivirus[14-16]. [CAUTIONARY NOTE] The viral production and manipulation should be carried out according to the NIH guidelines.

The adenovirus does not integrate into the host genome. It has a very high efficiency of transduction in most cell types, including dividing cells and non-dividing cells, as well as primary cells and established cell lines. This makes the adenovirus a reliable vector for gene expression. High levels of the protein encoded by the adenovirus vector develop within 48 hr following transduction, and they can last for several weeks[17]. However, one drawback to using an adenovirus for protein expression is that the development of a recombinant adenovirus is both complicated and time consuming. This drawback explains in part why many researchers have been turning to lentiviruses for recombinant gene expression. Unlike adenoviral constructs, generating a lentiviral construct is quick and easy. Although lentiviruses generally have lower efficiencies of transduction than adenoviruses, in both dividing and non-dividing cells, they do integrate into the host genome. Consequently, expression of the transduced gene is more stable for lentiviruses than for adenoviruses.

Due to technological advances in the field of microscopy, it is much easier to capture live cell images of cells expressing recombinant proteins. This holds true even at video rate speeds of acquisition. This allows the investigator to determine how particular alterations in the protein of interest functionally impact the cell in real time. The confocal spinning disk microscope has several key features that make it an optimal technique for live cell imaging[18,19]. The Yokogawa spinning disc allows for much more rapid image acquisition, while at the same time utilizing far less laser power than point scanning confocal microscopes. Both of these special features are due to the spinning disk, which contains numerous confocal holes through which the laser passes simultaneously to the samples. During acquisition, the disk itself spins quickly and continuously[18-20]. By using the autofocus system of the microscope, stable focus is maintained over long periods of time. This allows researchers

to take well-focused live cell images. Acquired images are played back as a movie file. The images are analyzed using image analysis software such as ImageJ[21,22], FIJI[23], or other commercially available software.

Protocol

1. Isolation of rat neonatal cardiomyocytes

1. Use Dulbecco's modified Eagle's medium (DMEM) and minimum essential medium (MEM) supplemented with fetal bovine serum (FBS) and penicillin/streptomycin (P/S) as the culture medium. Add bromodeoxyuridine (BrdU) to the medium to prevent growth of fibroblasts for the first 2 days after the isolation.

	Base medium	FBS	BrdU (mM)	P/S (U/ml)
selection	DMEM	10%	0	20
The first day	DMEM	10%	0.1	10
The next day	DMEM/MEM	5%	0.1	10
Further culture	DMEM/MEM	5%	0	10

Table 1: Medium for rat neonatal cardiomyocytes. Use this media for culturing rat neonatal cardiomyocytes. DMEM/MEM is 1:1 mix of DMEM and MEM medium.

1.1) Cardiomyocyte isolation, Day 1 (Estimated required time, about 1 hr)

NOTE: For work with neonatal rodents, refer to local university guidelines and rules set by animal care programs, and adhere to institutionally approved animal protocols. All methods described in this protocol have been approved by the Institutional Animal Care and Use Committee of the Yale Animal Resource Center.

1. Prepare reagents and tools: calcium- and magnesium-free Hank's balanced salt solution (CMF HBSS), 30 ml x 2, 10 ml x 2, on ice; 1 mg trypsin, reconstituted with 2 ml of CMF HBSS, on ice; autoclaved tools; 70% ethanol (EtOH) in 250 ml beaker; four sterilized 10 cm plastic dishes, three for the isolation of hearts and one for trypsinization.
2. Order 1 litter of 1-to-2-day-old rat pups (about 10 pups) with their dam from an experimental animal distributer.
3. **Day1,** Transfer pups to small cage and euthanize dam with a ketamine/xylazine cocktail (Ketamine, 200ml/10g and xylazine 20ml/10g) overdose.
4. Take one pup and sterilize its entire body with 70% EtOH. Decapitate pup with well-maintained sharp surgical scissors on a sterilized 10 cm plastic dish at a location isolated from other pups.
   NOTE: In order to collect the samples as quickly as possible, it is best to use the most rapid and efficient method for terminating the life of the rodent. Decapitation when properly used by skilled professionals with well-maintained equipment may result in less fear and anxiety for the animal and be more rapid and practical than other forms of euthanasia. It is consistent with the recommendations of the American Veterinary Medical Association Guidelines for the Euthanasia of Animals.
5. Remove beating heart with sterilized tools, and put heart in 30 ml ice chilled CMF HBSS in 50 ml conical tube. NOTE: Remove the hearts as quickly as possible and put them on ice immediately. After removing the hearts from body, all procedures should be done on ice.
6. Collect 5 hearts in 30 ml of ice chilled CMF HBSS and the remaining 5 hearts in another 30 ml of ice chilled CMF HBSS. Swirl the tube to rinse the hearts. NOTE: After this step, perform all procedures in a laminar flow hood.
7. Discard the supernatant and transfer the hearts to a new sterilized 10 cm plastic dish on ice. NOTE: Be careful not to aspirate hearts with the aspirator.
8. Remove large vessels and/or undesired tissues. Add 10 ml of ice chilled CMF HBSS to the dish to rinse the hearts.
9. Transfer all hearts to a new sterilized 10 cm plastic dish on ice. Mince the hearts with scissors to less than 1 mm^3 on ice.
10. Add 9 ml of chilled CMF HBSS. Add 1 ml of chilled trypsin reconstituted with CMF HBSS (final 50 ug/ml).
11. Seal the dish with parafilm and cover it with aluminum foil, then place it in cold room (4 ˚C) overnight.

1.2) Cardiomyocyte isolation, Day 2 (Estimated required time, about 4 hrs)

1. Prepare reagents and tools: 50 ml conical tube x 2; 2 mg trypsin inhibitor, reconstituted with 1 ml of CMF HBSS, on ice; 1500 units collagenase, reconstituted with 5 ml of Leibovitz L-15, at room temperature (RT); Leibovitz L-15, reconstituted with 1 liter of sterilized water and filter through a 0.22 micron filter, 8 ml at RT; 10 mg/ml (32.6 mM) BrdU in water; P/S; DMEM + 10% FBS; MEM; two 10 cm plastic cell culture dishes; 3.5 cm glass bottom dishes.
2. **Day2,** Preparing gelatin coated dishes. Add 1 ml of 0.1% gelatin solution to coat a 3.5 cm glass bottom dish for an imaging experiment. Incubate dishes at 37 ˚C for several hours, then aspirate and discard gelatin solution before use.
   NOTE: A 3.5 cm glass bottom dish is suitable for an imaging experiment using a fluorescent microscope. For extracting proteins from primary cardiomyocytes to detect protein expression by western blotting (WB), 0.1% gelatin coated plastic cell culture dishes can be used.
3. Transfer all heart tissue digested by trypsin overnight with buffer to a 50 ml conical tube on ice in the sterilized hood. Add 1 ml of trypsin inhibitor in CMF HBSS (final 182 ug/ml). Close lid of 50 ml conical tube tightly to prevent contamination.
4. Incubate the tube for about 30 min in a 37 ˚C incubator to warm tissue and buffer to 30-37 ˚C. Add 5 ml of collagenase in Leibovitz L-15 (final 94 unit/ml). Incubate at 37 ˚C for 45 min with gentle shaking (170-200 rpm shaker in 37 ˚C incubator).
   NOTE: This step can be done outside of the laminar flow hood. All subsequent steps should be done at RT.
5. Disinfect the outside of a 50 ml conical tube with 70% EtOH and put it in the sterilized hood.
6. Triturate tissues using auto pipette with pipetting at 3 ml/sec of speed for 20 times. Allow tissue residue to settle for 3 min and filter the supernatant with a 70 um cell strainer.

NOTE: A regular size 10 ml pipette can be used for the trituration.
7. Add 5 ml of the Leibovitz L-15 to the remaining tissue clumps and triturate and filter the supernatant again. Wash cell strainer with 2 ml of the Leibovitz L-15.
8. Place filtered cell solution in a hood for 20 – 60 min to allow collagenase to digest the partially degraded collagen.
9. Sediment cells at 100 x *g* for 5 min. Re-suspend cells in 20 ml of DMEM containing 10% FBS and high concentration P/S (20 U/ml).
10. Pre-plate cells on two 10 cm plastic cell culture dishes for 1 hr in CO_2 incubator at 37 ˚C for cardiomyocyte selection. NOTE: Fibroblasts attach more readily to the bottom of the dish than cardiomyocytes.
11. While waiting, prepare DMEM containing 10% FBS, P/S (10 U/ml) and 0.1 mM BrdU. Add 1 ml of 10 mg/ml BrdU to 325 ml of DMEM containing 10% FBS and P/S.
12. Swirl dishes gently and collect the cardiomyocyte containing supernatant from two 10 cm dishes.
NOTE: Most cardiomyocytes will still be floating in the supernatant after 1 hr of incubation. To avoid contaminating with fibroblasts that are lightly attached to the dish, do not collect the supernatant by pipetting.
13. Optional> Count cells in the supernatant with and without 0.2% trypan blue to check cell viability.
14. Sediment cells at 100 x *g* for 5 min. Re-suspend cells in DMEM containing 10% FBS, P/S (10 U/ml) and 0.1 mM BrdU. Plate cells at 2 x 10^5 cells/dish in gelatin-coated 3.5 cm glass bottom dishes for observation using the microscope. NOTE: Do not disturb cells after plating for at least 24 hrs in CO_2 incubator at 37 ˚C.
15. **Day 3,** Change medium 24 hrs after plating to DMEM/MEM containing 5% FBS, P/S and 0.1 mM BrdU.
16. **Day 4,** Change medium 48 hrs after plating to DMEM/MEM containing 5% FBS and P/S.
NOTE: Change medium every 2-3 days with DMEM/MEM containing 5% FBS and P/S.

2. Lentiviral transduction

2.1) Packaging of lentiviral plasmids

NOTE: Please refer to other sources for further in-depth information on this subject [24-26]. It will take about 3 days to prepare the lentiviral solution. It is best to use fresh lentiviral solution to achieve higher transduction efficiency. Start the packaging of lentiviral plasmids and isolation of rat neonatal cardiomyocytes in parallel. Instead of using polyethyleneimine (PEI) [27] for packaging of lentiviral plasmids, a commercially available transfection reagent can be used. Follow the manufacturer's instructions.

1. Prepare reagents and tools; HEK 293T cells, 10 cm plastic cell culture dishes, lentiviral plasmid solutions (pMDLg/pRRE, pRSV-Rev, pMD2.G, lentiviral transfer vector, 1.0 mg/ml each), 1 g/l PEI, serum-free medium, 20 mM chloroquine in water, 10% bleach, 1% SDS in 70% EtOH
2. **Day 0**, Plate 2-2.5 x 10^6 HEK 293T cells per 10 cm dish the day before transfection.
NOTE: 30-60% confluence at transfection is optimal.
3. **Day 1**, Preparing PEI transfection solution. Add 30 ul of 1 g/l PEI to 960 ul serum-free medium in a 1.5 ml tube. Add 4 plasmids into the PEI transfection solution in the 1.5 ml tube, and mix with tapping.

Name of plasmid	Note	Size (kbp)	volume added (ml)	Final amount in 10 cm dish (mg)	Final concentration in 10 cm dish (pM)
Lentiviral transfer vector	encodes gene to be packaged	9-13	3.6-5.2	3.6-5.2	60
pMDLg/pRRE	expresses HIV-1 GAG/POL	8.8	1.76	1.76	30
pRSV-Rev	expresses HIV-1 REV	4.1	0.82	0.82	30
pMD2.G	expresses VSV G	5.8	1.16	1.16	30

Table 2: The amount of plasmids for lentiviral packaging. Use these plasmid amounts to transfect HEK293 cells in 10 cm dishes. Final amount of lentiviral transfer vector per dish may differ according to its size, maintain final concentration per dish at 60 pM. Average molecular weight of one base pair of double stranded DNA is 660 daltons.

4. Discard old medium from 10 cm dish of HEK 293T cells, and gently add 9 ml of new medium (DMEM + 10% FBS, without P/S).
5. Add PEI-DNA mixture gently drop-wise onto the plate and gently swirl to mix with medium. Add 10 ul of 20 mM chloroquine (final 20 uM) to 10 ml medium. NOTE: Chloroquine is thought to reduce the degradation of plasmid-containing transfection complexes through partial neutralization of the pH within lysosomal compartments [28].
6. Incubate in CO_2 incubator at 37 ˚C for 6 hrs. Then, remove medium containing the PEI-DNA mixture and add 10 ml of new medium (DMEM + 10% FBS + 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH7.4 + P/S). NOTE: After incubation, virus will be produced in the medium. Aspirating Pasteur pipettes and/or tips must be decontaminated by 10% bleach. Always decontaminate the biological safety cabinet surface and potentially contaminated equipment with 70% EtOH regardless of whether supernatant has been spilled or not. If spilling has occurred, thorough decontamination with 1% SDS in 70% EtOH is necessary.
7. **Day 2-3**, Incubate in CO_2 incubator at 37 ˚C for 48-72 hrs. **Day 4**, collect lentiviral solution (continue to **Section 2.2**).
NOTE: Medium can be changed at 48 hrs after transfection. Collecting virus supernatant 48 hrs and 72 hrs twice may achieve to obtain higher titer virus solutions.

2.2) Collection of lentiviral solution and transduction

1. Prepare reagent and tools: 15 ml conical tubes, 1 M HEPES, pH7.4 (0.22 um filtered)
2. **Day 4**, Collect supernatant containing lentivirus particles (lentiviral solution) with 10 ml sterilized Luer-Lok syringe, then filter the supernatant through 0.45 um filter into a 15 ml conical tube. Add 1/100 volume of 1 M HEPES pH7.4 to filtered lentiviral solution (final 10 mM).

NOTE: For filtering, use only cellulose acetate or polyethersulfone (PES) (low protein binding) filters. Do not use nitrocellulose filters. Nitrocellulose binds surface proteins on the lentiviral envelope and destroys the virus.

3. Remove medium from 3.5 cm dishes of rat neonatal cardiomyocytes, obtained at the last step of **Section 1.2**, add 1 ml of filtered lentiviral solution to the 3.5 cm dish.
4. <Optional> Add hexadimethrine bromide (final concentration 4-6 ug/ml).
   NOTE: Hexadimethrine bromide may enhance lentiviral transduction efficiency. Concentration of hexadimethrine bromide should be optimized.
5. Incubate dish for 6 hrs in CO_2 incubator at 37 ˚C for lentiviral transduction, then change medium to new DMEM/MEM containing 5% FBS and P/S. Incubate dish in CO_2 incubator at 37 ˚C for 3 days.
   NOTE: Integration of the exogenous gene into the host genome by lentivirus is considered to be completed by 72 hrs.
6. **Day 7**, Use cells from step 2.2.5 for the next step. NOTE: Protein expression from the lentiviral plasmid can be confirmed by WB or immunocytochemistry (ICH) after 48-72 hrs of transduction in order to determine the relative expression level (Figure 1a and 1b).

2.3) Concentration of lentiviral solution

NOTE: It is best to use fresh lentiviral solution in order to achieve highest transduction efficiencies, in case the lentiviral solution titer is not high enough, the lentiviral solution can be concentrated by polyethylene glycol (PEG) precipitation [29].

2.3.1) Preparation of 4x PEG solution (32% PEG6000, 400 mM sodium chloride (NaCl), 40 mM HEPES pH7.4)

1. For 125 ml 4x PEG solution, weigh 40 g of PEG6000, then dissolve it in 60 ml water.
   NOTE: The number after PEG is the average molecular weight (*i.e.* Average molecular weight of PEG6000 is 6,000.)
2. Slowly add 10 ml of 5 M NaCl. Slowly add 5 ml of 1 M HEPES pH7.4. Adjust pH to 7.4 using 2 M sodium hydroxide.
3. Add water to 125 ml. Sterilize 4x PEG solution by membrane filtration with a 0.22 um filter and store it at 4 ˚C.

2.3.2) Lentivirus concentration

1. Filter lentiviral transduced supernatant into new 50 ml tube using a 0.45 um filter.
2. Add 4x PEG solution 1:3 ratio (PEG solution:supernatant = 1:3). Store at 4 ˚C for 16-48 hrs.
3. Centrifuge at 2,600 x *g* at 4 ˚C for 30 min. Discard supernatant and centrifuge at 2,600 x *g* at 4 ˚C for 5 min.
4. Discard supernatant carefully to avoid disturbing the pellet. Re-suspend pellet in serum-free medium using 1/2 to 1/25 volume of the original supernatant volume.
5. Use directly or freeze using liquid nitrogen then store at -80 ˚C

3. Adenoviral transduction

NOTE: Please refer to other sources for methods for the construction and propagation of adenoviral constructs [13]. Aspirating Pasteur pipettes and/or tips must be decontaminated by 10% bleach. Always decontaminate the biological safety cabinet surface and potentially contaminated equipment with 70% EtOH regardless of whether supernatant has been spilled. If spilling has occurred, thorough decontamination with 1% SDS in 70% EtOH is necessary.

1. Adenoviral titration by 50% tissue culture infectious dose ($TCID_{50}$)
   NOTE: Before transduction, it is necessary to titrate the adenoviral stock solution. $TCID_{50}$ is one of the best methods to titrate the viral solution. Because the method evaluates a titer by an infectious capacity to HEK 293T cells, calculated $TCID_{50}$ reflects actual infectability of the adenovirus stock. The PFU (plaque-forming units) is proportional to the $TCID_{50}$ with a factor of about 0.56 [30].
   1. Prepare cells and tools: HEK 293T cells, 10 cm cell culture dishes, 96-well flat-bottom cell culture plate, 8-channel multi pipette
   2. Prepare 70-90% confluent HEK 293T cells in one 10 cm dish.
   3. Perform a pre-dilution of $1/10^4$ of the virus stock. Add 50 ul of DMEM + 10% FBS to each well of a 96-well flat-bottom cell culture plate. Add 25 ul of the pre-diluted virus stock into the first row, from A to H.
   4. Mix well and transfer 25 ul from the first row of wells to the second row with an 8-channel multi pipette. Repeat the 1/3 dilution of viral solution to each row of wells up to and including the 11th row and discard last 25 ul.
   5. Triturate HEK 293T cells into 10 ml of DMEM + 10% FBS. Add 50 ul of cell solution into each well from rows 1 through 12.
   6. Incubate cells at 37 ˚C in CO2 incubator for 11-13 days. Add 50 ul of DMEM + 10% FBS after 4-5 days and 7-8 days of plating.
   7. Measure the cytopathic effects in each well at 11-13 days following infection.

lane	1	2	3	4	5	6	7	8	9	10	11	12
Row A	d	d	d	d	d	d	d	d	d			
Row B	d	d	d	d	d	d	d	d	d			
Row C	d	d	d	d	d	d	d	d	d			
Row D	d	d	d	d	d	d	d					
Row E	d	d	d	d	d	d	d	d	d	d	d	
Row F	d	d	d	d	d	d	d					
Row G	d	d	d	d	d	d	d	d				
Row H	d	d	d	d	d	d	d	d	d	d	d	

the number of positive wells per row	8	8	8	8	8	8	8	6	5	2	2	0
the ratio of positive wells per row	1	1	1	1	1	1	1	0.75	0.63	0.25	0.25	0

d	Displaying over 50% cytopathic effects
	Displaying under 50% or no cytopathic effects

Lane 1, dilution 3^1x10^4; lane 2, dilution 3^2x10^4; … ; lane 11, dilution 3^2x10^{11}; lane 12, control.
S = the sum of the ratios of positive wells per row
= 1+1+1+1+1+1+0.75+0.63+0.25+0.25 +0= 8.875
$TCID_{50}$ = 3 x 10^4 x 3 x (8.875-0.5) = 2.97 x 10^8
$TCID_{50}$/ml = 5.94 x 10^9

Table 3: Example of an infected 96 well-plate after 10 days incubation. Measure the cytopathic effects in each well and sum the ratios of positive wells per row.

8. Calculate the titer according to Kaerber's formula [31].
   $TCID_{50}$ = (dilution of first lane) x $(dilution)^{(S-0.5)}$
   S = the sum of the ratios of positive wells per row
   NOTE: For this protocol, $TCID_{50}$ = (30000) x $(3)^{(S-0.5)}$. Because 50 ul of viral solution is used in this protocol, divide $TCID_{50}$ by 0.05 to calculate $TCID_{50}$/ml.

3.2) Adenoviral transduction

formula	example
MOI = PFU of virus stock / number of cells	100 (MOI) = 6 (ml) x 5.94 x 10^6 ($TCID_{50}$/ml) x 0.56 /
	2 x 10^5 (cells)

The PFU is proportional to the TCID50 with a factor of about 0.56.
Titer of virus stock for example = 5.94 x 109 (TCID50/ml) = 5.94 x 106 (TCID50/µl)
Example: Add 6 ml of virus stock with a titer of 5.94 x 109 (TCID50/ml) to a dish containing 2 x 105 cells to achieve an MOI of 100.

Table 4

1. Calculate required amount of adenoviral solution from its titer (PFU/ml, viral particle/ml or $TCID_{50}$/ml). Use the formula to calculate MOI (multiplicity of infection).
2. Add calculated amount of virus solution to serum-free medium (see Table 5). Mix well and incubate for 20 min at RT.

MOI	Volume from stock (ml)	Volume to add serum free medium (ml)	Volume to add to dish (ml)
1	3	197	2
3	3	197	6
10	3	197	20
30	3	197	60
100	6	194	200

Table 5

3. **Day7**, Take dish obtained at the last step of **Section 2.2**, and replace medium with 750 ul of new DMEM/MEM containing 5% FBS and P/S.
   NOTE: Reduction of medium volume at transduction to about 50% of the original volume is critical for high transduction efficiency.
4. Add diluted virus solution to dish. Incubate dish for 4-8 hrs in CO_2 incubator at 37 ˚C. Then, replace with fresh medium.
5. Incubate dish for 24-48 hrs in CO_2 incubator at 37 ˚C. At day **8-9**, Use cells for the live-cell imaging experiment. NOTE: Protein expression from the adenoviral plasmid can be confirmed by WB or ICH after 24-48 hrs of transduction in order to determine the relative protein expression level (Figure 1c and 1d).

4. Live cell imaging

4.1) Image acquisition using a confocal spinning disk microscope with a temperature controlled chamber and a CO_2 environmental system (optional)

1. Turn the temperature control system on at least 1 hr prior to acquisition, so that all devices equilibrate to 37 ˚C prior to the start of acquisition. Turn on confocal spinning disk microscope system (PC for operation, microscope, spinning disk unit, CCD camera, lasers, fluorescent light source, normal light source, motorized stage, and automatic shutters).
   NOTE: More than 1 hr is necessary to achieve the complete temperature equilibration of all devices.
2. Change medium in 3.5 cm glass bottom dishes to desired medium for the acquisition, if necessary. Place the dish on the stage of confocal spinning disk microscope in a temperature controlled chamber.
   NOTE: Due to spherical aberration for optimal observation by microscopy use of a 3.5 cm glass bottom dish with No. 1.5 cover glass (about 0.16–0.19 mm thickness) is ideal. If the objective being used has a correction collar, images obtained with cover glasses thicker than 0.17 mm can be corrected. Also, Phenol red has a slight fluorescence. Sometimes it is preferable to use a phenol red free medium, such as DMEM without phenol red. If there is no device to control CO_2 concentration on the microscope stage, it is better to use a CO_2 independent medium or medium buffered by HEPES for long term time-lapse imaging.
3. Select the light path to the eyepieces.
4. Turn the shutter of the bright-field light source on. Adjust the focus to cells under the microscope through the eyepieces. Turn the shutter for the bright-field light source off.
5. Turn the shutter for the fluorescent light source on. Find cells expressing fluorescent proteins under the microscope through the eyepieces. Turn the shutter for the fluorescent light source off.
6. Optional> Press the activation button for the perfect focus system in front of the microscope to turn the perfect focus system on in order to keep focus steady during the time-lapse acquisition.
7. Change the light path to the spinning disk confocal microscope. Adjust settings for acquisition appropriately using the operating software, checking images displayed on the screen. (*e.g.* Focus, laser power, exposure time, CCD camera gain and off-set)
8. Set settings for the fluorescent image acquisition using operating software. Example: Select “Change channels using light paths” and “Channel 1: EGFP” to acquire fluorescent signal from EGFP for channel one.
9. Set the frequency between time-points by configuring time-lapse settings in order to take time-lapse images using the operating software. Example: To acquire one time-point every 5 minutes, select “Minutes per Time-point” from the drop-down menu, and enter 5 into the text field.

   NOTE: Select “Maximum Speed” to make a movie at actual speed. If expression of the fluorescent protein is low, the fluorescence may bleach quickly during time-lapse acquisition. In that case, it is better to reduce the number of acquisition time-points.
10. Start the time-lapse acquisition by clicking the “start” button to acquire an image sequence.
    NOTE: It is better not to use the multiple-points acquisition function. It may cause loss of focus or movement of the imaging field during time-lapse imaging.

4.2) Analysis of acquired images

NOTE: Acquired images can be played back as a movie file and analyzed using the analysis software.

1. Select the acquired images to be included in a movie. If necessary, crop regions of interest from images and select interesting time-points to reduce file size of the movie using the operating software.
2. Export images as a movie file in AVI or MOV format. Select a movie format from “Format” drop-down menu and the timing required for the finished movie, then click “Export”. NOTE: Time-lapse images acquired at one time-point every 5 minutes can be played back as a movie at 10 frames per second, which is 3,000 times faster than its actual speed.

Representative Results

To illustrate the technique, a lentivirus encoding EGFP (enhanced green fluorescent protein)-tagged Cx43 (Connexin43) or a mutated Cx43 [32] was used to express EGFP-tagged proteins in cells, and an adenovirus encoding FGFR1DN (fibroblast growth factor receptor 1 dominant negative) was used to shut down FGF signaling in the cell [33-35]. Three days following the isolation of cardiomyocytes, the isolated cardiomyocytes were transduced with lentivirus in order to express EGFP-tagged proteins in the cells. Then after 3 days of lentiviral transduction, the isolated cardiomyocytes were further transduced with an adenovirus encoding FGFR1DN to eliminate FGF signaling in the cells. After allowing enough time for cells to express the transduced exogenous genes, a confocal spinning disk microscope was used to capture live cell images. Acquired images are played back as a movie file and analyzed using image analysis software. For more detailed representative results please see Sakurai *et al*[32].

The expression of EGFP tagged proteins following lentiviral transduction was confirmed by confocal spinning disk microscopy 72 hr following lentiviral transduction **Figures 1A-B**. The expression of FGFR1DN by adenoviral transduction was confirmed by WB and ICC after 24 hr of adenoviral transduction **Figures 1C-D**. The time of adenoviral addition to 3.5 cm dishes was defined as time 0 and time-lapse images were acquired with a 40x air NA 0.9 objective lens every 5 min for 6 hr using a chamber heater to maintain the temperature at 37 ˚C. Time-lapse images of EGFP-tagged proteins in the primary cardiomyocytes taken by confocal spinning disk microscopy are shown in **Supplemental Movies 1-2**.

In cases where the temperature equilibration was not adequate, or the multiple-acquisition mode was not functioning properly, images captured might move in the x-y plane **Supplemental Movie 1**. However when working properly the autofocus system stably maintained focus and this did not occur. As shown in **Supplemental Movie 2**, when the temperature equilibration was adequate and only one region of interest was acquired, the images captured are of very high quality.

The beating of cardiomyocytes was assessed after long-term time-lapse live cell imaging in order to confirm cell viability. Even after 16 hr of time-lapse imaging EGFP expressing cardiomyocytes maintained their functional properties and beat rhythmically **Supplemental Movie 3**.

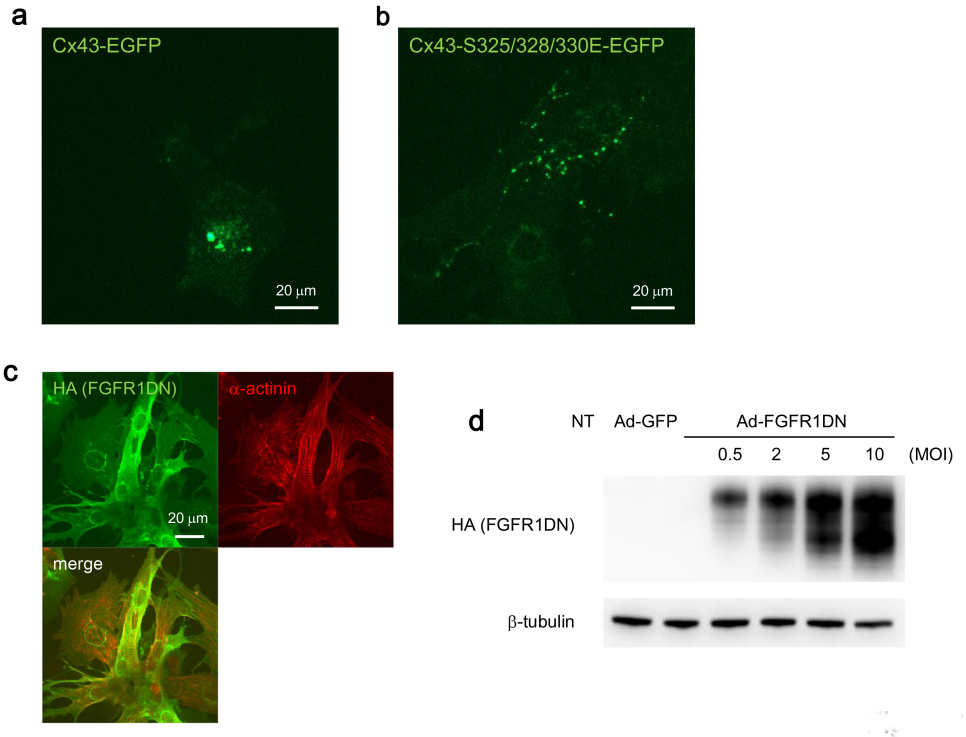

Figure 1. Confirmation of protein expression following lentiviral or adenoviral transduction. (A) Isolated rat neonatal cardiomyocytes expressing Cx43-WT-EGFP transduced with Ad-FGFR1DN 3 days after transduction. See also **Supplemental Movie 1**. **(B)** Isolated rat neonatal cardiomyocytes expressing Cx43-S325/328/330E(3SE)-EGFP transduced with Ad-FGFR1DN 3 days after transduction. See also **Supplemental Movie 2**. **(C)** Protein expression following adenoviral transduction detected by ICC 24 hr after transduction. Hemagglutinin (HA) protein-tagged FGFR1DN was detected by anti-HA antibody using conventional ICC methods, alpha-actinin was used as a marker for cardiomyocytes. **(D)** Expression of FGFR1DN protein following adenoviral transduction detected by WB 24 hr after transduction. HA-tagged FGFR1DN was detected by anti-HA antibody by WB, beta-tubulin was used as loading control.

Supplemental Movie 1. Time-lapse imaging of isolated rat neonatal cardiomyocytes expressing Cx43-WT-EGFP transduced with Ad-FGFR1DN. The time-lapse images were acquired with a 40x objective lens every 5 min for 6 hr with a confocal spinning disk microscope. Acquired images were played back as a movie at 10 frames per sec. This movie has been modified from Sakurai *et al*[32]. Please click here to view this video.

Supplemental Movie 2. Time-lapse imaging of isolated rat neonatal cardiomyocytes expressing Cx43-S325/328/330E-EGFP transduced with Ad-FGFR1DN. The time-lapse images were acquired with a 40x objective lens every 5 min for 6 hr with a confocal spinning disk microscope. Acquired images were played back as a movie at 10 frames per sec. This movie has been modified from Sakurai *et al*[32]. Please click here to view this video.

Supplemental Movie 3. Time-lapse imaging of isolated rat neonatal cardiomyocytes played back at actual speed. The time-lapse images of isolated rat neonatal cardiomyocytes expressing Cx43-EGFP transduced with Ad-FGFR1DN were acquired with a 40x objective lens every 200 msec for 10 sec with a confocal spinning disk microscope following 16 hr of time-lapse acquisition. Acquired images were played back as a movie at 5 frames per sec at actual speed. Please click here to view this video.

Discussion

Primary cardiomyocytes isolated from neonatal rats have long been used to study cardiomyocyte functions *in vitro*. This protocol describes a method for the isolation of neonatal cardiomyocytes from rat pups using a two-step enzyme digestion method, first digesting with trypsin O/N at 4 °C and then purified collagenase. One advantage of employing the purified collagenase step is that the same grade of enzyme is used for all isolations. Thus, the quality and amount of isolated cells is consistent from experiment to experiment.

Given the higher transduction efficiencies associated with adenovirus, if a high efficiency of transduction of the cell culture is necessary for a given experiment, it is better to use an adenovirus. However, if high levels of transduction are not necessary, a lentiviral vector can be used. Having the option of using either adenoviral or lentiviral vectors makes it easier to achieve appropriate transduction and expression levels for different types of genes. If the fluorescent intensity detected by live-cell imaging using the confocal spinning disk microscope or ICC is very low after lentiviral transduction, using another lentiviral transfer vector that uses a different promoter to express the gene of interest may resolve this

problem. With adenoviral vectors, if the band detected by WB is weak after adenoviral transduction, this may indicate a low titer viral stock. In that case, it is better to prepare a higher titer adenoviral solution by propagation in cells before proceeding.

One limitation of this technique is that the yield of isolated viable cardiomyocytes is not highly consistent from experiment to experiment, even when using purified collagenase. In order to perform reproducible imaging experiments it is best to count viable cells after the selection of isolated cardiomyocytes in the supernatant at step 1.2.13. in the protocol section. Then, seed cardiomyocytes on glass bottom dishes at varying dilutions. Once the cells have attached, select dishes with the appropriate cell concentration for the experiment planned.

There are many advantages of using neonatal cardiomyocytes, but there are also some disadvantages. The major drawback is their clear difference from adult cardiomyocytes. Fully differentiated adult cardiomyocytes are rod-shape and isolated adult primary cardiomyocytes maintain their rod-shaped morphology, whereas isolated neonatal primary cardiomyocytes spread in all directions[36]. The phenotypic differences between isolated adult and neonatal cardiomyocytes are also reflected in their patterns of gene expression [37]. Therefore, for experiments in which differentiated cardiomyocytes are required, a protocol for the isolation of adult cardiomyocytes will be needed[4].

Employing adenoviral and lentiviral transduction of recombinant proteins into primary rat neonatal cardiomyocytes in combination with confocal spinning disk microscopy allows us to analyze the functions of proteins in cultured live primary cells. Relative to existing methods, which utilize transfection to introduce the gene of interest into cells, a significant advantage of this protocol is that adenoviruses and lentiviruses are very efficiently taken up by the cells resulting in the expression of the gene of interest in almost all cells in the culture. Furthermore, the use of two different types of viruses for gene expression makes it easier to achieve appropriate transduction rates and expression levels for multiple genes. siRNAs and chemical agonists and antagonists can also be used in this system to further perturb and analyze the functions of genes and the proteins they encode.

Preparation of the hearts is an extremely important step in the protocol. Remove the hearts from the body as quickly as possible. Be sure to put them on ice immediately to keep cell viability high. Another important step is the preparation of high quality, and titer, lentiviral/adenoviral stocks. If poor quality viral stocks are used, transduction and expression levels of the gene of interest will be low. Therefore, they may not be suitable for imaging studies.

An important future application of this technique will be its' use in mouse cardiomyocytes. Pilot studies testing whether this method can be used for the isolation of mouse neonatal cardiomyocytes have yielded promising results. Due to the relatively small size of mouse hearts compared to those of rats, less tissue is obtained initially. Thus, fewer cells are isolated in a single procedure using the same number of animals but simply using more mice for the isolation should eliminate this obstacle. The ability to isolate mouse cardiomyocytes allows researchers to investigate the functions of cells isolated from genetically modified mice (transgenic, knock-out, knock-in) that have been produced in order to study a particular form of the gene of interest.

One of the major hurdles in cardiovascular research has been the difficulty associated with investigating the expression and function of a given gene expressed in heart tissue *in vivo* in real time. However the ability to isolate functional beating cardiomyocytes, modulate cell functions using viral transduction, and then study them in real time using the confocal spinning disk microscope helps to shed light on the role of cardiomyocytes *in vivo* and bridge this gap. This method provides a simplified system for understanding cardiomyocyte function at the cellular level. It allows for real time analysis of how perturbations in gene expression alter cardiomyocyte function. Live cell imaging of cardiomyocytes will provide further insights into cardiomyocyte function. Such insights will lead to advances in basic cardiovascular research, resulting in novel therapies for the treatment of cardiovascular disease.

Disclosures

The authors have nothing to disclose.

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