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Generating Primary Cultures of Murine Cardiac Myocytes and Cardiac Fibroblasts to Study Viral Myocarditis

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Abstract

Viruses can induce direct damage to cardiac myocytes and cardiac fibroblasts resulting in myocarditis and impaired cardiac function. Cardiac myocytes and cardiac fibroblasts display different capacities to support viral infection and generate a protective antiviral response. This chapter provides detailed protocols for generation and characterization of primary cultures of murine cardiac myocytes and cardiac fibroblasts, offering a powerful tool to probe cell type-specific responses that determine protection against viral myocarditis.

Keywords

Cardiac myocyte; Cardiomyocyte; Cardiac fibroblast; Myocarditis; Primary cell culture; Reovirus

1 Introduction

Viral myocarditis (cardiac damage) [1–3] is the second leading cause of sudden death in young adults [4]. Most virus families have been implicated in the human disease, although enteroviruses such as coxsackievirus B3 and adenoviruses are generally the most frequently cited [1–3, 5]. While immune-mediated damage is likely a major component of enterovirus-induced myocarditis, the virus also induces direct cytopathic effect [2]. Importantly, immunosuppressive therapy has proven only minimally beneficial for human patients, leaving the role of immune-mediated damage in viral myocarditis unclear [3, 6]. Reovirus induces a direct cytopathic effect in cardiac cells [7] and induces myocarditis in mice lacking B and T cells [8]. Thus, reovirus-induced murine myocarditis provides an outstanding model to study the direct effects of viral infection in the heart and cardiac cells [9–11]. This chapter describes generation of primary cultures of murine cardiac myocytes and cardiac fibroblasts to study those direct viral effects.

While each year brings new technologies to stimulate cardiac cell repair [12, 13], the unfortunate reality is that adult cardiac myocytes are largely nondividing and unable to self-renew after cardiac damage [14–17]. Cardiac fibroblasts are the other major cell in the heart and, in addition to maintaining their capacity for cell division, provide a variety of critical functions [18, 19]. Uninfected cardiac myocytes and cardiac fibroblasts express different levels of the antiviral cytokine interferon- β , providing an integrated network of protection for

³¹Cultures are generally inoculated with a small volume, incubated for 1 h to allow cell attachment, and then overlaid with additional media.

the heart before the virus infects [11]. During infection, cardiac myocytes and cardiac fibroblasts display cell type-specific differences in susceptibility to infection and in the ensuing protective response [9, 11]. Indeed, the capacity for different reoviruses to induce myocarditis correlates with their capacity to induce cytopathic effect in cardiac myocytes but not cardiac fibroblasts, confirming cell type-specific differences in the cardiac cell response to viral infection, and validating the use of these primary cultures [7]. The primary cell cultures described in this chapter offer a powerful tool to probe cell type-specific responses that determine protection against viral myocarditis.

2 Materials

2.1 Mice (See Note 1)

1. Timed-pregnant mice can be purchased or generated in-house from wild-type or transgenic colonies (see Note 2).
2. Use a sufficient number of timed-pregnant females to generate 30–150 fetal and neonatal (<24 h old) mice. The fraction of each is random (see Note 3).

2.2 On Lab Bench (See Note 4)

1. Bench paper.
2. Large dissection scissors to open pregnant mice, small dissection scissors to remove fetuses, small dissection scissors to remove hearts. Sterilize by autoclaving in advance or by dipping in ethanol and flaming immediately before starting.
3. Two plastic Petri dishes each containing ~20 ml sterile PBS (see Subheading 2.5).

2.3 In Tissue Culture Hood (See Note 4)

1. Two plastic Petri dishes each containing ~20 ml sterile PBS (see Subheading 2.5).
2. Disposable sterile plastic transfer pipettes.
3. 5, 10, and 25 ml sterile pipettes.

¹All procedures, including housing mice, mating mice, and euthanizing mice and fetuses, must be approved in advance by the appropriate Institutional Animal Care and Use Committee (IACUC). Timed-pregnant mice are available from commercial sources such as Charles River Laboratories.

²Cardiac myocyte cultures derived from adults do not thrive for more than approximately 1 day, and therefore it is necessary to generate cultures from fetuses and/or neonates. If mating mice in-house and using females that have not been synchronized for estrus, house two females per male and separate after 2–4 days, the shorter time yielding a smaller fraction of pregnancies. It is convenient to mate mice on Monday or Tuesday and separate them on Friday to generate cultures on a Monday (20 or 21 days after initiating the mating).

³Mouse strains vary dramatically in litter size. Plan accordingly. Cultures do not appear to differ regardless of the fraction of fetuses vs. neonates, and can be 100 % one or the other.

⁴Cardiac myocyte cultures are extraordinarily sensitive to contaminating residues that can build up in autoclaves. Use disposable plastic rather than glass whenever possible. If autoclaved glass must be used, be sure that the autoclave is used only for clean items rather than disposal of waste.

4. Forceps, sterilized by autoclaving in advance or by dipping in ethanol and flaming immediately before starting.
5. Micropipettes (e.g., Eppendorf, Pipetman) and both large and regular orifice sterile tips.
6. Two sterile small “flea” (7 × 2 mm) spin bars (VWR #58948-976, autoclaved in glass test tubes).
7. Two 50 ml disposable sterile plastic conical tubes (“50 ml tubes”), each with one of the sterilized small spin bars (above).
8. Rack to hold two 50 ml tubes.
9. Stirrer/hot plate (set temperature lowest possible; see more about temperature below).
10. 1,000 ml beaker (to hold trypsin flask and one or two 50 ml tubes) with thermometer (to maintain 36–37 °C).
11. Sterile Whatman filter paper in Petri dish (stored as autoclaved batch of filters in sterile covering).
12. BD Falcon cell strainer #352340 (BD Biosciences, San Jose CA, USA).
13. Ice bucket with two to four 50 ml disposable sterile plastic conical tubes each containing 21 ml trypsin inhibitor (*see* Subheading 2.5); generally kept directly adjacent to tissue culture hood (*see* ^{Note 5}).

2.4 Additional Equipment and Supplies

1. Refrigerated tabletop centrifuge set at 4 °C.
2. Hemocytometer and Eppendorf tubes for cell counts.
3. Multiple 50 ml disposable sterile plastic conical tubes (“50 ml tubes”).
4. Multiple 6-well tissue culture clusters.
5. Multiple 96-well, 48-well, or 24-well tissue culture clusters (based on desired plating; *see* below).

2.5 Reagents (*See* ^{Note 4})

1. Ethanol for sterilizations.
2. PBS (without divalent cations, e.g., Mediatech #45000-446).
3. 60 or 120 ml 37 °C 0.05 % trypsin-PBS for heart dissociations (diluted from 10× [e.g., Gibco #15400-054] *see* ^{Note 6}).

⁵The number of trypsin inhibitor tubes will depend on the number of tubes used for trypsinizations (*see* Note 18).

⁶Use 60 ml if trypsinizations are in a single tube; use 120 ml if two tubes. Mix 6 or 12 ml 10× (0.5 %) trypsin/EDTA (Gibco # 15400-054) with 54 or 108 ml PBS. It is convenient to place this in a T75 tissue culture flask to fit in the beaker bath in the tissue culture hood; keep it at 36–37 °C.

4. Completed DMEM = cDMEM (DMEM [Corning/Cellgro # 10-017-CV] + 7 % fetal calf serum [FCS; e.g., Atlanta Biologicals Premium Select #S11550] + 10 µg/ml gentamicin (e.g., Sigma #G1272)) (*see* ^{Note 7}).
5. Cold trypsin inhibitor (cDMEM + additional 13 % FCS) (*see* ^{Note 8}).
6. 37 °C 0.05 % trypsin-PBS for fibroblast trypsinization (e.g., Mediatech # 25-051-Cl, 1×, *see* ^{Note 9}).

3 Methods

3.1 Euthanizing Mice and Extracting Neonatal and Fetal Hearts, on Benchtop with Bench Paper

1. Euthanize nonpregnant dams by IACUC-approved protocol.
2. Decapitate neonates by IACUC-approved protocol and dab briefly on paper towel to remove blood (*see* ^{Note 10}).
3. Use small dissection scissors to cut through ribs to open thoracic cavity.
4. Squeeze open gently to expose heart. If it is still beating, this can facilitate identification.
5. Use same small dissection scissors to cut base of heart and transfer to PBS dish (*see* ^{Note 11}).
6. Euthanize up to three pregnant females at a time (by IACUC-approved cervical dislocation; not CO₂), and spray abdomens generously with 70 % ethanol (*see* ^{Note 12}).
7. Working with a single euthanized female, use the large dissection scissors to cut the abdominal skin and peel it back. Spread the U-shaped uterus for good access (*see* Fig. 1).
8. Use small dissection scissors to cut one end of the U-shaped uterus.
9. Remove one fetus and use small dissection scissors to decapitate immediately by IACUC-approved protocol.
10. Gently roll the decapitated fetus back and forth across a paper towel to ensure that all uterine fluids are removed (*see* ^{Note 13}).

⁷Generally 500 ml DMEM (Corning/Cellgro # 10-017-CV; contains glutamine but not sodium pyruvate) + 38 ml FCS + 538 µl 10 mg/ml gentamicin.

⁸For each tube of 21 ml: 18.3 ml completed DMEM + 2,730 µl FCS, for final 20 % FCS.

⁹It is convenient to use Mediatech # 25-051-Cl, 1×.

¹⁰Two methods appear to be equally successful. Either decapitate a single neonate and remove its heart before proceeding to the next neonate or decapitate a group of five to ten neonates (depending on personal speed) and remove their hearts before proceeding to the next group.

¹¹The number of hearts per Petri dish should not exceed 100. The PBS dish will accumulate some blood (*see* Fig. 1f).

¹²CO₂ will enter the bloodstream and could affect the quality of cardiac cell cultures generated from fetal hearts. The ethanol serves to sterilize the abdomen surface but also to wet the fur and keep it from becoming airborne.

¹³It is essential that uterine fluids do not contaminate the hearts. These contaminants will result in a swirl of slime when centrifuging cardiac cells, and the resulting cultures will not thrive.

11. Proceed with next fetus until all fetuses have been removed from first female.
12. Change gloves and use fresh dissection scissors to open the fetal thoracic cavity and remove heart to PBS dish as for neonates (*see* Fig. 1) (*see* ^{Note 14}).
13. When all fetuses have been removed from first female, proceed with second female until all fetuses are removed.
14. When all hearts have been removed to PBS dish(es), transfer the dish(es) to tissue culture hood.

3.2 Dissociating the Hearts

1. Use a transfer pipette to remove most of the PBS from the Petri dish(es) with hearts.
2. Refill the dish with PBS and then remove most of this PBS to facilitate access to hearts.
3. Use sterile forceps to transfer the hearts to a fresh Petri dish containing PBS.
4. Use a transfer pipette to remove most of the PBS from this Petri dish with hearts.
5. Refill the dish with PBS and then remove most of this PBS to facilitate access to hearts.
6. Use the same sterile forceps to transfer a group of ~15 hearts to sterile PBS-wetted Whatman paper in a glass Petri dish. Gently roll the group a little bit being sure to remove blood clots, and then transfer them to a fresh Petri dish containing PBS (*see* ^{Note 15}).
7. Repeat for the remaining hearts, pooling all hearts in a single Petri dish containing PBS.
8. Remove most of the PBS to facilitate access to hearts.
9. Use the same sterile forceps to transfer the hearts ~equally to one or two 50 ml tubes containing only a spin bar. Use one tube for <70 hearts and two tubes for >80 hearts.
10. Add 6.5 ml 37 °C trypsin-PBS.
11. Stir in 36–37 °C beaker bath (~150 ml H₂O in 1,000 ml beaker) on stirrer/hot plate until suspension is only *barely* cloudy, ~1 min. Do not let temperature exceed 37 °C (*see* ^{Note 16}).

¹⁴Fetal and neonatal hearts can be put in the same dish, but there should be no more than 100 hearts per dish.

¹⁵Rolling hearts on wetted Whatman paper removes the extreme outer layer of the hearts and removes blood clots to increase the purity of the cardiac myocyte and fibroblast cultures. The paper should be barely damp. Do not roll them too much; the goal is to remove all blood clots (with forceps facilitation) but minimal cardiac tissue.

12. Remove tube(s) from the beaker bath to a rack in the hood. The hearts will quickly settle.
13. Use a transfer pipette to carefully remove the supernatant to the tube(s), and discard this supernatant (it contains mainly fibroblasts).
14. Add 6.5 ml 37 °C trypsin-PBS to each tube.
15. Replace the tube(s) in the 37 °C beaker bath.
16. Stir 5 min (*see* ^{Note 16} again).
17. Remove the tube(s) from the beaker bath to a rack in the hood. The hearts will quickly settle.
18. Note the appearance of the supernatants and hearts, and use a fresh transfer pipette to transfer the supernatant from the tube of hearts to the first cold trypsin inhibitor tube. Invert the trypsin inhibitor tube to mix and replace it on ice. Save this transfer pipette and use it for all subsequent transfer steps to avoid losing cells (*see* ^{Note 17}).
19. Add 6.5 ml 37 °C trypsin-PBS to each tube of hearts.
20. Replace the tube(s) in the 37 °C beaker bath.
21. Repeat **steps 16–20**, until four supernatants (~6 ml each) have been pooled in the first cold trypsin inhibitor tube (four trypsinizations if using one heart tube; two trypsinizations if using two heart tubes; not including the first trypsinization from which the supernatant was discarded).
22. Repeat **steps 16–21** for a maximum of eight total trypsinizations (not including the first discarded supernatant), using the remaining cold trypsin inhibitor tubes (*see* ^{Note 18}).
23. Centrifuge in a tabletop centrifuge at $\sim 300 \times g$ for 10 min at 4 °C ($\sim 1,200$ rpm in a Sorvall tabletop centrifuge).
24. Gently decant the supernatants into an equal number of sterile 50 ml tubes (save the cell pellets for **step 26**) (*see* ^{Note 19}).
25. Centrifuge the supernatants retrieved in the previous step in a tabletop centrifuge at $\sim 300 \times g$ for 10 min at 4 °C (*see* ^{Note 20}).

¹⁶The stirring speed should be sufficient to generate a gentle vortex down the center of the tube, and the tube should be angled so that the spinbar is “beating” the hearts physically.

¹⁷It is highly advisable to take good notes about the appearance of the supernatant (cloudiness) and hearts (clumped or not; easy or difficult to disperse), to help troubleshoot if culture preparations are not reproducible.

¹⁸Note the cloudiness of the supernatants each time; after peaking it should decrease. In later trypsinizations, the supernatant nearest the hearts may become “gooey.” It is critical that none of this “goo” gets into the cold trypsin inhibitor tubes. If it is not possible to remove cells from the trypsinization tubes without also retrieving this “goo,” stop the trypsinizations. Generally, eight trypsinizations (not including the first from which the supernatant is discarded) are sufficient. If there is excessive “goo” (*see* above), then stop earlier.

¹⁹After centrifugation, carefully hold the 50 ml tubes up to the light and look for a “swirl” of “slime” emanating from the pellet. If there is a minor “swirl,” proceed as planned. If there is a major, dominant swirl, consider discarding this entire tube of cells. While this will reduce the yield of cells, contaminating slime may jeopardize the culture. One cause of “slime” is uterine fluids that contaminate gloves or scissors when they touch the hearts during their removal.

²⁰A significant fraction of cells do not pellet in the first centrifugation; this second centrifugation captures them.

26. Meanwhile, aliquot ~150 ml cDMEM into a flask or bottle as a reservoir. Use a 5 ml pipette to transfer 2.5 ml cDMEM from the reservoir to the pellet in the first tube, take up the pellet gently to keep it intact, transfer it to the second tube with a pellet, and continue transfers if additional tubes exist. Use the same cell-laden pipette to retrieve 2.5 ml cDMEM from the reservoir, rinse the first “empty” tube, serially rinse any remaining tubes and pool this rinse with the cell suspension (final total volume ~5 ml). Holding the pipette so that the tip is flat against the tube bottom, use maximum force to retrieve and eject the suspension to disperse the pellet. Repeat this “up and down” ~7 times to disperse the pellet. Place tube on ice while waiting to proceed to **step 27**.
27. Gently decant the supernatants from **step 25** into a waste beaker to discard.
28. Handle the pellets and washes exactly as for **step 26**, and pool with the cell suspension in **step 26**. The final volume will be ~10 ml total.
29. Continue to pipette up and down (~5 ml volumes) ~7 times until there is no further obvious disaggregation. Estimate the volume.
30. Remove two independent 10 µl samples to Eppendorf tubes with 90 µl PBS and use a hemocytometer to count cells that are not Red Blood Cells [RBCs] (*see* ^{Note 21}).
31. Calculate the number of cells (*see* ^{Note 22}).

3.3 Separating Cardiac Myocytes from Cardiac Fibroblasts

1. Calculate the number of 6-well clusters to plate at $\sim 1.25 \times 10^6$ cells/well as a “pre-plating” to separate the rapidly adhering fibroblasts from the more slowly adhering myocytes.
2. Add cDMEM to the cell suspension so the final volume will result in 2 ml suspension per well.
3. Dispense 2 ml per well in 6-well clusters. Rinse suspension tube with very small volume cDMEM and add to several already-plated wells.
4. Incubate for 3 h in a 37 °C, CO₂ incubator to allow fibroblasts to adhere (*see* ^{Note 23}).
5. Use a P1000 micropipette set at 700 µl with a large orifice tip to use media in the first well to vigorously rinse that well seven to eight times, squirting

²¹The capacity to distinguish cardiac myocytes from fibroblasts by size and shape is controversial, but some would say that fibroblasts appear smaller and have a jagged edge. The protocol is successful regardless of whether both myocytes and fibroblasts or only myocytes are counted at this step. RBCs are smaller, extremely round, and have a “donut-like” appearance. Do not count RBCs. Count at least 100 cells per loading.

²²Calculate: the average number of cells per square $\times 10^4$ cells/ml $\times$ tenfold dilution in PBS $\times$ total ml. There should be $\sim 2\text{--}5 \times 10^5$ non-RBC cells per heart.

²³By eye, at the end of the incubation the wells should look like they have a thick “monolayer” which starts to “rip” as you swirl the cluster. The myocytes are not adherent at this stage; just aggregated.

directly onto the “monolayer.” Transfer 700 µl of cell suspension to a 50 ml tube, leaving ~1.3 ml in the well (*see* ^{Note 24}).

6. Similarly remove 700 µl from each of the five remaining wells in that cluster, pooling all in the same 50 ml tube (leaving ~1.3 ml in each well) and rocking the plate after each removal to ensure that cells do not dry out.
7. Repeat **steps 5** and **6** for that first cluster, pooling the suspension in the same 50 ml tube and leaving ~600 µl in each well.
8. Remove the remaining ~600 µl from all six wells and pool in the same 50 ml tube.
9. Using a 10 ml pipette, retrieve ~6 from the cDMEM reservoir and add ~1 ml to each well.
10. Repeat **steps 5** and **6** for that first cluster, pooling the suspension in the same 50 ml tube and leaving ~300 µl in each well.
11. Remove the remaining ~300 µl from all six wells and pool with the cell suspension.
12. Retrieve ~2 ml from the cDMEM reservoir and add ~1 ml to first wells in top and bottom rows.
13. Use a P1000 micropipette with a large orifice tip to transfer media across the rows of wells and pool with the cell suspension.
14. Using a 10 ml pipette, retrieve ~6 from the cDMEM reservoir and add ~1 ml to each well (*see* ^{Note 25}).
15. Repeat the entire procedure for the remaining clusters. Aim to generate a single tube of cell suspension if possible, by slightly adjusting volumes above as necessary. If a single tube would require major volume adjustments, generate two tubes of cell suspension.
16. Centrifuge in a tabletop centrifuge at $\sim 300 \times g$ for 10 min at 4 °C ($\sim 1,200$ rpm in a Sorvall tabletop centrifuge).
17. Meanwhile, incubate clusters containing fibroblasts and 1 ml overlying media in a 37 °C, CO₂ incubator until ready to trypsinize.
18. Gently decant the supernatants into an equal number of sterile 50 ml disposable sterile plastic conical tubes (save the cell pellets for **step 20**).
19. Centrifuge the supernatants retrieved in the previous step in a tabletop centrifuge at $\sim 300 \times g$ for 10 min at 4 °C (*see* ^{Note 20} again).

²⁴The wash here should be quite vigorous. The fibroblasts are fairly firmly attached, and disengagement of overlying myocytes requires direct disruption.

²⁵Check by microscope after removing the myocytes. The well should contain mainly adherent cells with some rounded cells but no “rips” across the monolayer.

20. Meanwhile, resuspend pellets from **step 18** and wash tubes exactly as for **step 26** in previous section.
21. Gently decant the supernatants from **step 19** into a waste beaker to discard.
22. Handle the pellets and washes exactly as for **step 20**, and pool with the cell suspension in **step 20**.
23. Pipette the cell suspension through a cell strainer into a fresh 50 ml tube (using small volumes to avoid losing cells along the length of pipette), carefully measuring volumes of each addition.
24. Rinse the source tube with 1 ml cDMEM and add to the strainer.
25. Calculate the final volume.
26. Remove two independent 10 μ l samples to Eppendorf tubes with 90 μ l PBS and use a hemocytometer to count cells that are not RBCs.
27. Calculate the number of cells (*see* ^{Note 26}).
28. Add cDMEM to 5×10^5 cells/ml.

3.4 Plating Cardiac Myocytes

1. Using large orifice micropipette tips, plate cardiac myocytes (*see* Table 1).
2. Incubate in a 37 °C, CO₂ incubator overnight.

3.5 Plating Cardiac Fibroblasts

1. Use a transfer pipette to remove and discard media from all wells in one 6-well cluster.
2. Add 1 ml PBS to each well.
3. Repeat for up to two additional 6-well clusters.
4. Use a transfer pipette to remove PBS from all wells in first 6-well cluster.
5. Add 100 μ l 0.05 % trypsin-PBS to each well.
6. Repeat for up to two additional 6-well clusters.
7. Incubate in a 37 °C, CO₂ incubator for ~7 min.
8. Remove cluster(s) and tap sides to release cells.
9. Check by microscope to ensure cells are rounded and floating. If not, incubate several minutes longer.
10. Add 1,000 μ l cDMEM to each well.
11. Use overlying media to rinse wells and pool in 50 ml tube.

²⁶Calculate: the average number of cells per square $\times 10^4$ cells/ml $\times$ tenfold dilution in PBS $\times$ total ml. There should be $\sim 1 \times 10^5$ – 3×10^5 myocytes per heart.

12. Centrifuge in a tabletop centrifuge at $\sim 300 \times g$ for 10 min at 4 °C ($\sim 1,200$ rpm in a Sorvall tabletop centrifuge).
13. Decant supernatants into waste.
14. Use 5–10 ml cDMEM to resuspend the pellet.
15. Do not count the cells. Instead, resuspend fibroblasts in two times the volume of media used for cardiac myocytes (*see* ^{Note 27}).
16. Incubate in a 37 °C, CO₂ incubator overnight.

3.6 Infecting Cultures 2 Days Post-plating

1. After incubating cultures overnight, assess by light microscopy (*see* ^{Notes 28 and 29}).
2. Incubate overnight and assess by light microscopy again (*see* ^{Note 30}).
3. Remove overlying media and infect the cultures (*see* Table 2) (*see* ^{Notes 30–32}).

3.7 Assessing Culture Purity by Immunofluorescent Microscopy

1. Plate cultures on Poly-D-Lysine-coated 8-well chamber slides (BD Biosciences, #354632) (*see* Table 1) (*see* ^{Note 33}).
2. One day post-plating, assess by light microscopy.
3. Two days post-plating, assess by light microscopy and then carefully aspirate media and wash once with PBS.
4. Add 200 μ l 4 % paraformaldehyde in PBS to each chamber and incubate for 20 min at room temperature.
5. Carefully remove paraformaldehyde and wash three times with ice-cold PBS (*see* ^{Note 34}).

²⁷This generates fibroblasts that remain slightly subconfluent 2 days after plating. If fewer fibroblasts are needed, simply harvest fewer wells and calculate volume of media accordingly. For example: if two 6-well clusters are pre-plated during procedure to separate myocytes from fibroblasts, harvest only one 6-well cluster to plate fibroblast cultures.

²⁸Cardiac myocyte cultures usually beat after 1 day, but not vigorously. Usually only one or several myocytes per field beat. The culture is very temperature sensitive and beating will decrease dramatically as the cluster is examined at room temperature. After returning the cluster to the incubator, it may take several hours for it to resume its original beating frequency. Cardiac myocyte cultures should have only minimal contaminating RBCs. If there are many RBCs, modify procedure for better removal of blood clots when rolling across Whatman filter. Cultures can be washed gently 1 day post-plating to remove RBCs, but be aware that this introduces a variable.

²⁹Cardiac fibroblast cultures are difficult to visualize by light microscopy because the cell borders are difficult to discern. Decreasing the light can help to increase the contrast and visibility.

³⁰After 2 days, cardiac myocyte cultures should beat well, either as individual cells or as synchronous fields. Cardiac fibroblasts will still be difficult to visualize, but should be subconfluent.

³²Cultures should not be maintained for longer than approximately 1 week post-infection. Contaminating dividing cardiac fibroblasts in cardiac myocyte cultures may obscure myocyte-specific phenotypes. Cardiac fibroblasts may change their phenotypes as they divide post-plating.

³³Cardiac myocytes do not adhere well to glass slides. Collagen binding is known to stimulate signal transduction pathways, and therefore is a suboptimal coating. Poly-D-Lysine-coated slides (BD Biosciences, #354632) enhance adherence and are inert to our knowledge. Cardiac fibroblasts are plated on Poly-D-Lysine slides as well to allow direct comparisons.

³⁴Paraformaldehyde should be disposed of properly as hazardous waste.

6. Snap off chamber top. Never let chamber areas dry throughout the rest of this protocol.
7. Add 0.25 % Triton X-100 in PBS and incubate for 10 min at room temperature.
8. Use a transfer pipette to wash slide well with ice-cold PBS, bang slide edge on paper towel to remove excess PBS, and use strips of Whatman filter paper to wick off remaining PBS near cells.
9. Add 60 μ l 5 % normal serum from the host of the secondary antibody (*see below*) in PBS to each chamber area and incubate for 60 min at room temperature.
10. Wash with ice-cold PBS as for **step 8**.
11. Add 60 μ l 300 nM DAPI (Sigma, no. D8417, diluted in PBS) to each chamber area and incubate for 15 min at room temperature protected from light.
12. Wash with ice-cold PBS as for **step 8**.
13. Add 60 μ l mouse anti-alpha skeletal muscle actin (Abcam #ab28052, diluted 1:50 in PBS 0.1 % BSA [IgG- and protease-free; Jackson ImmunoResearch #001-000-161]) to each chamber area and incubate for 60 min at room temperature.
14. Wash with ice-cold PBS as for **step 8**.
15. Add 60 μ l fluor-tagged appropriate secondary antibody (e.g., Alexa 488-conjugated goat anti-mouse, Invitrogen # A11029, diluted 1:1,000 in PBS 0.1 % BSA) to each chamber area and incubate for 60 min at room temperature (*see* ^{Note 35}).
16. Wash with ice-cold PBS as for **step 8**.
17. Add 60 μ l chicken anti-vimentin (Thermo Scientific # PA1-16759, diluted 1:5,000 in PBS 0.1 % BSA) to each chamber area and incubate for 60 min at room temperature.
18. Wash with ice-cold PBS as for **step 8**.
19. Add 60 μ l fluor-tagged appropriate secondary antibody (e.g., Alexa 594-conjugated goat anti-chicken, Invitrogen # A11042, diluted 1:1,000 in PBS 0.1 % BSA) to each chamber area and incubate for 60 min at room temperature.
20. Wash with ice-cold PBS as for **step 8**.

³⁵Cardiac myocytes may autofluoresce, particularly in the red channel (excitation ~590 nm, emission ~620 nm) but rarely if ever in the green channel (excitation ~495 nm, emission ~519 nm) or far-red channel (excitation ~650 nm, emission ~665 nm). Because use of red fluors is generally necessary for double staining, be sure to include appropriate negative controls (e.g., cardiac myocytes incubated with primary but not fluor-tagged secondary antibody) to monitor autofluorescence, which appears as one to five perfectly round bodies per cell ~half the size of the nucleus.

21. Use large orifice micropipette tip to add 60 µl ProLong Gold antifade mounting reagent (Invitrogen # P36930) directly to ~4 chamber areas (i.e., ~15 µl per area) (*see* Note 36).
22. Place a large glass coverslip on the slide, starting at an angle to prevent bubbles. Remove excess mounting reagent surrounding coverslip.
23. Use nail polish to seal the right and left (not the top or bottom) edges of the coverslip on the slide.
24. Examine slides using a microscope (inverted or confocal) with wide-field ultraviolet light illumination and appropriate filters.
25. Store slides at 4 °C protected from light. Slides will last for at least 1 year.

3.8 Assessing Virus Effects in Cardiac Cell Cultures

1. At the appropriate time post-infection (depending on the virus and assay), cultures are suitable for quantitation of: frequency of virus infection (e.g., immunocytochemistry), virus subcellular localization (e.g., confocal microscopy; *see* Note 35 again), generation of infectious virus (e.g., plaque assay), generation of virus DNA or RNA (e.g., qPCR or qRT-PCR) or protein (e.g., immunoblot), virus-induced cytokine secretion (e.g., ELISA of supernatants), virus-induced cytopathic effects (e.g., MTT assay; *see* Note 37), and other viral parameters (*see* Note 38).

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³⁶Using more than ~15 µl ProLong Gold per chamber will leave slides too wet such that the nail polish in **step 23** will mix with the ProLong Gold and cover the chamber areas.

³⁷Assays for apoptosis can be employed, but cardiac myocyte cultures may have high rates of spontaneous apoptosis. Be sure to include appropriate control cell cultures.

³⁸For any assay, be sure to normalize results to internal controls appropriately. Primary cultures may generate greater well-to-well variability than cell lines do. Keep in mind that cardiac fibroblasts divide while cardiac myocytes do not. Phenotypes are likely to be cell type-specific; therefore it is important to ensure that cultures are monitored for purity.

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Fig. 1.

Removing fetal mouse hearts. (a) Abdomen of euthanized pregnant mouse is sprayed with ethanol, skin and membrane are pulled back, and U-shaped uterus is exposed. (b and c) Decapitated fetal mouse is held between fingers. Thoracic cavity is cut, taking care to keep scissor tips pointed outward away from mouse to avoid the heart. (d) Middle finger is gently pressed against back of the fetus to splay open the thoracic cavity and better expose the heart for removal. (e) Scissor tips are used to cut heart away from mouse and transfer to Petri dish. (f) A maximum of 100 hearts are transferred to a Petri dish containing PBS. Some contaminating blood is common

Table 1

Plating cardiac myocyte cultures

Vessel	Volume of cell suspension to plate	Cell number	Example of purpose
96-well cluster	300 µl per well	1.5×10^5 myocytes	Viral replication by plaque assay
48-well cluster	1,000 µl per well	5×10^5 myocytes	RNA or protein harvest (small)
24-well cluster	2,000 µl per well	1×10^6 myocytes	RNA or protein harvest (large)
8-well chamber slide	700 µl per well	3.5×10^5 myocytes	Microscopy

Table 2

Infecting cardiac myocyte and cardiac fibroblast cultures

Vessel	Volume of inoculum	Final volume including overlay (μl)
96-well cluster	100 μl per well	200–250
48-well cluster	350 μl per well	1,000–1,200
24-well cluster	700 μl per well	1,700–2,000
8-well chamber slide	200 μl per well	400–600