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Isolation and Culture of Adult Mouse Cardiac Myocytes

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Summary

Cardiac myocytes are activated by hormonal and mechanical signals and respond in a variety of ways, from altering contractile function to inducing cardio-protection and growth responses. The use of genetic mouse models allows one to examine the role of cardiac-specific and other genes in cardiac function, hypertrophy, cardio-protection, and diseases such as ischemia and heart failure. However, studies at the cellular level have been hampered by a lack of suitable techniques for isolating and culturing calcium-tolerant, adult mouse cardiac myocytes. We have developed a straightforward, reproducible protocol for isolating and culturing large numbers of adult mouse cardiac myocytes. This protocol is based on the traditional approach of retrograde perfusion of collagenase through the coronary arteries to digest the extracellular matrix of the heart and release rod-shaped myocytes. However, we have made modifications that are essential for isolating calcium-tolerant, rod-shaped adult mouse cardiac myocytes and maintaining them in culture. This protocol yields freshly isolated adult mouse myocytes that are suitable for biochemical assays and for measuring contractile function and calcium transients, and cultured myocytes that are suitable for most biochemical and signaling assays, as well as gene transduction using adenovirus.

Key Words: Cardiac myocyte; cell isolation; cell culture; hypertrophy; cell signaling; apoptosis; adenovirus; β-adrenergic.

1. Introduction

Cardiac myocytes respond to a variety of hormonal, neural, mechanical, and electrical stimuli by altering their force and rate of contraction *(1)*. In addition, cardiac myocytes hypertrophy as a compensatory response to either physiological stimuli, such as exercise, or pathological stimuli, such as hypertension *(2)*. The signaling pathways regulating cardiac myocyte function, hypertrophy, and death encompass a wide array of signaling molecules and pathways, including G protein-coupled receptors, cytokine receptors, and a plethora of intracellular signaling molecules *(3–6)*, and these processes have important clinical relevance *(7)*.

From: *Methods in Molecular Biology, vol. 357: Cardiovascular Proteomics: Methods and Protocols*
Edited by: F. Vivanco © Humana Press Inc., Totowa, NJ

The advent of transgenic and knockout mouse technology has advanced our ability to examine the role of many cardiac-specific and other genes in cardiac function, hypertrophy, cardio-protection, ischemia, and heart failure. However, it would be extremely beneficial to study these genetic manipulations at the myocyte level. Although techniques for isolating and culturing myocytes in other animals, such as rat or rabbit, have been refined, the isolation and culture of adult mouse cardiac myocytes remains challenging.

This chapter describes a straightforward protocol for the isolation and the short-term (24 h) and long-term (72 h) culture of adult mouse cardiac myocytes. The protocol is an adaptation of procedures used to isolate myocytes from other animals, such as rats and rabbits, and prior work in the mouse *(8–10)*. Briefly, the heart is excised rapidly and mounted on a perfusion apparatus. The heart is then perfused with a calcium-free buffer to arrest contraction, followed by a collagenase-based enzyme solution to digest the extracellular matrix of the heart. After digestion, myocytes are dispersed into a single-cell suspension and calcium is reintroduced. Once the myocytes are equilibrated (calcium-tolerant), they are plated in culture dishes, allowed to attach, and finally washed and cultured overnight.

Using this protocol, we routinely isolate roughly 1–1.5 $\leftrightarrow 10^6$ rod-shaped myocytes per heart *(11)*, which is approximately two- to threefold higher than other published reports in the mouse heart *(8–10)*. The goal of the protocol is rod-shaped myocytes, to mimic cells in the intact heart, rather than the round myocytes ("meatballs") that are seen when the myocytes hypercontract. Freshly isolated myocytes are highly useful for biochemical and other assays, because this preparation eliminates "contamination" by the many nonmyocytes assayed when the intact heart is used. The short-term (24 h) culture protocol allows the myocytes to recover and stabilize after the isolation procedure, and is suitable for signaling assays and early growth and death end points. The long-term (72 h) culture is a platform for late growth responses and genetic manipulation by adenovirus, or by other approaches. We have used the freshly isolated myocytes to measure contractile function and calcium transients. We have used the short-term cultured myocytes in a wide variety of applications, including measuring production of second messengers like cAMP, activation of signaling proteins using phospho-specific antibodies, and changes in gene expression *(11,12)*. Finally, we have used the long-term cultured cells for high-efficiency gene transduction with adenovirus *(11)*. The following describes the protocol and illustrates these various applications.

2. Materials

See details of solutions in **Tables 1–6**. All solutions should be made with culture-grade 18.2 MΩ H_2O.

Table 1
Perfusion Buffer (1X and 10X Recipes)[a]

Compound	Mol. wt (g/mol)	Final conc. (m*M*)	1X add g/L	10X add g/L
NaCl	58.4	120.4	7.03	70.3
KCl	74.6	14.7	1.1	11
KH_2PO_4	136.1	0.6	0.082	0.82
Na_2HPO_4	142	0.6	0.085	0.85
$MgSO_4$-$7H_2O$	246.5	1.2	0.30	3
Na-HEPES[b]	1 *M*	10	10 mL	100 mL
$NaHCO_3$	84	4.6	0.39	
Taurine	125.1	30	3.75	
BDM	101.1	10	1	
Glucose	180.2	5.5	1	

[a]All reagents are from Sigma Chemical (St. Louis, MO), except Na-HEPES (Gibco-BRL, Bethesda, MD). For 1X perfusion buffer made fresh from powder at the time of use, dissolve ingredients in 990 mL of 18.2 MΩ H_2O and filter-sterilize. Adjust the pH to 7.0 with sterile HCl as needed. For 10X perfusion buffer, dissolve in 900 mL of 18.2 MΩ H_2O, add 100 mL of Na-HEPES, and store at 4°C for up to 1 wk. To make 1X perfusion buffer, add $NaHCO_3$, taurine, BDM, and glucose fresh on the day of use, and filter-sterilize. Adjust the pH to 7.0 with sterile HCl as needed.

[b]Na-HEPES comes as a 1 *M* liquid.

Table 2
Myocyte Digestion Buffer (50 mL/Heart)[a]

	Final conc.	Amount
Perfusion buffer		50 mL
Collagenase II	2.4 mg/mL	120 mg

[a]Prepare fresh for each heart and add to the reservoir just prior to digestion, to prevent heat inactivation of the enzyme. The absolute amount of enzyme will vary from lot to lot and from different manufacturers (*see* **Note 7**).

2.1. Myocyte Isolation

1. Perfusion buffer (*see* **Table 1**): reagents are from Sigma Chemical (St. Louis, MO), except Na-HEPES (Gibco-BRL, Bethesda, MD), and buffer is optimally made fresh each day, using 18.2 MΩ H_2O, and sterile-filtered with a 0.22-µm filter. The buffer pH should be 6.9–7.0; if not, adjust the pH to 7.0 with sterile HCl after filtration. For simplicity, if many preparations are to be done in a single week, a 10X buffer without $NaHCO_3$, taurine, 2,3-butanedione monoxime (BDM), and glucose can be made and stored at 4°C, then when ready for use these four reagents are added fresh, the solution is filtered, and the pH is adjusted to 7.0.

Table 3
Myocyte Stopping Buffer (20 mL/Heart)

	Final conc.	Volume
Perfusion buffer		18 mL
Calf serum	10%	2 mL
100 m*M* $CaCl_2$	12.5 µ*M*	2.5 µL

Table 4
Myocyte Plating Medium

	Final conc.	Volume (mL)
MEM (Eagle's w/HBSS)		42.5
Calf serum	10%	5
BDM	10 m*M*	1
Penicillin	100 U/mL	0.5
Glutamine	2 m*M*	0.5
ATP	2 m*M*	0.5

Table 5
Myocyte Culture Medium[a]

	Final conc.	Volume (mL)
MEM (Eagle's w/HBSS)		48.5
BSA	1 mg/mL (0.1%)	0.5
Penicillin	100 U/mL	0.5
Glutamine	2 m*M*	0.5

[a]This myocyte culture medium is for short-term culture (24 h). For long-term culture (72 h), the myocyte culture medium is also supplemented with 10 m*M* BDM and ITS medium supplement (*see* **Table 6** and **Note 17**). Even in the short-term culture, addition of a low concentration of BDM (1 m*M*, 100 µL of BDM stock) can improve myocyte morphology and survival, without any detectable effect on the assays shown in this chapter. BSA in the short-term culture can be reduced to 0.1 mg/mL (0.01%) without detectable negative effects.

2. Myocyte digestion buffer (*see* **Table 2**): add to perfusion buffer 120 mg per 50 mL crude Collagenase Type II for a final 2.4 mg/mL (Worthington Biochemical, Lakewood, NJ). Note that the amount of collagenase will vary with the particular lot being used. After adding the collagenase, this buffer should be sterile-filtered again with a 0.22-µm filter to remove any undissolved particulate.
3. Myocyte stopping buffer (*see* **Table 3**): add to perfusion buffer 10% (v/v) calf serum (CS) (HyClone, Logan, UT) and 12.5 µ*M* $CaCl_2$ (Sigma Chemical). CS is not heat-inactivated.

Table 6
Stock Solutions[a]

Calf serum: (HyClone, cat. no. SH30073). Store the CS in 25-mL aliquots in sterile 50-mL tubes at −20°C.

Bovine serum albumin (endotoxin and lipid-free): (Sigma, cat. no. A-8806). Prepare 100 mg/mL stock in H_2O (100X, 10%, 5g in 50-mL) and sterile-filter. Store BSA in 5-mL aliquots at −20°C.

$CaCl_2$ (1 *M*): (Sigma, cat. no. C-7902). Prepare 1 *M* by adding 14.7 g/100 mL H_2O. Filter with 150-mL filter unit (Fisher, cat. no. 09-740-28E) and store at room temperature. Make 1:10 dilution to get 100 m*M* stock for the stop buffer and calcium reintroduction.

BDM: 2,3-Butanedione monoxime: (Sigma, cat. no. B-0753). Prepare 500 m*M* stock in H_2O (50X, 2.25 g/50 mL H_2O). Warm the solution to dissolve BDM and sterile-filter into a 100-mL filter bottle. Store in 5-mL aliquots at 4°C. Warm before use to dissolve any precipitate.

Penicillin-G: (Sigma, cat. no. P-7794, powder). Penicillin powder is 1600 U/mg (6.25 mg/10,000 U). Prepare 10,000 U/mL stock in H_2O (100X, 312.5 mg/50 mL H_2O) and sterile-filter. Store in 5-mL aliquots at −20°C.

L-Glutamine: (Gibco, cat. no. 25030-81). This is 200 m*M* (100X) stock supplied in a 50-mL bottle. Store in 5-mL aliquots at −20°C.

Laminin coating solution: (BD Bioscience, cat. no. 354232). The stock solution is 2 mg/mL, and is stored in 100-µL aliquots at −70°C. Dilute 1:200 in PBS (Ca/Mg-free) for working solution of 10 µg laminin/mL PBS. Do not re-use diluted laminin. Unused coated dishes can be saved overnight, but this is not advised.

Heparin: (ICN/MP Biomedicals, cat. no. 101932). The stock solution is 1000 IU/mL, and is stored at room temperature. Dilute 1:10 in PBS (Ca/Mg-free) for working solution of 100 IU/mL. Do not re-use diluted heparin.

Na-ATP: (Sigma, cat. no. A-6419). Prepare 200 m*M* stock (100X, 1 g/9 mL H_2O), sterile-filter, store 0.5-mL aliquots at −20°C. ATP is not stable and is added fresh to the medium just prior to use, 0.5 mL in 50 mL MEM.

ITS medium supplement (insulin, transferrin, selenium): (Sigma, cat. no. I-1884). Make a 100X stock solution from the lyophilized powder (which contains 25 mg insulin, 25 mg transferring, and 25 µg Na selenite) by adding 50 mL H_2O. Sterile-filter the ITS, and store 1-mL aliquots at −20°C. Add 1 mL to 100 mL MEM for long-term myocyte culture, for final 5 µg/mL insulin, 5 µg/mL transferrin, and 5 ng/mL selenium (*see* **Note 17**).

[a]Note that all water is culture-grade 18.2 MΩ H_2O.

4. Myocyte plating medium (*see* **Table 4**): plating medium is minimum essential medium (MEM) with Hank's Balanced Salt Solution (HBSS) (Gibco-BRL), containing 10% (v/v) CS, 10 m*M* BDM, 100 U/mL penicillin, 2 m*M* glutamine, and 2 m*M* Na-ATP. ATP is not stable and is added fresh to the medium prior to use from a 200 m*M* sterile stock solution that is stored at −20°C.

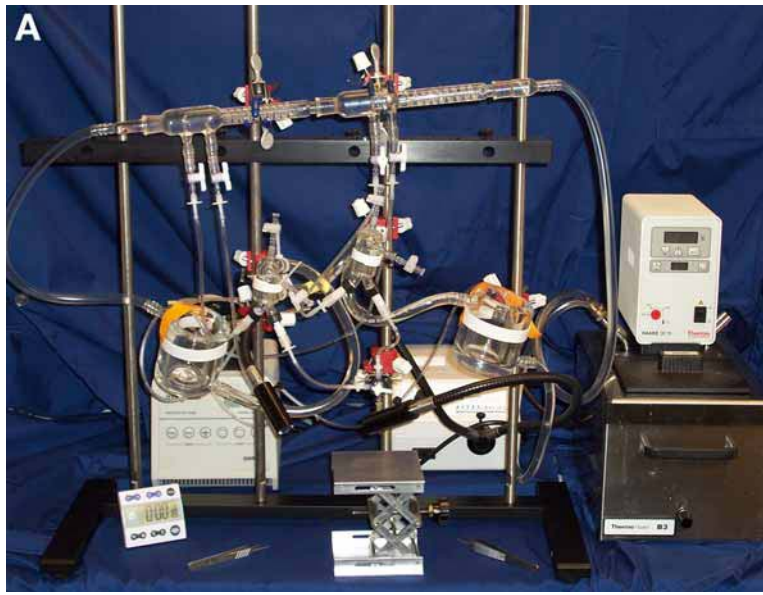


Fig. 1. Perfusion system. The perfusion system has two halves, one for the perfusion buffer and one for the digestion buffer. Each half contains a water-jacketed reservoir for warming solutions to 37°C, from which fluid is pumped through a peristaltic pump, through a heat exchanger to maintain solution temperature, through a bubble trap, then finally out through the cannula. Fluid flow is switched from perfusion buffer to digestion buffer by clamping the appropriate tube at the pump, and turning the three-way valve above the cannula. To maintain the perfusate a 37°C, the water-jacketed circulation system is maintained at 42°C (but this must be empirically determined). **(A)** Overview of the perfusion system. The peristaltic pump and pump tubing are from Rainin Instruments (Oakland, CA); the circulating water bath is from Haake (VWR Instruments); the illuminator, light pipes and focusing lens, and the thermocouple thermometer and probe are from Cole Parmer Instruments; and finally the ring stand is from Radnotti Glass.

5. Myocyte culture medium (*see* **Table 5**): culture medium is MEM with HBSS (Gibco-BRL), containing 1 mg/mL bovine serum albumin (BSA) (endotoxin- and lipid-free), 100 U/mL penicillin, and 2 m*M* glutamine).
6. Laminin-coated dishes: laminin (*see* **Table 6**) (BD Biosciences, San Jose, CA) is stored at −70°C as 100-µL aliquots of a 2 mg/mL stock. Thaw the laminin in the refrigerator, and add 20 mL of ice-cold phosphate-buffered saline (PBS; $CaCl_2$/$MgCl_2$-free, Gibco-BRL) to the laminin stock, for a final concentration of 10 µg/mL. For each heart to be isolated, coat 15 to 20 35-mm dishes or 5 to 7 60-mm dishes. To coat 35-mm dishes, cover the bottom with 1 mL of laminin coating solution; to coat 60-mm dishes, add 3 mL. Incubate at room temperature for at least 1.5–2 h with gentle shaking on a rocker platform (do not use an orbital shaker).

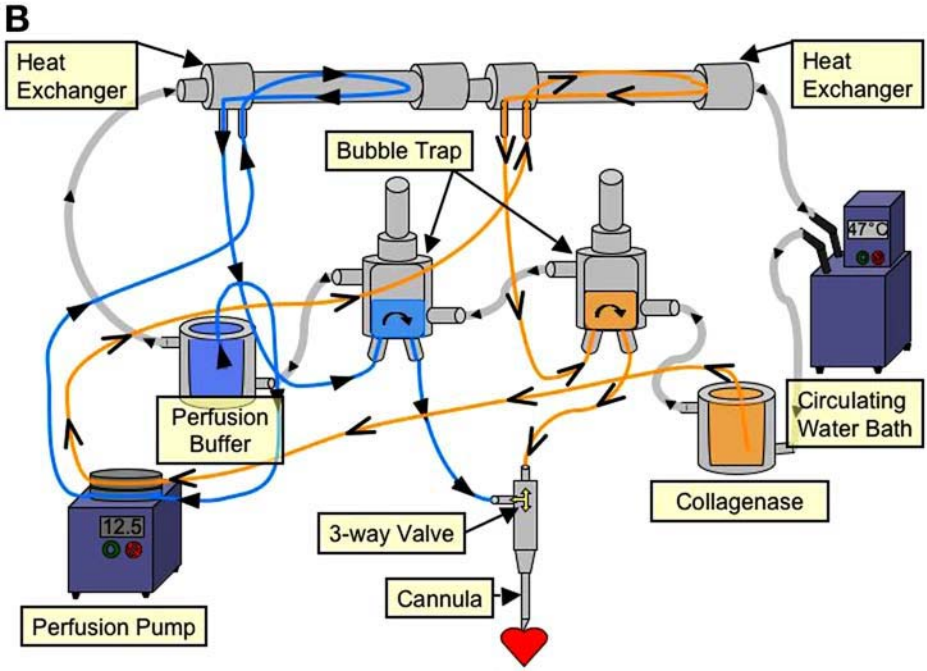


Fig. 1. (*continued*) **(B)** Schematic diagram of the perfusion system and fluid flow. Perfusion buffer or myocyte digestion buffer are loaded in the water-jacketed reservoir and pumped through the peristaltic pump, through the water-jacketed heat exchanger, through a water-jacketed bubble trap, through a three-way valve that controls flow from each half of the system and out through the cannula. The water-jacketed reservoir and heat exchanger are from Ace Glass (Vineland, NJ); the bubble trap, ring clamps, tubing, and tubing adaptors are from Radnotti; and the one-way and three-way valves are from Cole Parmer.

Remove the laminin coating solution just prior to plating the myocytes. It is highly preferable to use the dishes on the day they are made. However, if the plates are not used on the day they are prepared, wrap the dishes individually in parafilm, and store in the refrigerator overnight. Do not refreeze laminin or reuse the coating solution.

7. Perfusion system (*see* **Fig. 1**): the perfusion system is shown in **Fig. 1**. The perfusion system has two halves, one for the perfusion buffer and one for the digestion buffer. Each half contains a water-jacketed reservoir for warming solutions to 37°C, from which fluid is pumped through a peristaltic pump, through a heat exchanger to maintain solution temperature, through a bubble trap, then finally out through the cannula. Fluid flow is switched by changing the pump head attachment and diverting flow from one side to the other, at the three-way valve above the cannula. To maintain the perfusate at 37°C at the outflow of the cannula, the

Table 7
Equipment and Supplies

Culture incubator and metal trays, laminar flow culture hood with vacuum aspirator, centrifuge, phase contrast microscope, light microscope.
Peristaltic pump and pump tubing (Rainin Instruments, Oakland, CA); circulating water bath (Haake, VWR Instruments); iluminator, light pipes, focusing lens, thermocouple thermometer, and probe (Cole Parmer Instruments); ring stand (Radnotti Glass); water-jacketed reservoir and heat exchanger (Ace Glass); bubble trap, ring clamps, tubing, and tubing adaptors (Radnotti); one-way and three-way valves (Cole Parmer); Digi-Sense thermometer and insulated wire probe (Cole Parmer); cannula made of 20G needle, with the point removed and filed flat, and notches 1 and 2 mm above the tip as reference points.
Isoflurane and oxygen; atomizer and induction chamber (Vetland), nose cone made from a small funnel.
Surgical tools: clip, scissors, retractor, foreceps, and Dumont microsurgery fine-tip forceps with microblunted, atraumatic tips angled at 45° (Fine Science Tools) (autoclaved before use); 6-0 surgical silk (about 15 cm); sterile 1-mL syringes with needles (for heparin injection); 70% ethanol
Sterile 60-mm bacterial culture dishes (Valmark).
Sterile culture plastics: transfer pipettes with 2-, 1.5-, and 1-mm openings (Fisher Scientific); 5- and 10-mL pipets; culture dishes, 35- and 60-mm (Fisher Scientific, Falcon 35-3001 and 35-3004); conical polypropylene tunes, 15-mL and 50-mL.
Hemocytometer (VWR, cat. no. 15170-079).

water-jacketed circulation system is maintained at 42°C to 48°C (which must be empirically determined). A Digi-Sense thermometer and insulated wire probe (Cole Parmer, Vernon Hills, IL) are used to measure perfusate temperature. *See* **Subheading 3.10.** for cleaning the perfusion system.

8. Cannula: the cannula for the perfusion system is a 20-gage (G) needle, with the point removed and filed flat. To aid in the cannulation, we make notches 1 and 2 mm above the tip as reference points.

2.2. Removal and Cannulation of the Heart

See **Table 7** for list of special materials and equipment.

1. Heparin: 1000 IU/mL (CN/MPBiomedicals), prepare at 100 U/mL in PBS (Ca^{2+}/Mg^{2+}-free; Gibco-BRL).
2. Surgical tools: all of our surgical tools and a small clip used to hold the aorta to the cannula are from Fine Science Tools (FST, Foster City, CA). For the fine-tip forceps, we recommend the Dumont microsurgery forceps with micro-blunted, atraumatic tips angled at 45° (FST).
3. Isoflurane atomizer, including the induction chamber (Vetland, Louisville, KY).

2.3. Heart Perfusion and Enzyme Digestion

1. Calcium chloride: 100 m*M* $CaCl_2$ in H_2O, sterile filtered with a 0.22-µm filter.

2.4. Myocyte Dissociation

1. Valmark 60-mm dish: this is a sterile bacterial culture dish made by Valmark (Midwest Scientific, St. Louis, MO) to which myocytes will not adhere. Although we assume other nonculture treated dishes would work, we have not tried other vendors.
2. Sterile transfer pipets with 2-, 1.5-, and 1-mm openings (Fisher Scientific, Pittsburgh, PA).

2.5. Calcium Reintroduction

1. ATP: 200 m*M* Na-ATP (Sigma) made in H_2O, stored frozen in aliquots at −20°C, thawed immediately prior to use.
2. Calcium chloride: 100 m*M* $CaCl_2$ in H_2O, sterile-filtered with a 0.22-µm filter.

2.6. Plating Myocytes and Culture

1. Incubators: our incubators are kept at 37°C with 2% CO_2. When MEM with HBSS, which contains 0.35 g/L $NaHCO_3$, is placed in a 2% CO_2 incubator, the resultant pH of the medium is about 6.9–7.0.
2. Falcon culture dishes, 35-mm (15–20 per heart) or 60-mm (5–7 per heart), coated with laminin as above.
3. Myocyte culture medium (*see* **Table 5**).
4. Hemocytometer.

2.7. Long-Term Culture (72-h) of Adult Mouse Myocytes

1. Long-term myocyte culture medium: myocyte culture medium (*see* **Table 5**), with additional 10 m*M* BDM, and insulin, transferrin, and selenium (ITS Medium Supplement, Sigma) (*see* **Table 6** and **Note 17**).
2. Falcon culture dishes, 35- or 60-mm, coated with laminin.

3. Methods

The heart perfusion is done on an open lab bench, but maintaining clean technique and using sterile solutions. The work is transferred to a laminar-flow culture hood at **Subheading 3.4., step 1**.

3.1. Preparation for Myocyte Isolation From One to Two Hearts

This section describes the initial preparation for myocyte isolation. Briefly, all the buffers are prepared, culture dishes are coated with laminin, and the perfusion system is primed.

1. Prepare 500 mL of perfusion buffer for each heart (50 mL will be used to make the myocyte digestion buffer).

2. Prepare 50 mL of myocyte digestion buffer for each heart, and store each 50 mL in a separate 50-mL tube. Do not warm the enzyme for each heart to 37°C until ready to isolate myocytes; exposure to prolonged high temperature might inactivate the enzyme.
3. Prepare myocyte stopping buffer, used to inactivate the collagenase and other proteases in the crude mixture.
4. Prepare myocyte plating medium and myocyte culture medium. Equilibrate both at 37°C in a 2% CO_2 incubator for at least 2 h to adjust temperature and pH.
5. Prepare laminin-coated culture dishes.
6. Prepare the perfusion apparatus (*see* **Fig. 1**). Set the circulating water bath so that the outflow from the tip of the cannula is 37°C, as measured with a digital thermometer. Check the flow rate of the pump and adjust to 4 mL/min (*see* **Note 1**).
7. Run 100 mL of purified water through the perfusion system.
8. Add perfusion buffer (50 mL) and myocyte digestion buffer (50 mL) to the correct reservoirs, prime the perfusion system with buffers (run perfusion buffer and myocyte digestion buffer through system for 5 min), eliminate air bubbles, and allow time to warm to 37°C (about 10 min).
9. Add 10 mL of room temperature perfusion buffer to a 60-mm culture dish for heart collection. Add 10 mL of room temperature perfusion buffer to another 60-mm culture dish for heart cannulation, and place on an adjustable stage under the perfusion apparatus.
10. Position the cannula with the tip close to the surface of the perfusion buffer in the 60-mm dish.
11. Cut a small piece of 6-0 surgical silk (about 15 cm), knot loosely, and place on the adjustable stage (this will be used to secure the aorta to the cannula).

3.2. Removal and Cannulation of the Heart

This section describes the removal and the subsequent cannulation of the heart on the perfusion system. Briefly, the mouse is injected with heparin to prevent coagulation of blood in the coronary arteries. The mouse is then anesthetized, the chest opened and the heart rapidly removed and cannulated. Perfusion with the calcium-free perfusion buffer is started immediately and blood should rapidly clear from the coronary arteries, indicating proper cannulation and good perfusion.

1. Anesthetize the mouse with isoflurane and 100% O_2. Set the isoflurane atomizer dial to 3% (scale 1 to 5% of total flow), turn the O_2 valve to 0.5 L/min, and place animal inside the induction chamber. When the mouse is anesthetized, it will lose consciousness and roll over on its side. Check with a toe pinch to ensure that the mouse is fully anesthetized. Transfer the mouse to the surgery/perfusion area and place under a nose cone connected to the anesthesia system (*see* **Note 2**).
2. Once the mouse is anesthetized, inject the mouse intraperitoneally with 0.5 mL heparin, diluted in PBS to 100 IU/mL. Injecting after anesthesia reduces stress to the animal and improves the isolation.

3. Wait a few minutes for the heparin to circulate. Wipe the chest with 70% ethanol. Adjust the isoflurane (usually 1.5%) as necessary to ensure proper level of anesthesia (movement indicates that the anesthesia is too shallow, whereas irregular respiration indicates that it is too deep). Check with a toe pinch to ensure that the mouse is fully anesthetized.
4. Open the peritoneal cavity and chest with small scissors and use forceps to peel back the rib cage to expose the heart. Lift the heart gently using forceps. Identify and cut the pulmonary vessels, which will make it easier to identify and cut the aorta. Cut the transverse aorta between the carotid arteries, cut the right carotid artery at the same time, and immediately place the heart in a 60-mm dish containing 10 mL of perfusion buffer at room temperature (*see* **Note 3**).
5. Remove extraneous tissues (thymus and lungs), if necessary, and transfer heart to the second 60-mm dish with perfusion buffer at room temperature.
6. Working under magnification (*see* **Note 4**), cannulate the heart using fine-tip forceps to slide the aorta onto the cannula so that the tip of the cannula is just above the aortic valve (check the 1-mm notch on the cannula to ensure proper cannulation; *see* description of cannula in **Subheading 2.1., step 8**). Attach a small clip to the end of the aorta on the cannula to prevent the heart from falling. Start the perfusion immediately (4 mL/min). Tie the aorta to the cannula with 6-0 silk thread. Total time to cannulate the heart should be less than 1 min (*see* **Note 5**).

3.3. Heart Perfusion and Enzyme Digestion

This section describes the enzymatic digestion of the heart. Briefly, the heart is perfused with a collagenase solution to digest the extracellular matrix. During the digestion, the heart will initially become very hard to the touch, owing to a large increase in vascular resistance upon introduction of enzyme. As the perfusion continues the heart will become pale, swollen, and flaccid, indicating a good digestion.

1. Once cannulated, perfuse the heart with perfusion buffer for 4 min at 4 mL/min, to flush blood from the vasculature and remove extracellular calcium to stop contractions (*see* **Note 6**). Measure the temperature of the heart with an insulated wire probe attached to a digital thermometer, placing the temperature probe into ventricle to ensure that the temperature is 37°C. This does not need to be done each time, but should be done periodically to ensure reproducibility.
2. After 4 min, switch to myocyte digestion buffer and perfuse for 3 min at 4 mL/min. Collect the myocyte digestion buffer and discard (*see* **Notes 7** and **8**).
3. After 2–3 min, add 15 µL of 100 m*M* $CaCl_2$ to the myocyte digestion buffer in the reservoir and continue to digest for 8 min at 4 mL/min, although digestion times can vary from heart to heart. At this point, the calcium concentration of the myocyte digestion buffer is roughly 40 µ*M* (15 µL of 100 mm $CaCl_2$ in roughly 35 mL of enzyme buffer). From this point on, the myocyte digestion buffer can be collected and added back to the reservoir for reuse until the digestion is completed. The total digestion time is usually about 11 min. If the heart is well-perfused during

the enzyme digestion, the heart will become swollen and turn slightly pale, and separation of muscle fibers on the surface of the heart might become apparent. Digestion should be terminated if the heart feels spongy when gently pinched.

3.4. Myocyte Dissociation

This section describes the dissociation of myocytes following enzymatic digestion of the heart. Briefly, the heart is removed from the cannula and gently teased apart. A buffer containing serum is added at this point to stop the enzyme digestion and prevent overdigestion. Finally, an initial count of the isolated cells is made to evaluate the digestion of the heart.

1. Once enzyme digestion of a heart is complete (heart appears swollen, pale, and flaccid), cut the heart from the cannula just below the atria using sterile, fine scissors. Place the ventricles in a sterile 60-mm Valmark dish containing 2.5-mL of myocyte digestion buffer. From this point forward, all subsequent steps are performed under a laminar flow culture hood using sterile technique (*see* **Note 9**).
2. Tease the ventricles into 10–12 small pieces with fine-tip forceps. Add 5 mL room temperature myocyte stopping buffer to the dish. Pipet gently several times with a sterile plastic transfer pipet (2-mm opening). This process takes 60–90 s. The tissue should be very flaccid, almost falling apart on its own, and require very little force to dissociate, which will indicate a good digestion (*see* **Note 10**).
3. Transfer the cell suspension to a 15-mL polypropylene conical tube. Rinse the plate with 2.5 mL of myocyte stopping buffer, and combine with the cell suspension for a final volume of 10 mL. Myocyte stopping buffer contains serum to inactivate proteases; the final CS concentration is 5%.
4. Continue to dissociate the heart tissue gently, using sterile plastic transfer pipets with different sized openings (1.5- and 1-mm diameters), until all the large pieces of heart tissue are dispersed in the cell suspension. Avoid vigorous agitation to minimize shearing of the cells (*see* **Note 10**). This process should take 3–5 min. Bring the final volume of cell suspension to 10 mL with myocyte stopping buffer.
5. Transfer 80 µL cell suspension to a microcentrifuge tube, and use duplicate 10 µL aliquots to count rod-shaped and round myocytes in a hemocytometer (VWR, cat. no. 15170-079). Calculate the total number of myocytes, the number of rod-shaped myocytes, and the percent of rod-shaped myocytes. Record these values as the initial number of cells obtained (*see* **Note 11**). For example, 10 µL of the cell suspension is loaded onto each side of the hemocytometer, five grids (center, 4 corners) are counted on each side, and the two sides are averaged. If 60 rod-shaped myocytes and 30 round myocytes are counted, this would give a yield of 1.8 million total myocytes, with 1.2 million rod-shaped (67%):
   a. $[(60 \text{ rod} + 30 \text{ round})/5 \text{ grids}] \leftrightarrow 10^4 \text{ (a constant)} = 1.8 \leftrightarrow 10^5$ cells/mL or $1.8 \leftrightarrow 10^6$ myocytes in the original 10 mL
   b. Rod-shaped myocytes are $1.2 \leftrightarrow 10^6$ (67% rods) by the same formula
6. The important numbers for determining the quality of the isolation are the total number of myocytes (rod and round) and the total number of rod-shaped myocytes

(or the percentage of rod-shaped myocytes). If at this point, the total myocyte yield from a heart is low (<1 million), or the percent of rod-shaped myocytes is low (<50%), this would indicate a less then optimal isolation and a decision as to whether to continue can be made.
7. While counting, allow the remaining myocytes to sediment by gravity for a few minutes at room temperature in the 15-mL tube.
8. Centrifuge for 3 min at 20*g* (400 rpm on Precision Durafuge 100 benchtop centrifuge). Gently resuspend the pellet in 10 mL myocyte stopping buffer (final calcium concentration 12.5 μ*M*).

3.5. Isolation of Myocyte From Multiple Hearts

Often, one might wish to isolate myocytes from more than one heart, sometimes with different genotypes. We have isolated myocytes sequentially from up to four hearts of the same genotype or two hearts with different genotypes.

1. If two hearts of different genotypes are to be digested sequentially, resuspend the combined pellet from the first heart in 10 mL of myocyte stopping buffer, and leave at room temperature while the second heart is digested. Once the second heart is in 10 mL of myocyte stopping buffer, proceed to the calcium reintroduction with both preparations.
2. If two hearts of the same genotype are to be digested sequentially, resuspend the combined pellet of the first heart in 5 mL myocyte stopping buffer, and leave at room temperature while the second heart is digested. Resuspend the combined pellet of the second heart in 5 mL myocyte stopping buffer and combine with cells from the first heart so that the total volume of myocyte stopping buffer is 10 mL. Conduct calcium reintroduction procedures on the combined preparations.

3.6. Calcium Reintroduction

This section describes the gradual reintroduction of calcium to produce calcium-tolerant myocytes. Briefly, the myocytes are incubated in buffer with increasing concentrations of calcium, finally achieving a concentration of 1.2 m*M* Ca^{2+} as in our culture medium.

1. Prior to the calcium reintroduction, add 100 μL of 200 m*M* ATP to the tube, so that the final concentration of ATP in the buffer is 2 m*M* (*see* **Note 12**).
2. At this point, the calcium concentration is 12.5 μ*M*. A three-step calcium reintroduction is done at room temperature to bring the calcium concentration to 1.2 m*M*.
3. Prepare three 15-mL tubes containing 10 mL myocyte stopping buffer:
   a. 100 μ*M* calcium: 10 μL of 100 m*M* $CaCl_2$ in 10 mL myocyte stopping buffer.
   b. 400 μ*M* calcium: 40 μL of 100 m*M* $CaCl_2$ in 10 mL myocyte stopping buffer.
   c. 900 μ*M* calcium: 90 μL of 100 m*M* $CaCl_2$ in 10 mL myocyte stopping buffer.
4. Centrifuge the myocytes for 3 min at 20*g*. Remove the supernatant, which contains nonmyocytes and some round myocytes, and to the pellet, carefully introduce the contents of tube 1 by mixing very gently with a 1.5-mm plastic pipet.

5. Let the myocytes stand for 2 min, then repeat **step 4**; continue in this fashion through tube 3.
6. Resuspend the final pellet in 5 mL of myocyte plating medium at 37°C for a final calcium concentration of 1.2 m*M*.
7. Count rod-shaped and round myocytes using a hemacytometer. Count both sides of a hemocytometer (10 grids, take mean of 5 grids), with duplicate cell aliquots. Calculate the total number of myocytes, the number of rod-shaped myocytes, and the percent of rod-shaped myocytes (*see* **Subheading 3.4., step 5** for a sample calculation). Record these values as the number of myocytes for plating. The most important number is the total number of rod-shaped myocytes, and the goal is at least 1 million. If the total myocyte number is low (total cells less than 1 million), or if myocyte quality is poor (less than 60% rod-shaped myocytes), a decision may be made as to whether to continue with the preparation or to start over, depending on the judgment of the technician.
8. For electrophysiological studies or measurements of contraction and calcium transients, myocyte may be suspended in any suitable buffer, as in **Subheading 3.6., step 6**.

3.7. Plating Myocytes, Myocyte Attachment, and Culture

This section describes myocyte plating and short-term culture (24 h). Briefly, the myocytes are plated on laminin-coated dishes and allowed to attach. After attachment, the nonattached cells, mostly round myocytes and a few rod-shaped myocytes, are washed away. Typically, we get more than 70% plating efficiency; that is, more than 70% of the rod-shaped myocytes initially plated actually attach. After washing, the cultures are about 90% rod-shaped in the dish, and we retain roughly 90% of these rod-shaped myocytes overnight in culture ***(11)***.

1. Myocyte plating medium, which is MEM containing calf serum, BDM (a contraction inhibitor), glutamine, ATP, and penicillin (*see* **Table 4**), should be equilibrated for 2–3 h at 37°C in a 2% CO_2 incubator. Myocyte culture medium should also be equilibrated for the same time. Calculate the total number of rod-shaped myocytes and determine the volume of myocyte plating medium required to adjust the concentration of rod-shaped myocytes to 25,000 rod-shaped myocytes/mL in a 50-mL tube. Make sure that the myocytes are resuspended well by gently pipetting, using a 10-mL pipet.
2. Set up trays to plate no more than six dishes per tray at a time to avoid pH changes as medium is exposed to air. Plate the appropriate amount of rod-shaped myocytes in the desired culture dishes: 2 mL (containing 50,000 rod-shaped myocytes) in a laminin-coated, 35-mm dish, and 6 mL (containing 150,000 rods) in a 60-mm laminin-coated dish. This is a density of roughly 52 rod-shaped myocytes per mm^2. Use a 5-mL pipet to plate two 35-mm dishes at once, to prevent myocytes from settling in the pipet during plating. Use a 10-mL pipet to plate single 60-mm dishes. During the plating procedure, gently resuspend the myocytes constantly, to ensure

that they do not settle to the bottom of the tube, which will cause variation in plating density. Once the myocytes are plated, distribute the myocytes evenly in each dish by gently sliding the tray forward and backward and side-to-side in a cross-like pattern three to four times on the surface of the culture hood. Never swirl the medium in the dish or the myocytes will clump in the center of each dish. Immediately place finished trays in a 2% CO_2 incubator at 37°C. Incubate for 1 h to allow myocyte attachment (*see* **Notes 13–15**).

3. After 1 h, gently aspirate the plating medium with a sterile Pasteur pipet into a vacuum flask. To remove any remaining unattached myocytes and debris, wash each dish with approx 1 mL pre-equilibrated myocyte culture medium, adding medium gently to the sides of the dish, not the bottom, then aspirate the wash. When changing the medium, do not remove more than one tray from the incubator at once, and wash one dish at a time. Also minimize the time that the incubator door is open, to maintain CO_2.
4. Add myocyte culture medium to the washed cells, using 1 mL medium for 35-mm dishes, and 3 mL medium for 60-mm dishes. Immediately return myocytes to the incubator. At this stage, the myocytes are in myocyte culture medium, which is MEM containing only 1 mg/mL BSA, glutamine, and penicillin.
5. After an additional hour, count the number of rod-shaped myocytes and round myocytes under an inverted microscope with a ↔20 objective. Count myocytes in three different dishes (from early, middle, and late in plating), and count at least three randomly selected fields. Average the count of the three fields for each dish, and then average the three dishes. Calculate the total number of myocytes, the number of rod-shaped myocytes, and the percent of rod-shaped myocytes. For example, in an experiment with 35-mm dishes:
   a. Total myocytes = (mean no. of myocytes counted/no. of fields) ↔ area factor
      Example: (120 myocytes/3 fields counted) ↔ 1052 = 42,080 myocytes/dish or 42,080 myocytes/962 mm^2 = 44 myocytes/mm^2.

      The "area factor" needs to be empirically determined for each microscope objective/eyepiece being used. To calculate the area factor, we use a stage micrometer to measure the diameter of the microscopic field for our ↔20 objective and eyepiece. We calculate the area of the circular microscopic field, and then divide the area of the dish (supplied by the manufacturer and measured directly) by the area of the field, to give the area factor. For our microscope, the diameter of the field at ↔20 is 1.08 mm, which is an area of 0.9145 mm^2, and the area of a 35-mm dish is 962 mm^2, therefore the area factor is 1052. Either total myocytes/mm^2 or total myocytes/dish can be calculated. Myocytes/mm^2 can be used to define the preparation and to calculate plating efficiency as a quality control among preparations. Total myocytes/dish allows determination of biochemical assays on a "per myocyte" basis.
   b. Total rod-shaped = (no. of rods counted/no. of fields) ↔ area factor
      Example: (105 rods/3 fields counted) ↔ 1052 = 36,820 rods
   c. Percent rod-shaped = (no. of rods/total no. of cells)
      Example: (36,820/42,080)*100 = 88%

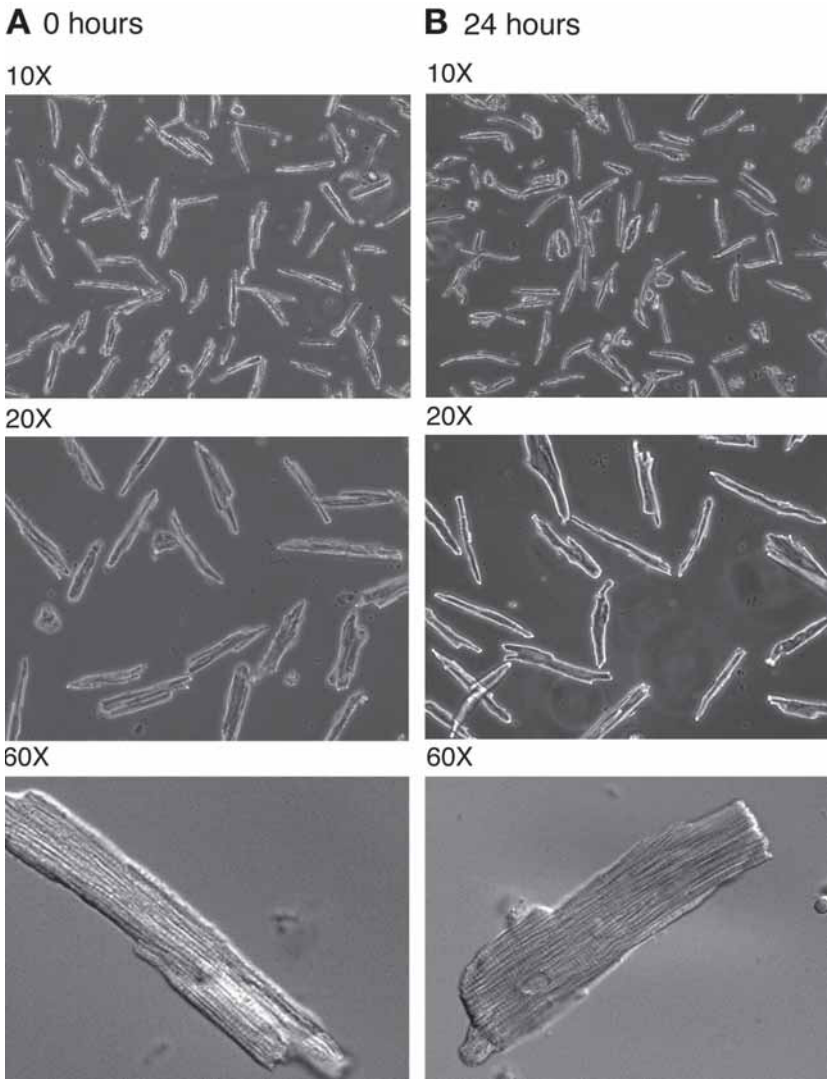


Fig. 2. Normal rod-shaped morphology of adult mouse cardiac myocytes cultured for 24 h. Myocytes were photographed under microscopy by phase contrast (↔100 and ↔200) and differential interference contrast (↔600) after culture in myocyte culture medium for **(A)** 0 h and **(B)** 24 h.

d. Plating efficiency 36,820 rods counted/50,000 plated = 74% plating efficiency. Record these values as the number of myocytes at T0.

6. Continue to incubate the myocytes at 37°C in 2% CO_2 until use. Count again at 24 h or at the time of use (18–24 h). **Figure 2** depicts myocytes after plating and medium change (0 h) and after 24 h in culture (*see* **Note 16**).

3.8. Long-Term Culture (72 h) of Adult Mouse Myocytes

The protocol described previously is for the short-term (24 h) culture of adult mouse cardiac myocytes. For long-term culture, we developed a separate culture medium that is supplemented with 10 m*M* BDM and insulin, transferring, and selenium (ITS Medium Supplement; *see* **Note 17**). **Figure 3** depicts myocytes after 0, 24, 48, and 72 h in culture with or without 10 m*M* BDM and ITS.

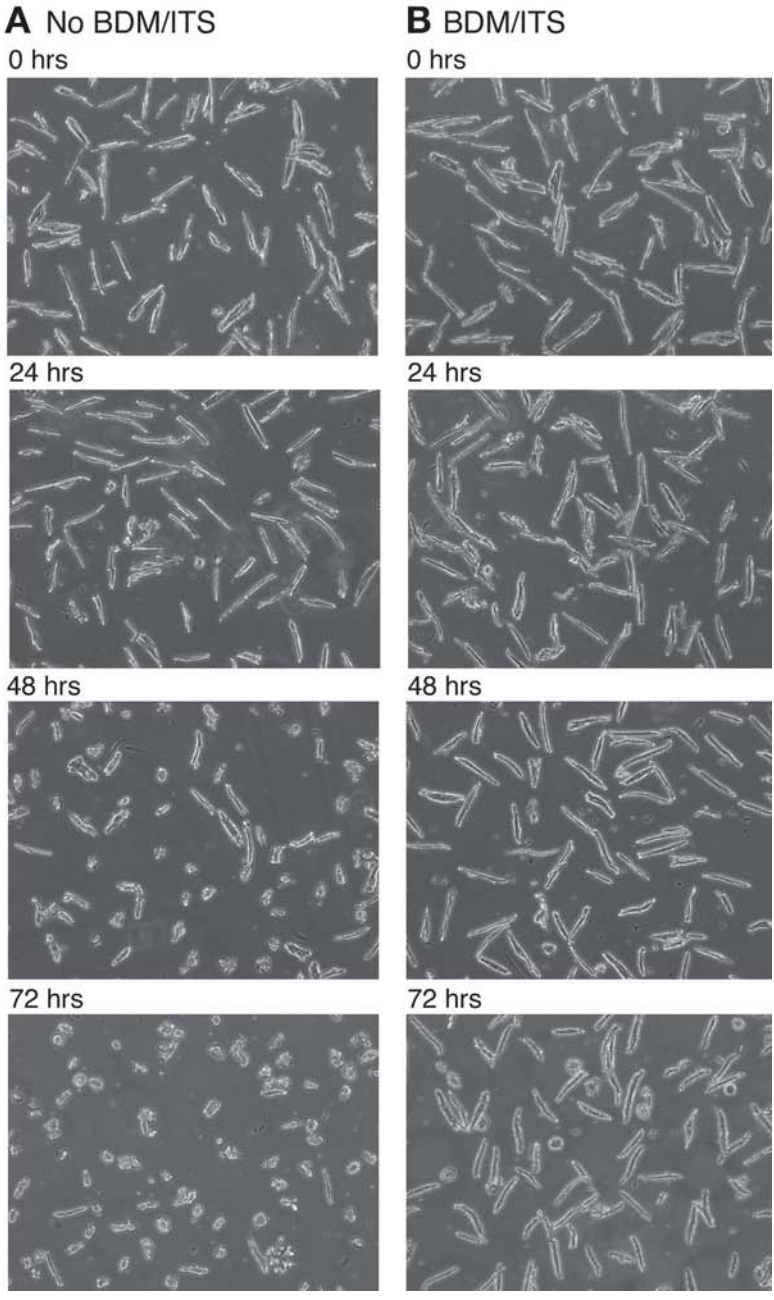
3.9. Applications of Freshly Isolated and Cultured Adult Mouse Myocytes

Recently, we have used freshly isolated and cultured myocytes in a number of experiments relevant to the study of cardiac function, hypertrophy, ischemia, and heart failure. Using freshly isolated myocytes, we measured myocyte shortening, and by loading myocytes with Fura-2AM, we also recorded calcium transients (*see* **Fig. 4**). In short-term cultured myocytes, we measured second-messenger generation (*see* **Fig. 5**) and signaling protein activation/phosphorylation by Western blot (*see* **Fig. 6**). We used freshly isolated myocytes to demonstrate that aortic constriction, a known hypertrophic stimulus, induces myocyte hypertrophy, quantified by measuring isolated myocyte size with a Coulter Multisizer, and fetal gene expression, shown by Western blot for β-myosin heavy-chain protein in isolated myocytes (*see* **Fig. 7**). In short-term cultured myocytes, we quantified myocyte apoptosis induced by hydrogen peroxide using Annexin V staining (*see* **Fig. 8**) (and TUNEL staining, not shown). Finally, we used adenoviral infection of long-term cultured myocytes to achieve high-efficiency gene transduction (*see* **Fig. 9**). These wide-ranging studies demonstrate the suitability of this culture model for studies of cardiac myocyte function, hypertrophy, signaling, and apoptosis.

3.10. Cleaning the Perfusion Rig

It is very important to have a routine protocol to clean the perfusion rig after each preparation, to minimize contamination. We use the following procedures.

1. Clean the profusion rig once a week with 1 *M* HCl. Perfuse and fill all parts of the rig with 1 *M* HCl, and incubate for 10 min, followed by three washes of sterile distilled water. Do not have the cannula attached when perfusing the rig with HCl, as the acid will corrode the metal part of the 20-G cannula.
2. Clean the rig every day with 70% ethanol before and after isolating a heart. Perfuse and fill all parts of the rig with 70% ethanol, and incubate for 10 min, followed by three washes of sterile distilled water.
3. Clean the cannula with 70% ethanol (do not use bleach) and autoclave with the surgical instruments before each isolation.


A No BDM/ITS
B BDM/ITS
0 hrs
0 hrs
24 hrs
24 hrs
48 hrs
48 hrs
72 hrs
72 hrs

4. Notes

1. The temperature of the perfusate and the flow rate of the pump should be checked routinely. Over time, peristaltic pump tubing will fail and will need to be replaced (every 2–3 mo or 20–30 preparations) to maintain consistent flow rates. These routine checks are essential to maintain consistency in preparations.
2. In this protocol, we use isoflurane to anesthetize mice prior to removing the heart. Older and more conventional anesthetics like pentobarbital or ketamine have a much longer onset and significantly reduce respiration, which greatly increases the risk of myocardial ischemia. Isoflurane, with its rapid onset and minimal effects on respiration, avoids these problems, and we found our isolation procedure was dramatically improved by switching to isoflurane ***(11)***.
3. Proper dissection of the heart is the key to a successful cannulation and perfusion. For optimal enzyme digestion of the heart and good myocyte yields, the aorta must be positioned on the cannula so that it does not pass through the aortic valve, which would prevent proper perfusion of the coronary arteries. When removing the heart, we routinely cut the transverse aorta between the carotid arteries. *See* Rokosh and Simpson for anatomy of the mouse aorta ***(13)***. Too long a section of aorta will make the aorta harder to identify and to lift onto the cannula. Conversely, too short a section of aorta will make it harder to tie off the aorta on the cannula and increase the likelihood of pushing the cannula through the aortic valve, preventing good perfusion. When isolating myocytes from a heart after pressure overload by transverse aortic constriction, the proximal aorta will be quite dilated.
4. It is very highly recommended to use a magnifying lense or a dissecting microscope when cannulating the aorta, to make the heart and aorta easier to visualize and cannulate.
5. When cannulating the aorta, a rapid cannulation is essential for optimal enzyme digestion and good myocyte yields. We typically try to cannulate the heart, from removing the heart to starting perfusion, in less than 45 s.

Fig. 3. *(Opposite page)* Myocyte culture medium supplemented with 2, 3-butanedione monoxime (BDM) and insulin, transferrin and selenium (ITS) preserves normal rod-shaped morphology of adult mouse cardiac myocytes for 72 h. Myocytes were plated at a density of 50,000 rod-shaped myocytes per 35-mm culture dish and were cultured for 72 h in myocyte culture medium with or without 10 m*M* BDM and ITS medium supplement (10 µg/mL insulin, 5.5 µg/mL transferrin, and 5 ng/mL selenium). Myocytes cultured in **(A)** myocyte culture medium alone, or **(B)** myocyte culture medium supplemented with BDM and ITS, were photographed under phase-contrast microscopy (↔100) after 0, 24, 48, and 72 h in culture. We consider rod-shaped myocytes to be the most physiological, because they mimic the cells in the intact heart. However, it should also be noted that "round" myocytes, sometimes called "meatballs" are not necessarily dead. In fact, many or most can be alive, which can be easily documented using vital cell stains.

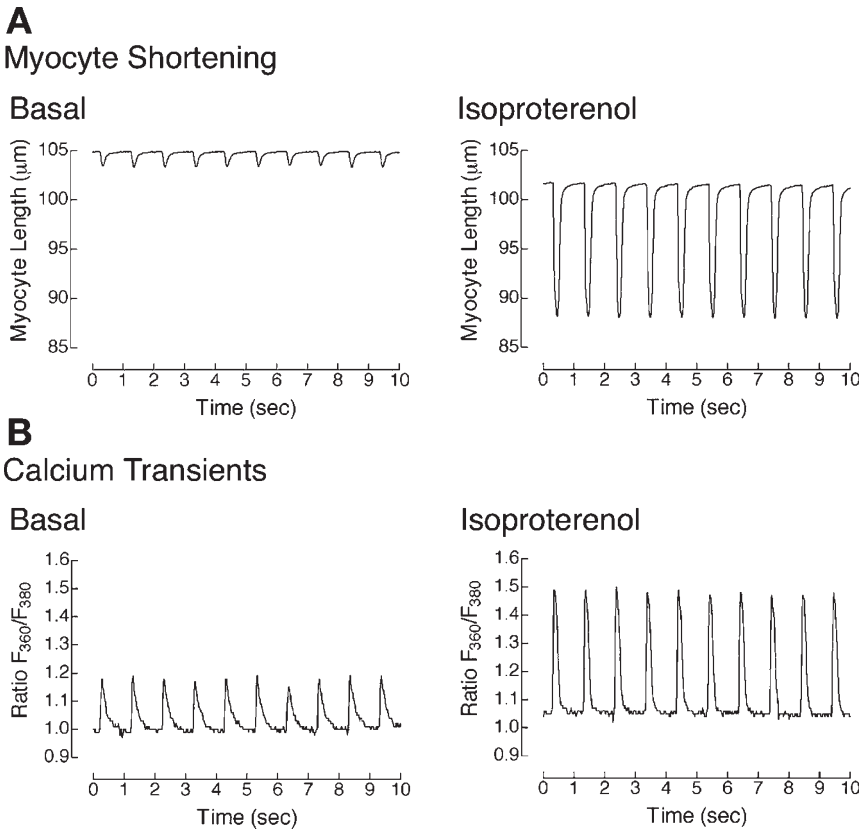


Fig. 4. β-Adrenergic stimulation with isoproterenol increases contraction and calcium transients in freshly isolated adult myocytes. Myocytes were plated and immediately loaded with the calcium indicator Fura-2AM at 25°C in HEPES buffer. For all measurements, myocytes were electrically paced (1 Hz, with 20 V pulse amplitude and 4 ms pulse duration) and basal and ligand-induced changes in length and calcium were recorded. **(A)** Contraction in a representative myocyte, measured as length change, is shown before and 5 min after treatment with isoproterenol (1 μ*M*). The *y*-axis shows myocyte length in μm and the *x*-axis shows time in seconds. **(B)** Calcium transients are shown in a single myocyte before and 5 min after treatment with isoproterenol (1 μ*M*). The *y*-axis shows Fura-2 emission ratios (360 nm/380 nm), and the *x*-axis shows time in seconds.

6. Note that none of the solutions are oxygenated. Other protocols use 95% O_2/5% CO_2 *(**10**)*, which, when used with a bicarbonate buffer, has the dual effect of oxygenating the solutions and maintaining pH. However, when the heart is arrested by perfusion with calcium free buffer, the oxygen demand of the muscle tissue is dramatically reduced. Therefore, we tested whether oxygenation of the perfusion solutions was necessary, and found that neither myocyte yields nor survival in

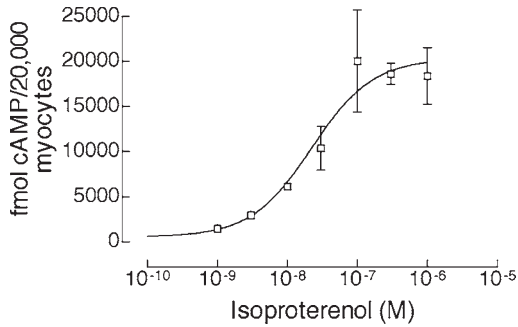


Fig. 5. β-Adrenergic stimulation with isoproterenol increases cAMP in cultured adult mouse myocytes. Myocytes were cultured for 18 h (overnight) in 35-mm dishes and then treated for 15 min with increasing concentrations of isoproterenol (1 n*M* to 1 μ*M*) in the presence of the phosphodiesterase inhibitor isobutyl-methyl-xanthine (1 μ*M*). After treatment, myocytes were lysed, and cAMP content was determined by ELISA (Amersham, Piscataway, NJ). The graph shows mean ± SEM, $n = 3$, with each measurement made in duplicate.

culture was affected if solutions were not oxygenated. The perfusion solution does contain a small amount of bicarbonate, as a metabolic requirement, but the pH is maintained by the Na-HEPES ***(11)***.

7. In this protocol, we use collagenase type II (Worthington Biochemicals) to digest the heart. However, we have also used Blendzyme type I (Roche Molecular Biochemicals, Indianapolis, IN) with trypsin (Sigma), and a combination of collagenases B and D (Roche) ***(11)***. In our experience, the yields using crude collagenase preparations are higher, but vary between lots, requiring testing different lots of enzyme and fine-tuning enzyme concentrations. On the other hand, Blendzyme, which is a recombinant collagenase mixture, is much more standardized, reducing variability between lots, but is more expensive and gives slightly lower yields.
8. In developing this protocol, we compared a constant flow perfusion system with a constant pressure system (70 mmHg) for myocyte isolation. Although both systems were workable, we found that the constant flow system was easier to use. This is because as the enzyme starts to digest the heart, coronary pressure increases, which significantly reduces flow and hinders digestion. We found that maintaining a constant flow at 4 mL/min reduced this problem and made the digestion process easier ***(11)***.
9. After digestion the heart should appear pale and flaccid, and one might be able to see some muscle fiber dissociation on the surface of the heart. When taken down and initially cut into small pieces for further mechanical disruption with the 2-mm and 1.5-mm pipets, very little force should be needed and the heart should almost fall apart on its own.
10. The myocyte stopping buffer contains 10% CS. In the past, we tried BSA to inactivate the collagenase and other proteases, but found that serum was better ***(11)***.

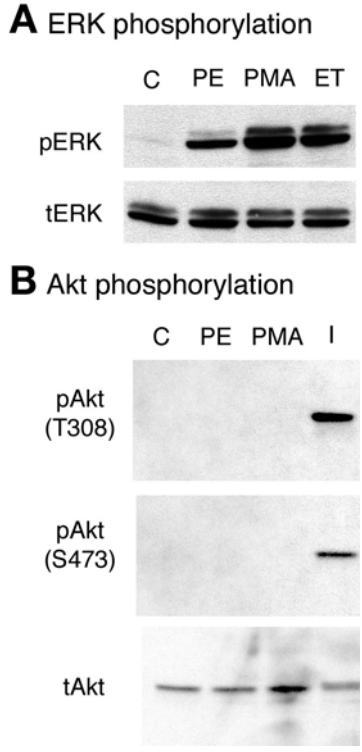


Fig. 6. Hypertrophic agonists phosphorylate (activate) kinases by Western blot in cultured adult mouse myocytes. Myocytes were cultured for 18 h (overnight) in 35-mm dishes and then treated for 15 min with phenylephrine (PE; 20 μ*M*, plus 2 μ*M* Timolol), phorbol 12-myristate-13-acetate (PMA; 100 n*M*), endothelin (ET; 10 n*M*), or insulin (I; 6 μ*M*). After 15 min, myocytes were washed once with PBS and lysed directly in Laemeli's sample buffer. Western blots were done to detect **(A)** phosphorylated ERK (pERK) and total ERK (tERK), and **(B)** phosphorylated Akt (pAkt, threonine 308, or serine 473) and total Akt (tAkt). Antibodies were from Cell Signaling (Beverly, MA).

Collagenase is also inactivated by dilution, and a generous volume at this step additionally prevents myocyte damage during dissociation with the transfer pipets.

11. When myocytes are counted on the hemocytometer, great care must be taken when loading the counting chambers. The myocytes are very large, making it difficult to get an even dispersion in the counting chamber.
12. We found that adding ATP during the calcium reintroduction and to the plating medium improves the maintenance of rod-shaped morphology. We hypothesize that the ATP improves the metabolic state of the myocytes and helps to maintain calcium homeostasis while the cells are recovering from isolation and attaching to the culture dish.

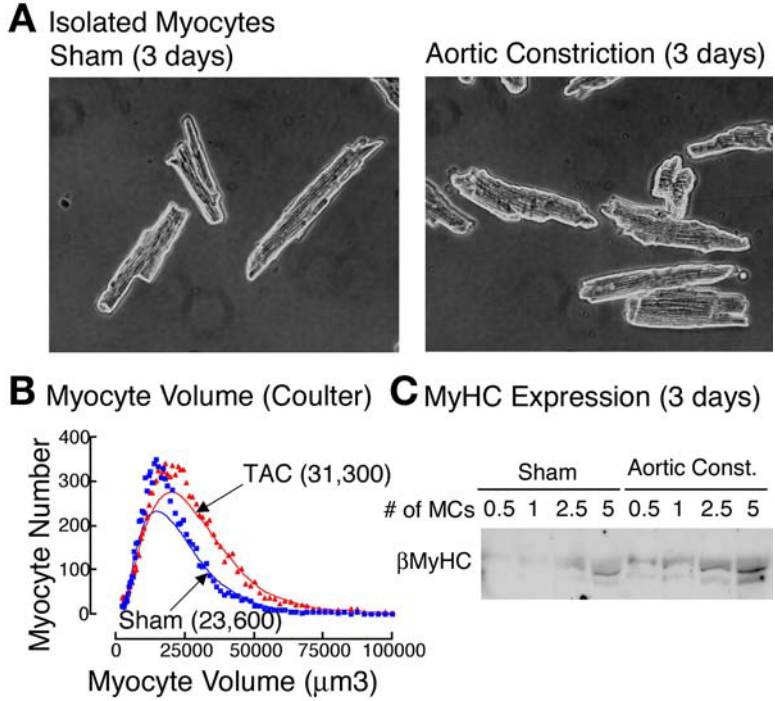


Fig. 7. Freshly isolated adult mouse myocytes document hypertrophy and fetal gene induction following pressure overload. Transverse aortic constriction (TAC) surgery was performed on mice to induce pressure overload hypertrophy ***(15)***. After 3 d, myocytes were isolated and fixed immediately in 4% paraformaldehyde in phosphate-buffered saline (pH 7.4). **(A)** Myocytes from sham and TAC mice were photographed under phase microscopy at ↔400. **(B)** Myocyte volumes were measured using a Coulter Multisizer ***(11,12)***. The myocyte volume distribution compares myocyte volume (μm^3) vs myocyte number, demonstrating the right shift to increased cell volume after TAC. In parentheses are the median volume for sham myocytes (23,600 μm^3) and pressure-overloaded myocytes (TAC, 31,300 μm^3). **(C)** Freshly isolated myocytes were counted and lysed in 1.5X Laemeli's sample buffer, and 0.5 to 5 ↔ 10^3 myocytes were used in Western blot for β-myosin heavy chain, a classical fetal hypertrophic marker gene, using the L2 antibody ***(14)***, showing the increased β-myosin-heavy chain after TAC.

13. We plate the myocytes on laminin-coated dishes. However, we tried other attachment matrices, including collagen IV, fibronectin, poly-L-lysine, and gelatin. Only collagen IV performed as well as laminin, and could be substituted for laminin ***(11)***.
14. To maintain rod-shaped morphology, we culture the myocytes in slightly acidic culture medium, pH approx 7.0. The myocyte culture medium base is MEM with HBSS, which contains 0.35 g/L of sodium bicarbonate. Normally, this medium is used in a 1% CO_2 incubator where the medium pH is 7.4, but when placed in a 2% CO_2 incubator, the medium pH is 6.9–7.0.

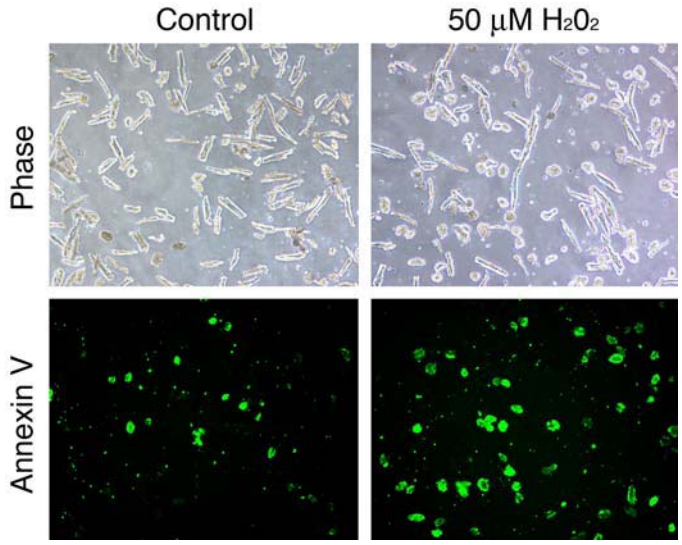


Fig. 8. Oxidative stress with H_2O_2 induces apoptosis by annexin V staining in cultured adult mouse myocytes. Myocytes were cultured for 18 h (overnight) and then treated for 2 h with hydrogen peroxide (H_2O_2, 50 μ*M*), which is known to induce myocyte apoptosis. Myocytes were stained with FITC-Annexin V (Roche) by adding the Annexin V directly to the culture medium, to identify apoptotic cells as Annexin V-positive. Myocytes were photographed under phase and fluorescent microscopy at ↔200, where Annexin V-positive myocytes are bright against the dark background (lower panels).

15. The myocytes are allowed to attach for 1 h. We have tried longer attachment times, but this did not improve the plating efficiency. We have also tried times as short as 20 min, but plating efficiency was not as good as 1 h. Plating time might require adjustment in one's own laboratory.
16. For short-term (24 h) culture of myocytes, we tested medium with and without 10 m*M* BDM and found that BDM caused a modest improvement in maintenance of rod-shaped morphology overnight. We found no effect of culturing myocytes in BDM on several different assays, including accumulation of cAMP, the phosphorylation of several signaling proteins including ERK and Akt, and the contractile response to β-adrenergic stimulation.
17. To maintain viable rod-shaped myocytes for more than 24 h, we tested several medium additives, including BDM, which inhibits spontaneous contractions; insulin, transferrin, and selenium medium supplement (ITS); ascorbic acid, an anti-oxidant; MnTMPyP, a superoxide dismutase inhibitor; caspase inhibitors; bongkrekic acid, an anti-apoptotic agent; verapamil, a calcium channel antagonist; and low-calcium medium. For optimal survival to 72 h, we found that the best results were obtained when myocyte culture medium was supplemented with 10 m*M* BDM and full-strength ITS medium supplement, which contained 1 μg/mL insulin, 0.55

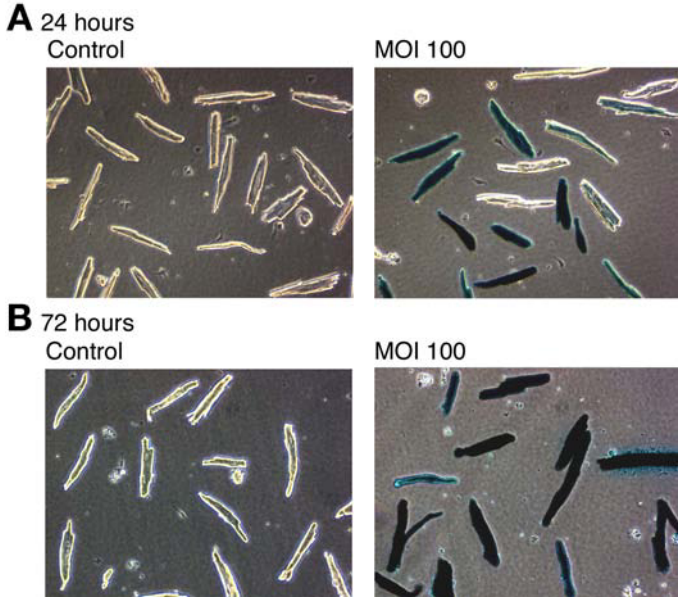


Fig. 9. High-efficiency infection of cultured rod-shaped adult mouse myocytes with adenovirus expressing β-galactosidase. Myocytes were infected at 0 h with adenovirus containing a β-galactosidase transgene at an MOI of 100, then cultured for 72 h in myocyte culture medium with 2, 3-butanedione monoxime and insulin, transferrin, and selenium. β-Galactosidase activity (dark-stained cells) was assayed at **(A)** 24 h and **(B)** 72 h. Myocytes are photographed under phase contrast microscopy (↔100).

μg/mL transferrin, and 0.5 ng/mL selenium) ***(11)***. We also found that 2.5 and 5 m*M* BDM were nearly as good as 10 m*M* BDM at maintaining rod-shaped morphology to 72 h, and were as good as 10 m*M* BDM at 48 h. In addition, the final concentration of the ITS used in our original work ***(11)*** could be reduced 100-fold for studies using insulin treatment. The current version of ITS (*see* **Table 6**) has slightly lower concentrations than our original ***(11)***.

Acknowledgments

Marietta Paningbatan, Philip Swigart, Yuan Huang, and Luyi Li provided expert technical assistance. Keng-Mean Lin, PhD, at the Alliance for Cellular Signaling (AfCS) Laboratory at the University of Texas, Southwestern, did the experiments with myocyte contraction and calcium ***(11)***. Maike Krenz and Jeff Robbins generously provided the β-myosin heavy-chain antibody (**Fig. 7**; **ref. *14***). The Department of Veterans Affairs Research Service, the National Institutes of Health, and the American Heart Association, Western States Affiliate, provided support.

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