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Gene Transfer of Calcium-Binding Proteins into Adult Cardiac Myocytes

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Abstract

Heart failure is the leading cause of combined morbidity and mortality in the USA with 50% of cases being diastolic heart failure. Diastolic heart failure results from poor myocardial relaxation and inadequate filling of the left ventricular chamber caused in part by calcium-handling dysregulation. In this chapter we describe methods to investigate new approaches of novel human Ca^{2+} binding protein motifs to restore normal Ca^{2+} handling function to diseased myocardium. Gene transfer of parvalbumin into adult cardiac myocytes has been studied as a potential therapeutic, specifically as a strategic Ca^{2+} buffer to correct cardiac mechanical dysfunction in disease. This chapter provides protocols for studying wild-type parvalbumin isoforms and parvalbumins with strategically designed EF-hand motifs in adult cardiac myocytes via acute adenoviral gene transfer. These protocols have been used extensively to optimize parvalbumin function as a potential therapeutic for failing heart muscle.

Keywords

Parvalbumin; Calcium; Adult cardiac myocyte; Contractility; Calcium imaging; Gene transfer

1 Introduction

Heart failure is the leading cause of combined morbidity and mortality in this country with an estimated five million Americans affected and up to 600,000 new cases per year [CDC/AHA statistics]. Heart failure can be subdivided clinically into systolic and diastolic heart failure. Systolic heart failure is a progressive and largely intractable clinical syndrome characterized by an overall decline in the pumping function of the heart. Diastolic heart failure is just as common as systolic heart failure and results from poor myocardial relaxation and inadequate filling of the left ventricular chamber. Diastolic heart failure is increasing in prevalence with the majority of patients being women and the elderly. There is no cure for heart failure except heart transplantation, which is woefully inadequate for the millions of heart failure patients. Thus there is an urgent need to discover and implement new approaches to treat this disease.

There is extensive scientific literature demonstrating myocardial intracellular Ca^{2+} dysregulation as a key element underlying defective heart performance in failure [1]. We and others have therefore used this foundation as a starting point to investigate new approaches of novel human Ca^{2+} binding protein motifs to restore normal Ca^{2+} handling function to

diseased myocardium. The growing burden of cardiovascular disease in this country and throughout the world necessitates unwavering vigor directed at the mechanistic basis of disease with the goal of identifying new therapeutic targets and effective treatment modalities. The focus here on novel physiologically optimized EF-hand Ca^{2+} binding motifs offers an exciting new therapeutic approach for the diseased and failing heart: specifically, strategic Ca^{2+} buffering to correct cardiac mechanical dysfunction in disease. We agree with the premise that accelerating Ca^{2+} transient decay would be corrective for diastolic dysfunction and would be beneficial to heart performance in the long term, providing excessive SR Ca^{2+} load is avoided. We have designed novel EF-hand Ca^{2+} binding motifs that facilitate fast Ca^{2+} transient decay while maintaining Ca^{2+} transient peak amplitude [2].

Parvalbumin is a cytosolic calcium buffer endogenously expressed in glycolytic skeletal muscle [3]. Due to parvalbumin's affinities for Mg^{+2} and Ca^{+2} and the cytosolic contents of these cations, parvalbumin almost exclusively binds Mg^{+2} at rest [4]. Upon calcium-induced calcium release from the sarcoplasmic reticulum, parvalbumin binds Ca^{+2} which allows contraction to occur and facilitates faster relaxation. Gene transfer of parvalbumin into adult cardiac myocytes has been studied as a potential therapeutic in diastolic heart failure where there is slow Ca^{+2} reuptake and relaxation [5–7]. This chapter provides protocols for studying parvalbumin and mutants in adult cardiac myocytes via adenoviral gene transfer [1]. These protocols have been used extensively to study parvalbumin and mutants to optimize parvalbumin function for cardiac myocytes and the heart [2, 8–10].

2 Materials

2.1 Isolation of Adult Cardiac Myocytes and Adenoviral-Mediated Gene Transfer

2.1.1 Instruments and Perfusion Apparatus

1. Gather surgical instruments and glassware (Fig. 1). Clean thoroughly and autoclave.
2. Surgical instruments: Large scissors, small scissors, small hemostat, large forceps, needle nose forceps, curved forceps, and 14 gauge luer lock compatible cannula.
3. Wide-bore glass Pasteur pipettes siliconized (sigma, SL2) for trituration.
4. 4–0 surgical silk.
5. The perfusion apparatus used for adult cardiac myocyte isolation (Fig. 2) consists of two solution reservoirs placed above and connected to a double-barreled warming coil with changeover stopcock (Harvard “Baker” perfusion). Circulating 37 °C water bath should be turned on ahead of time to warm up the perfusion apparatus.
6. The reservoirs of Ca^{2+} -free and Ca^{2+} +KHP solutions are placed at around 65 cm from the bench level and oxygenated.
7. The perfusion apparatus is cleaned three times with 70% ethanol then rinsed three times with sterile ddH₂O before and after each rat preparation.

2.1.2 Solutions

1. Ca^{2+} -free Krebs-Henseleit buffer (Ca^{2+} -free KHB): 118 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4 , 2 mM MgSO_4 , 25 mM HEPES, and 11 mM glucose.
2. Ca^{2+} Krebs-Henseleit buffer (Ca^{2+} -KHB): add 1.0 mM CaCl_2 to Ca^{2+} -free KHB (see Note 1).
3. Ca^{2+} -free KHB with collagenase: 30 mg of type 2 collagenase from Worthington to solve in 80 mL Ca^{2+} -free KHB during Ca^{2+} -free KHB retrograde perfusion (see Note 2).
4. Ca^{2+} -KHB with 2% bovine serum albumin: solve 30 mg BSA in 15 mL Ca^{2+} KHB.
5. Culture media: M199 (M3769–1 L from Sigma) + 1× Pen Strep.
6. Plating media: supplement M199 culture media with 5% heat-inactivated fetal bovine serum.
7. Filter sterilize all solutions using a 0.2 μm filter (see Note 3).

2.2 Analysis of Calcium-Handling Protein Expression and Localization

2.2.1 Western Blot Solutions

1. Lysis buffer (RIPA buffer): 50 mM Tris, pH 8.0; 150 mM NaCl; 0.1% Triton X-100; 0.5% sodium deoxycholate; 0.1% sodium dodecyl sulfate (SDS); protease inhibitors tablet.
2. Loading buffer: 62.5 mM Tris-HCl, pH 6.8; 2% SDS; 25% glycerol; 0.01% bromophenol blue.
3. Running buffer (Tris/glycine/SDS): 25 mM Tris, pH 8.3; 192 mM glycine; 0.1% SDS.
4. Tris-buffered saline with Tween 20 (TBST) buffer: 20 mM Tris, pH 7.5; 150 mM NaCl, 0.1% Tween 20.
5. Transfer buffer: 25 mM Tris base, 190 mM glycine, 20% methanol.

2.2.2 Immunofluorescence Solutions

1. Phosphate-buffered saline (PBS): 137 mM NaCl; 2.7 mM KCl; 8 mM Na_2HPO_4 ; and 2 mM KH_2PO_4 .
2. 3% paraformaldehyde in PBS: 3 g of paraformaldehyde to 100 mL of PBS.
3. Blocking buffer: 1× PBS containing 20% normal goat serum (NGS) and 0.5% Triton X-100.
4. Antibody dilution buffer: 1× PBS containing 2% NGS and 0.5% TX-100.

¹. Prepare and use KHB on the same day.

². Weigh collagenase and keep in Erlenmeyer flask covered with thin foil until needed.

³. Only add warm 37 °C media to cells. M199 media can be stored at 4 °C for weeks.

2.3 Cellular Functional Analysis

2.3.1 Solutions

1. Tyrode's solution: 140 mM NaCl, 0.5 mM MgCl₂, 0.33 mM NaH₂PO₄, 5 mM Hepes, 5.5 mM glucose, 1.8 mM CaCl₂, 5 mM KCl, pH to 7.4.
2. Tyrode's solution no Na⁺, no Ca²⁺: Same as Tyrode's solution with no CaCl₂ added and LiCl replaces NaCl.
3. Tyrode's solution no Na⁺, no Ca²⁺ with caffeine: Same as Tyrode's solution no Na⁺, no Ca²⁺ with addition of 20 mM caffeine.

2.3.2 Equipment

1. Ionoptix contractility and calcium imaging system.
2. Myocam.
3. HyperSwitch filter box.
4. Xenon arc lamp.
5. Fluorescence Systems Interface.
6. Fura-2 filter cubes and PMT.
7. Inverted microscope with 40× objective.
8. MyoPacer.
9. Heated stage adapter temperature control unit.
10. Stimulation chamber.
11. Motorized stage and recall software.
12. IonWizard analysis software.
13. Perfusion apparatus and vacuum pump.

3 Methods

3.1 Adult Rat Ventricular Cardiac Myocytes Isolation

The following protocol includes heart extraction and retrograde perfusion using a modified Langendorff apparatus for an initial digestion. The ventricles are then minced, and cardiomyocytes are separated by gentle trituration. Ca²⁺ is gradually added to make the isolated cardiomyocytes Ca²⁺-tolerant prior to plating.

3.1.1 Terminal Thoracotomy and Heart Excision

1. Adult female Sprague Dawley rat (225–275 g) is initially injected intraperitoneally with 0.3 cc of heparin (1000 IU/mL). After 10–15 min, anesthetize rat with 0.7 cc of sodium pentobarbital (diluted to 50 mg/mL). Note: use isoflurane to shortly anesthetize the rat and deliver the IP injection.

2. For all subsequent steps, a surgical mask and sterile gloves should be worn to prevent potential bacterial and mycobacterial contamination. Sterile surgical instruments and glassware should be used throughout the isolation procedure. *See Fig. 3* for a timeline.
3. Once the rat becomes unresponsive (30–60 s after pentobarbital injection), spray 70% ethanol over the chest and abdomen.
4. Cut open the thorax. Rapidly and carefully pull the heart upward using curved forceps, and excise above the aortic arch.
5. Place the heart in ice-cold 10 mL Ca^{2+} -free KHP solution (in 100 mm dish placed on ice bucket), trim excess tissue, and mount the heart on the cannula. Tie surgical silk twice to secure the aorta to the cannula. The tip of the cannula should be above the aortic valve. If the cannula tip is placed into the left ventricle, the coronary perfusion won't be successful.
6. Slowly flush the heart with Ca^{2+} -KHP in the cannula-mounted syringe. The heart will resume beating. Note: the cannula is fixed to syringe filled with Ca^{2+} -KHP and flushed prior to mounting the heart to avoid formation of air bubbles.

3.1.2 Retrograde Perfusion and Enzymatic Digestion

1. Start a slow drip of oxygenated Ca^{2+} -KHP, and then transfer the cannulated heart to the perfusion apparatus. Note: watch for bubbles; make sure there are no bubbles in the running perfusion apparatus before mounting the cannulated heart.
2. Increase the flow rate and wait for the solution to run clear (about 30 s). In the meantime, open the side valve to dispose of unoxygenated Ca^{2+} -free KHP solution setting in tubing.
3. Switch the stopcock to open oxygenated Ca^{2+} -free KHP, and allow the heart to perfuse until the ventricles stop contracting (less than 1 min). Simultaneously, dissolve 30 mg of collagenase type 2 from Worthington in 80 mL Ca^{2+} -free KHP by swirling.
4. Once the heart stops contracting, drain as much Ca^{2+} -free KHP through the side valve as possible, and then add the 80 mL of Ca^{2+} -free KHP with collagenase solution to the Ca^{2+} -free side. Adjust O_2 flow to avoid foam formation. At the end of the procedure, the final volume of collagenase solution should be close to 80 mL.
5. Once the collagenase solution is added, place 50 mL beaker under the heart, so it is immersed in collagenase solution, and start the timer. Flow rate should be approximately 10 mL/min. Recirculate the collagenase solution during the remaining time of the procedure.
6. After 7 min of perfusion with the collagenase solution, add 150 μL of 100 mM CaCl_2 to the collagenase solution (final $[\text{Ca}^{2+}] = 0.1875 \text{ mM}$). Let the heart perfuse for 7 more min, and add 150 μL of 100 mM CaCl_2 to the collagenase solution (final $[\text{Ca}^{2+}] = 0.375 \text{ mM}$). After another 7 min, add 150 μL of 100 mM

CaCl₂ to the collagenase solution (final [Ca²⁺] = 0.625 mM), and continue the perfusion for an additional 7 min for a total of 28 min of perfusion with collagenase solution. Note: it is important to not perfuse the heart for more than 30 min with the collagenase solution as this tends to decrease the final yield (*see* Note 4).

7. Remove the heart and cannula from perfusion apparatus, and collect all the collagenase solution from the perfusion apparatus in 250 mL Erlenmeyer flask.

3.1.3 Trituration and Induction of Ca²⁺ Tolerance

1. Transfer the heart to a plastic petri dish with 2–3 mL of collagenase solution. Using small scissors and needle nose forceps, remove the atria and aorta. Cut ventricles into 1–2 mm pieces, and gently transfer into sterile 50 mL beaker with 15–20 mL collagenase solution (*see* Note 5).
2. Gently swirl the tissue suspension at room temperature, then pour off supernatant (fraction 1), and add 15–20 mL of collagenase solution recovered in the Erlenmeyer flask.
3. Centrifuge fraction one tube at 40 × *g* for 15 s, recover supernatant in a new 50 mL conical, and discard fraction one cell pellet as cell viability is usually very low (around 1–10%).
4. Using wide-bore siliconized Pasteur pipette, gently triturate the minced tissue for 2–3 min. Recover supernatant in 15 mL conical labeled fraction 2, add 15–20 mL collagenase solution to the minced tissue, and place on the shaker.
5. Centrifuge fraction two tube at 40 × *g* for 15 s, recover supernatant in the 50 mL conical, and discard fraction two cell pellet (*see* Note 6).
6. Gently triturate the minced tissue for 2–3 min. Recover supernatant in 15 mL conical labeled fraction 3, add 15–20 mL collagenase solution to the minced tissue, and place on the shaker (*see* Note 7).
7. Centrifuge fraction three tube at 40 × *g* for 15 s; recover supernatant in the 50 mL conical. Resuspend cell pellet in 1–2 mL of Ca²⁺-KHP with 2% BSA.
8. Assess cell viability by looking at a drop of the cell suspension under the microscope. If 70% or more of the cells from the fraction are viable (rod shaped), place the tube on the shaker and save the fraction.
9. Repeat step 6, 7, and 8 to obtain fraction 4–15 (*see* Note 8).

⁴.During retrograde perfusion visualize the heart to verify that it is not turning white. This is a sign of ischemic tissue most likely due to a small bubble blocking perfusion.

⁵.It is important to prevent the heart from drying out while being cut down and minced.

⁶.The supernatant can be recovered in a 50 mL conical tube to be reused if one runs out of the collagenase solution in the Erlenmeyer flask.

⁷.The amount and velocity of trituration are critical as too forceful and fast will result in dead myocytes, while too slow and weak will result in not many myocytes.

⁸.The cell pellet color is also a good indicator of cell viability; a pink pellet likely contains a large number of viable cells, whereas in a white or slightly gray pellet, most cells are probably dead. Typically, cell pellets get pink (>70% viable cells) around fraction 5.

10. Combine the different fractions, and bring to a final volume of Ca^{2+} -KHP with 2% BSA to 10 mL in a 15 mL conical with a sterile plastic transfer pipette (wide mouth).
11. Increase Ca^{2+} concentration to 1.75 mM by adding 3–25 μL aliquots of 100 mM CaCl_2 at 5 min intervals. Incubate tube on the shaker at room temperature in between Ca^{2+} additions.
12. After final Ca^{2+} addition and incubation, centrifuge at $40 \times g$ for 15 s. In the tissue culture hood, aspirate supernatant, and gently resuspend cells in 10 mL of warm (37°C) M199 media supplemented with 5% FBS.
13. Use hemocytometer to count live cells in the homogenous cell suspension; make sure cells are well resuspended and not settling out.

3.2 Primary Culture and Adenovirus-Mediated Gene Transfer

3.2.1 Cultureware Preparation and Plating

1. In cell culture hood, place $1 \times 25 \text{ mm}^2$ coverslip per well (6well plate). Coat each coverslip with 100 μL laminin (40 $\mu\text{g}/\text{mL}$ from Life Technologies), and leave uncovered under UV for 10 min to sterilize. Cover plates and allow laminin to set for an additional 10 min prior to plating.
2. Adjust cell concentration to 1×10^5 myocytes/mL in M199 culture media supplemented with 5% FBS.
3. Remove excess laminin from coverslips by aspirating.
4. Using aerosol-resistant tips, carefully plate 200 μL cell suspension per coverslip (2×10^4 rod-shaped cells/coverslip).
5. Place plate in incubator carefully so as to not disturb cells (Fig. 4). Incubate for 2 h at 37°C (*see Note 9*).

3.2.2 Transduction: Gene Transfer

1. Just before the 2 h incubation is over, make the desired dilutions of the adenovirus which will be used for gene transfer. For parvalbumin use Flag-tagged versions for easy detection. Typical multiplicities of infection (MOI) for adult cardiac myocytes are between 100 and 500 without toxicity. This should be empirically determined for each virus. The virus should be thawed and kept on ice (do not allow it to warm to room temp). Resuspend the virus before diluting. Pipette up and down 20–25 times.
2. In a microcentrifuge tube, mix the virus with serum-free M199 media. A total volume of 200 μL per coverslip will be used. Mix well to get an even distribution of the virus.

⁹The surface tension should maintain the droplet on the coverslip. Make sure to not disturb the droplet when moving the plate to the incubator to avoid cells dispersion and to obtain a good yield of live cells.

3. At the end of the 2 h incubation, gently aspirate the plating media by carefully placing the Pasteur pipette tip at the edge of the bubble. Many of the unattached myocytes (dead) will be aspirated away.
4. Using an aerosol-resistant pipette tip, add 200 μ L of virus dilution to the coverslip, taking care that it stays on the coverslip. Carefully return the cells to the incubator for 1 h without disturbing the bubble.
5. After 1 h, add 2 mL M199 culture media to each well (without removing the virus-containing media). Try to aim delivery away from coverslip so that myocytes are not dislodged. Return the dish to the incubator.
6. 24 h later, aspirate the old media and replace with M199 culture media. Media should be changed every 2–3 days thereafter. Serum-free media is used for cardiomyocyte primary culture to minimize dedifferentiation of cardiomyocytes. Using the protocol described earlier, ventricular cardiomyocytes can be maintained for up to 7 days without detectable changes in contractile protein stoichiometry and isoform composition.

3.3 Analysis of Calcium-Handling Protein Expression and Localization

Parvalbumin, a cytosolic calcium-binding protein, can be characterized using both Western blot analysis and immunofluorescence staining. Western blot is a widely used technique to detect levels of expression of parvalbumin protein. Through this technique it is possible to measure the parvalbumin expression levels in biological tissues such as fast-contracting muscles, where levels are highest. Immunofluorescence is used to determine the localization and relative expression and allows visualization of the distribution of parvalbumin in the sample. Together, both techniques, which will be described next, determine the expression pattern and subcellular location of parvalbumin in biological samples.

3.3.1 Western Blot Analysis—Western blotting (WB), also called immunoblotting, is a common and well-accepted procedure to determine levels of protein expression in a cell or tissue extracted. This technique measures protein levels in a biological sample through antibody binding to a specific protein of interest. In this technique, previously separated proteins are transferred to a membrane, and antibodies specific to the epitopes on the separated proteins are then used to probe the membrane. The precise binding that occurs between an antibody and the protein epitope allows detection of the target protein. In this section we are going to describe how to use WB technique to detect parvalbumin protein expression.

3.3.2 SDS-PAGE—The first step on a SDS-PAGE procedure is the choice of which gel to use. The choice of which gel to use depends on several factors including the number of samples, the size of proteins you are looking at, and if you are attempting to separate proteins of similar molecular weights. The differences between the distinct types of gel are primarily the pH at which the acrylamide is polymerized and the pH at which is run. It is possible to make your own gel, or precast gels also can be used. The most common precast gel used in our lab is the Criterion (Tris-Glycine eXtended). Gel percentage of 12% is ideal for small proteins, such as parvalbumin.

1. After resuspending the sample in a lysis buffer, leave on ice for 30 min to 1 h.
2. Boil the sample for 2 min, and after that spin in microcentrifuge at maximum speed for 2 min.
3. Remove supernatant and transfer to a new tube. Take a small volume of lysate to perform a protein quantification assay. Use Bio-Rad BCA assay to determine the protein concentration for each sample lysate. This assay is sensitive and tolerates the presence of detergents.
4. To a volume of protein sample (cell or tissue lysate), add equal volume of loading buffer.
5. To reduce and denature, boil each sample at 95 °C for 5 min. Centrifuge at $16,000 \times g$ for 5 min. These samples can be stored at -80°C or can be used to procedure with gel electrophoresis.
6. Load equal amounts of protein into the wells of the SDS-PAGE gel, along with molecular weight marker. Load 50 μg of total protein from cell lysate or tissue homogenate.
7. Run the gel for 1–2 h at 100 V.

3.3.3 Transfer—The membrane used for transfer can be either nitrocellulose or PVDF (polyvinylidene difluoride). If you are going to use PVDF, activate this membrane with methanol for 1 min, and rinse with transfer buffer before use.

1. In preparation for transferring proteins, preincubate the filter paper, sponges, and membrane (either PVDF or nitrocellulose) in transfer buffer.
2. When gel is finished running, remove cartridge, and crack open sides using metal spatula.
3. Remove the gel from the plates, and place it into the transfer buffer for at least 15 min (*see Note 10*).
4. Building the transfer sandwich: Place a sponge (thin abrasive pads) topped by a piece of filter paper on to the black side of the folder. Next, carefully place the gel (pre-equilibrated in transfer buffer) on top of this. In a similar method, apply the membrane to the gel. It is particularly critical that air bubble is not introduced at this junction. Next, place a second piece of filter paper on top of the membrane. At this step, firmly roll out any air bubbles. Finally, place the second sponge on top of the assembly. Close the folder and secure the locking mechanism; it should be tight.
5. Place the sandwich into the transfer tank and fill with transfer buffer over the top. For tank transfers, it is important that the transfer buffer be stirred during the transfer.

¹⁰If using polyvinylidene fluoride (PVDF), pre-wet in methanol. This membrane is very hydrophobic, and water will not gain access to the filter unless it is pre-treated with an alcohol. In contrast, nitrocellulose is very soluble in high concentrations of alcohol and will dissolve if placed in these solutions.

6. Run at constant current (amps) at 350 mA for 1–5 h or at 250 mA overnight.

3.3.4 Antibodies

1. Immunodetection is performed by initially blocking nonspecific binding sites with Tris-buffered saline with Tween 20 (TBST) containing 5% (w/v) nonfat dry milk for 1 h at room temperature.
2. The membrane is then incubated in TBST + 5% milk containing the primary monoclonal antibody (for Flag-tagged parvalbumin you can use anti-Flag M2 antibody) overnight at 4 °C and then washed three times in TBST + 5% milk for 5 min each.
3. Incubate the membrane for 1 h at room temperature in TBST + 5% milk containing a secondary antibody conjugated to IR 680 or 800. Wash the membrane three times with TBST + 5% milk for 5 min each.
4. Antibody binding is detected by Licor Odyssey Imaging. Use image analysis software to read the band intensity of the loading control proteins. Anti-beta actin can be used as a loading control for parvalbumin detection by WB.
5. Use the loading control protein levels to normalize parvalbumin levels.

3.3.5 Immunofluorescence—Immunofluorescence (IF) is a powerful technique that combines the use of the specificity of antibodies to their antigen with optical microscopy imaging to visualize proteins within fixed cell or tissue samples. Through IF, parvalbumin can be detected using a two-step approach with unlabeled primary antibody followed by fluorophore-conjugated secondary antibody (called indirect detection). This technique can be useful to gain insight into parvalbumin localization allowing the identification of co-localization as well as changes in subcellular localization.

Fixation and Permeabilization

1. Fix cells in coverslips with 3% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min. Fixation is usually performed on ice to ensure better preservation and morphology, but fresh, cold fixative added during the fixation protocol works well.
2. Following the fixation, wash samples three times in ice-cold PBS for 5 min each.
3. For the permeabilization step, incubate the cells with 0.1% of TX-100 in PBS for 10 min.
4. Prepare humid chamber for blocking and antibody incubation steps. Line staining tray (or any other sealable plastic box) with gauze, saturate gauze with dH₂O, and then place cottonless swabs on top of gauze.
5. Drain off excess 1 × PBS and transfer the coverslips to humid chamber.
6. For the blocking step, add ~200 µL of PBS containing 20% normal goat serum (NGS) and 0.5% Triton X-100 (TX-100) on each coverslip. Incubate for 30 min

at room temperature. The PBS-TX + NGS should be first filtered through a 0.22 μ m syringe filter.

7. Gently aspirate off serum. Do not wash in 1 $\times$ PBS after this step, and do not allow sections to dry out at any point.

3.3.6 Antibodies

1. Immunodetection is performed by initially blocking nonspecific binding sites with Tris-buffered saline with Tween 20 (TBST) containing 5% (w/v) nonfat dry milk for 1 h at room temperature.
2. Wash samples three times in PBS containing 0.5% TX-100 for 5 min each.
3. Drain off excess liquid and place samples in humid chamber. Block nonspecific secondary antibody binding by incubating samples in PBS containing 20% NGS and 0.5% TX-100 for 30 min. Usually use 20% NGS unless having trouble with obtaining a positive stain.
4. Gently aspirate off serum. Incubate samples with secondary Ab (Alexa Fluor conjugated to goat anti-mouse IgG) diluted in PBS containing 2% NGS and 0.5% TX-100 for 1 h.
5. Wash samples three times in PBS containing 0.5% TX-100 for 5 min each.
6. If wanting to detect another protein with a second mouse primary antibody, follow the steps below. If only staining with one mouse primary, proceed to step 15.
7. Drain off excess liquid and place samples in humid chamber. Incubate samples in goat anti-mouse IgG overnight at 4 $^{\circ}$ C to neutralize unreacted sites on the first primary antibody and Alexa Fluor-conjugated secondary Ab.
8. Wash samples three times in PBS containing 0.5% TX-100 for 5 min each.
9. Drain off excess liquid and place samples in humid chamber. Incubate samples with goat anti-mouse Fab fragments for 1.5 h at room temperature. Fab fragments are diluted 1:20 in PBS-TX with 2% NGS. These steps eliminate any recognition by the second set of primary and secondary antibodies.
10. Wash samples three times in PBS containing 0.5% TX-100 for 5 min each.
11. Block samples with 20% NGS in 1 $\times$ PBS containing 0.5% TX-100 for 30 min at room temperature.
12. Drain off excess liquid and place samples in humid chamber. Add the second primary antibody diluted in PBS containing 2% NGS and 0.5% TX-100 for 1 h at room temperature. Wash three times in PBS containing 0.5% TX-100 for 5 min each.
13. Block samples in PBS containing 20% NGS and 0.5% TX-100 for 30 min at room temperature.

14. Apply second secondary antibody (Alexa Fluor-conjugated to goat anti-mouse IgG) diluted in PBS containing 2% NGS + 0.5% TX-100 for 1 h at room temperature.
15. Wash samples three times in PBS. Drain off excess liquid, and mount coverslip with a drop of mounting medium. Seal coverslip with nail polish to prevent drying and movement under microscope. Samples are ready for microscopic analysis.

3.4 Cellular Functional Analysis

Functional characterization of parvalbumin-transduced adult cardiac myocytes combines sarcomere length dynamics and calcium transient analysis using the Ionoptix contractility and calcium imaging system. Individual cardiac myocytes, 1–6 days post transduction, are analyzed for alterations in both contractility amplitude and kinetics and both calcium transient amplitude and kinetics. This analysis allows one to understand the impact of parvalbumin on excitation-contraction coupling.

3.4.1 Sarcomere Length Dynamics

1. Mount coverslips to temperature-controlled stage.
2. Apply a small amount of vacuum grease to the bottom of the stimulation chamber in a circle at the edge of where the coverslip will fit.
3. With a forceps remove one coverslip from the culture media, and quickly dry the bottom with a Kimwipe.
4. Gently attach to the bottom of the stimulation chamber with cell side up, and then press down on a flat surface to firmly attach.
5. Place in the heated stage and fill the chamber with warm Tyrode's solution.
6. With a forceps remove excess vacuum grease from the chambers edges.
7. Add temperature probe to the solution and monitor temperature until 37 °C.
8. Visualize the cells and start stimulation (usually 0.2 Hz to 1 Hz) (*see Note 11*).

3.4.2 Collection of Sarcomere Dynamics

1. Once the myocytes are at temperature and are stimulated, open IonWizard software and collect new experiment. Make sure the experiment includes sarcomere length detection.
2. Find a contracting myocyte through the microscope, and switch the light path to the MyoCam (*see Note 12*).
3. Position the myocyte in the video window so that the striations are vertical in the window.

¹¹-If the cells are not stimulating, make sure all grease is removed from the edges. If they still are not stimulating, check the pulse duration (should be 2–4 ms) and increase the voltage.

¹²-Make sure the myocyte is rod shaped and is pacing correctly with no random waves of contraction.

4. Drag the pink sarcomere length detection box on the myocyte, and position to get good signal. The more of the myocyte that is included, the higher is the signal to noise.
5. Verify the resting sarcomere length (healthy cells usually have sarcomere lengths in excess of 1.7 μm).
6. Start the experiment, and verify that the contraction is smooth and returning to baseline lengths. Collect at least ten contractions and then stop the experiment, and save the file for analysis.
7. If studying a drug such as isoproterenol, mark the location of the cell in the recall software to be able to recall that cell, and collect pairwise plus and minus drug (*see* Note 13).
8. Collect 10–15 cells per coverslip from 3 to 4 independent rat preps for statistical power.

3.4.3 Analysis in IonWizard 6.0

1. Open the file in IonWizard 6.0.
2. Average the contractions to one contraction under operations. The more contractions analyzed the better is the signal to noise in the average.
3. Under marks add a monotonic transient. Pay attention to the offset (how prior to the stimulation mark you want to analyze for baseline) and duration (make sure the duration is long enough to return 100% to baseline) (*see* Note 14).
4. Under operations select monotonic transient analysis. A fifth-order polynomial fit results with five colors fit over the average contraction. Make sure the five colors fit well with each other and with the curve otherwise change your offset or duration.
5. Export the trace and resulting data to graphing program for compiling all the myocytes collected (*see* Note 15).

3.4.4 Calcium Transient Analysis and SR Calcium Load

1. Reconstitute Fura-2 AM powder in DMSO to a final concentration of 1 mM. Use fresh DMSO for consistent results. Avoid repeat freeze-thaw cycles by aliquoting single day use amounts.
2. Add 1 μE Fura-2 AM to 1 mL of M199 in a six-well dish for a final concentration of 1 μM . Add 1 mL of M199 to two more wells of the dish for subsequent wash steps.

¹³Collecting pairwise increases the statistical power of the experiment therefore using a motorized stage, and recalling cells is optimal.

¹⁴If one is not getting good fits with the baseline or other portions of the transient, alter the duration and offset to match the trace.

¹⁵Common parameters to graph include baseline (BL), normalized peak height (bl% peak h), peak amplitude (peak h), times to peak, and times to baseline (usually 25%, 50%, and 75% for to peak and to bl). Be careful if you want to plot the exponential fits and tau. You need to make sure that those parameters fit well on the trace. This is not always the case.

3. Using forceps transfer a coverslip of cells to the Fura-2 AM M199 well. Leave at room temperature in the dark for 10 min.
4. Transfer the coverslip from the Fura-2 AM well to a M199 only well for 5 min, and repeat again to the next well for 5 more minutes.
5. Load the coverslip onto the stimulation chamber as above, and fill with Tyrode's solution.
6. Wait for an additional 5–10 min for de-esterification of the Fura-2 AM. The de-esterification time is 20 min total after removing from the Fura-2 AM well.
7. Verify correct temperature and start stimulating as above.

3.4.5 Collection of Calcium Transients

1. Collection of calcium data is similar to the above-described collection of sarcomere length dynamics with the exceptions below.
2. Make sure the experiment is set for calcium and sarcomere length (*see* Note 16).
3. After positioning a myocyte in the video window as above, reduce the window size to just smaller than the myocyte using the shutter on the MyoCam. Avoid cells that are adjacent to each other as the fluorescence can bleed through. Position the sarcomere length box to measure simultaneously.
4. Start the experiment, and monitor windows for the numerator, denominator, and the ratio as well as sarcomere length changes.
5. Again collect ten or more transients for analysis. The Fura-2 ratio is usually noisier than the sarcomere length curve so analyzing more transients can clean this up.
6. After collecting data from the cell, move to a portion of the coverslip nearby that is empty of cells, and collect background fluorescence for 5 s. This will be used in the analysis. Make sure this signal has no changes with stimulation and is truly background.

3.4.6 Caffeine Perfusion for SR Load Functional Assessment

1. Load cells with Fura-2 AM as above and mount to the stimulation chamber as above.
2. As cells are loading, set up a perfusion apparatus with flow control and in-line temperature control. Three 60 mL syringes work well for the three solutions that will be used. Fill one with Tyrode's; one with Tyrode's no Ca^{+2} , no Na^{+} ; and one with Tyrode's no Ca^{+2} , no Na^{+} plus caffeine.
3. Once coverslip is mounted, perfuse with Tyrode's solution, and allow media to come to temperature.

¹⁶Caution should be used for sarcomere length data in Fura-2 loaded myocytes as it can buffer Ca^{+} and affect contractility. The above loading procedure limits this, but every cell is different. That said, monitoring sarcomere length changes relative to Ca^{+2} changes is very informative.

4. Start pacing and set your window in IonWizard to bracket a cell as above.
5. Start collection (this is continuous collection of data throughout the experiment) of 10 contractions while monitoring the 340/380 nm ratio with Tyrode's.
6. Stop pacing and switch perfusion to Tyrode's no Ca^{+2} , no Na^{+} for 20 s.
7. Switch perfusion to Tyrode's no Ca^{+2} , no Na^{+} plus caffeine for 8 s.
8. Switch perfusion back to Tyrode's no Ca^{+2} , no Na^{+} to wash out caffeine until 340/380 nm ratio is back to baseline.
9. Switch to normal Tyrode's, and pace again to make sure the cell survived and is functioning normally (*see* Note 17).

3.4.7 Analysis

1. Analysis for calcium transients is as for sarcomere dynamics except for the background subtraction. Once averaged and monotonic transient analysis is complete, go to operations and click constants. Highlight background option, and enter the numerator background value and the denominator background value from the background file collected. This is done for every file.
2. Analysis for caffeine transients is manually entered marks to surround the caffeine peak by going to mark options in IonWizard. This will allow monotonic transient analysis of the caffeine peak as above.
3. Similar outputs to graph as sarcomere length dynamics are generally used.

3.5 Summary

The protocols above have been used extensively to study parvalbumin in adult cardiac myocytes [2,5,8–10]. The protocols allow one to study structure function of parvalbumin through mutagenesis and live cell function. In this way optimized parvalbumin mutants have been developed and will continue to be developed [9,10]. By following these protocols, one can engineer adult cardiac myocytes to adenovirally express parvalbumin, view localization and expression levels, and study live cell function through calcium imaging and contractility. In this way one can have a full understanding of the functional implications of parvalbumin in the cell.

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¹⁷·If perfusion is for an entire well, then only one cell is used per coverslip. Otherwise using micropipettes with a three-barrel end and a micromanipulator one can switch solutions at one cell, and use multiple cells on one coverslip.

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Fig. 1. Surgical instruments: Large scissors, small scissors, small hemostat, large forceps, needle nose forceps, curved forceps, and 14 gauge luer lock compatible cannula

Fig. 2.
Heart perfusion apparatus

Fig. 3.
Heart perfusion timeline

Fig. 4.

Cell plating: the surface tension should maintain the droplet on the coverslip. Make sure to not disturb the droplet when moving the plate to the incubator to avoid cells dispersion and to obtain a good yield of live cells