Video Article

Isolation and Culture of Neonatal Mouse Cardiomyocytes

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Abstract

Cultured neonatal cardiomyocytes have long been used to study myofibrillogenesis and myofibrillar functions. Cultured cardiomyocytes allow for easy investigation and manipulation of biochemical pathways, and their effect on the biomechanical properties of spontaneously beating cardiomyocytes.

The following 2-day protocol describes the isolation and culture of neonatal mouse cardiomyocytes. We show how to easily dissect hearts from neonates, dissociate the cardiac tissue and enrich cardiomyocytes from the cardiac cell-population. We discuss the usage of different enzyme mixes for cell-dissociation, and their effects on cell-viability. The isolated cardiomyocytes can be subsequently used for a variety of morphological, electrophysiological, biochemical, cell-biological or biomechanical assays. We optimized the protocol for robustness and reproducibility, by using only commercially available solutions and enzyme mixes that show little lot-to-lot variability. We also address common problems associated with the isolation and culture of cardiomyocytes, and offer a variety of options for the optimization of isolation and culture conditions.

Video Link

The video component of this article can be found at https://www.jove.com/video/50154/

Introduction

The earliest reports for the successful dissociation and culture of rodent heart cells dates back to the 1960's [1,2]. Even then, Harary and Farley noticed that cultured cardiomyocytes "may provide a unique system for the study of the requirements of the periodic contractility [, and may] provide a means of determining the contribution of various metabolic pathways for the [beating] process". Although Harary and Farley isolated and cultured cardiomyocytes from young rats, and the original protocol has been adapted and modified by many scientists over the years, the general isolation and culturing procedure has not greatly changed. However, better enzymes [3], standardized solutions [4,5], and the addition of the reversible channel and myosin ATPase inhibitor BDM to protect cells during the isolation procedure [6-9] has significantly improved cell-yield and viability.

Adult vs. neonatal cardiomyocytes

Cardiomyocytes isolated and cultured from neonatal mice or rats have several advantages over cultures of adult cardiomyocytes. Foremost, the isolation procedure for neonatal mouse or rat hearts is easier and less costly, when compared to the isolation of cardiomyocytes from adult mouse or rat [10]. Neonatal cardiomyocytes are far less sensitive to reintroduction into a calcium-containing medium after dissociation, greatly increasing cell-yield. Another big advantage is that neonatal mouse cardiomyocytes undergo a more rapid dedifferentiation - redifferentiation cycle that typically results in spontaneously beating cells 20 hr after plating, while adult cardiomyocytes typically require pacing to induce contraction. Neonatal cardiomyocytes are also more readily transfectable with liposomal transfection methods, whereas adult cardiomyocytes require viral vectors for successful delivery of transgenic DNA. In contrast to neonatal cardiomyocytes, culture of adult rodent cardiomyocytes [11-13] allows for investigations of myofibrillar degradation and eventual reestablishment of the contractile apparatus. These characteristic morphological changes in adult cardiomyocytes occur over periods of 1-2 weeks. The dedifferentiation - redifferentiation cycle is accompanied by reexpression of the fetal gene program, thereby mimicking pathological changes observed in human cardiomyopathies [14]. Another advantage of adult rat cardiomyocytes over the culture of neonatal cardiomyocytes is the ability to culture these cells for long periods of time.

Rat vs. mouse cardiomyocytes

The isolation and culture of rat neonatal cardiomyocytes has some benefits over that of mouse neonatal cardiomyocytes, including higher yields of viable cells and increased transfection rates. However, the wide usage of genetically modified mouse models for cardiac diseases (*e.g.* the muscle lim protein knockout mouse as model for dilated cardiomyopathy [15]) has led to the adaptation of the isolation procedure for

cardiomyocytes derived from neonatal mice. Although the protocols used to isolate neonatal rat and mouse cardiomyocytes are nearly identical, greater care must be taken in the selection of an appropriate enzyme mix for the latter. Indeed, neonatal mouse cardiomyocytes are generally more susceptible to overdigestion, resulting in a reduced cell-yield and viability. Moreover, the plating density should be adjusted, because cardiomyocytes derived from neonatal mice are somewhat smaller compared to cells derived from neonatal rat hearts.

With many uses for the investigation of morphological, electrophysiological, biochemical, cell-biological and biomechanical parameters as well as for the process of myofibrillogenesis, cultured neonatal cardiomyocytes have become one of the most versatile systems for the study of cardiac cell functions *in vitro*. The first step to a successful assay however, depends on an easy and reliable methodology to isolate neonatal mouse cardiomyocytes. Our protocol draws its methodology from many sources and was optimized for reproducibility and robustness. We discuss factors that influence cardiomyocyte-yield and viability, and provide a variety of options for the optimization of isolation and culture conditions.

Protocol

The following procedure describes a two-day protocol [16,17] for the isolation and culture of neonatal mouse cardiomyocytes. All solutions are sterile or sterile filtered. All tools are sterilized by surface sterilization with 75% ethanol. Except for the initial tissue extraction, all steps are performed in a sterile laminar flow cell culture hood. This protocol is intended for the isolation of neonatal mouse hearts from one-two litter(s) - approximately 5-14 pups, but may be adapted for larger litter sizes and rat neonatal cardiomyocytes. Scale media/enzyme usage as appropriate.

For work with neonatal rodents, refer to your local university guidelines and rules set forth by the legislature and/or animal care programs, and adhere to your institutionally approved animal protocol. All methods described in this protocol have been approved by the UC San Diego Institutional Animal Care and Use Committee (IACUC), and adhere to federal and state regulations.

Day 1.

1. Isolation of Cardiac Tissue from Neonatal Mice

1. Prepare 50 ml of 1x PBS (without Ca^{2+}, Mg^{2+}) supplemented with 20 mM BDM [6-8], and disperse into two sterile bacterial dishes placed on ice.
2. Prepare 10 ml of isolation medium in 50 ml sterile conical Falcon tube. Keep all solutions on ice. Sterilize scissors (one curved; one straight), and forceps (curved, Dumont No.7).
3. 1-3 days old neonatal mice are rinsed quickly in 75% ethanol solution for surface sterilization. Pups are decapitated using sterile scissors (straight), and the chest is opened along the sternum to allow access to the chest cavity and the heart (**Figure 1A**, **Supplemental Movie S1**). *Technical comment: Neonatal mice older than 3 days may be used, but result in fewer viable cells* [2].
4. Hearts are extracted from the body with curved scissors and transferred immediately into the bacterial dish containing 1x PBS (without Ca^{2+}, Mg^{2+}) with 20 mM BDM, on ice. All following steps are performed in the sterile cell-culture hood.
5. Remove lung tissue, larger vessels (and atria, if desired). Wash hearts in the 1x PBS solution (without Ca^{2+}, Mg^{2+}) with 20 mM BDM (on ice) to remove blood. Transfer washed hearts from first dish into the second bacterial dish containing 1x PBS (without Ca^{2+}, Mg^{2+}) with 20 mM BDM (on ice) using forceps or a perforated spoon (**Figure 1B**).
6. Transfer cleaned/washed hearts into a drop of isolation medium (approximately 250 µl; **Figure 1C**) in a third bacterial dish (on ice) and use the curved scissors to mince hearts into small pieces (approximately 0.5-1 mm^3, or smaller; **Figure 1D, 1E**).
7. Transfer minced hearts into a conical tube containing 10 ml of the isolation medium (on ice), and incubate with gentle agitation at 4 °C over night.

Day 2.

2. Enzymatic Tissue Digestion and Plating of Cells

1. Weigh in 15 mg of collagenase/dispase mixture (Roche), and dissolve the enzyme mix in 10 ml L15-medium [5] supplemented with 20 mM BDM (digestion medium). Sterile filter the digestion medium in the cell culture hood into a new sterile 50 ml Falcon tube.
2. Prepare 30 ml L-15 medium supplemented with 20 mM BDM, and plating medium.
3. Coat cell-culture plates with collagen solution (Sigma C-8919) for a minimum of 1 hr. Remove collagen solution (can be reused) and dry collagen coated cell-culture dishes in sterile laminar flow cell-culture hood.
4. Remove the conical tube containing the predigested hearts from 4 °C. Tissue fragments should be aggregated (**Figure 1G**). Let the tissue fragments sink to the bottom of the tube and remove the supernatant (make sure not to loose any tissue fragments; normally, approx. 1 ml of the isolation medium may remain in the tube). Add 5 ml of digestion medium and 5 ml of L-15 supplemented with 20 mM BDM to tissue fragments and oxygenate suspension using oxygen or air for 1 min.
5. Transfer the sealed conical tube containing the cardiac tissue fragments in digestion medium to 37 °C water bath for about 2 min to adjust temperature of the digestion solution.
6. Incubate cardiac tissue fragments at 37 °C with gentle agitation for 20-30 min (*e.g.* shaker at 37 °C, set to no more than 60rpm). Digestion times may greatly depend on enzyme mixture and lot number. ***Caution: longer incubation periods or higher enzyme concentrations may reduce cell viability.***

Technical comment: We recommend usage of a collagenase/dispase mixture from Roche (Cat.No.: 10269638001) that has very little lot-to-lot variability. The classical enzyme for the isolation of mouse cardiomyocytes is trypsin and/or collagenase type II [3,16-20] available from Worthington (Cat No CLS-2). However, performance of collagenase II may differ substantially from lot-to-lot. Worthington typically allows for the testing of several collagenase lots to optimize digestion times and enzyme usage. Alternatively, Worthington sells a cardiomyocyte isolation kit with a pre-tested enzyme mixture included that can be adapted for the isolation of mouse cardiomyocytes [17] (Cat No.: NCIS).

7. Place sterile cell-strainer (40-100 µm nylon mesh) in fresh sterile 50 ml conical falcon tube. Pre-wet cell strainer with 5 ml L-15 supplemented with 20 mM BDM. Gently triturate tissue fragments using a pre-wetted 10 ml cell-culture pipette for about 10-20 times. The tissue fragments should mostly disperse during this step, releasing the cells into suspension (**Figure 1H**).
8. Let larger tissue fragments sediment and transfer supernatant containing suspended cells into fresh conical tube through cell-strainer (**Figure 1I**).
9. Re-suspend the undigested tissue fragments in 5 ml digestion medium and incubate for an additional 5-10 min at 37 °C with gentle agitation.
10. After continuing digestion of remaining tissue fragments, gently triturate tissue for 10-20 times and add to conical tube containing cells from the first digest through cell strainer. Rinse cell-strainer with 5 ml L-15 supplemented with 20 mM BDM to allow passage of all digested cells.
11. Centrifugate conical tube containing suspended cardiomyocytes for 5 min at 300 rpm (approx. 50-100 x g). Remove the supernatant (containing mostly fibroblasts and endothelial cells) and re-suspend cell pellet in 10 ml plating medium.
12. Plate cells into 10 cm cell culture dish (**Figure 2A**) and incubate for 1-3 hr in cell culture incubator. This pre-plating step removes fibroblasts and endothelial cells (**Figure 2B**), which will adhere to the uncoated cell-culture dish (**Figure 2C**).
13. After incubation, wash non-adherent cardiomyocytes from 10 cm culture dish (re-suspend cells by repeatedly pipetting the plating medium over the dish), and transfer resuspended cells to a sterile conical Falcon centrifuge tube (15 ml or 50 ml).
    Optional: Repeat the pre-plating step to remove additional fibroblasts from cell-suspension. If desired, the adherent fibroblast and endothelial cells can be further cultured.
14. Count cells (*e.g.* by using Neubauer hemocytometer, trypan blue exclusion staining [21]).
15. Plate cells into collagen coated cell culture dishes with a density of approximately 1.5 x 10^5 cells per cm^2 (**Figure 2D**; see also comments in **Table 1** on plating and coating).
    Place dishes into cell culture incubator and leave undisturbed for 12-18 hr to allow for adherence and spreading of cardiomyocytes.

Day 3 - Culture of neonatal cardiomyocytes

1. Prepare maintenance medium and prewarm in 37 °C water bath. Refer to **Table 1** for the addition of proliferation inhibitors or chronotropic agents, or the procedure for the liposomal transfection of neonatal mouse cardiomyocytes.
2. One day after plating, cardiomyocytes should have adhered to the cell culture dish and optimally start spontaneously to contract (**Figure 2E, 2F**; **Supplemental Movie S2**; refer to **Table 2** for common problems during the isolation/culture procedure). Replace plating medium with maintenance medium, and culture for additional 1-5 days. Change medium as necessary.

Representative Results

Using this protocol, we isolated hearts from 8 one day old neonatal mice (**Figures 1A, 1B, Supplemental Movie S1**). After washing and mincing the hearts with scissors (**Figures 1C-1F**), tissue fragments were predigested in isolation medium over night at 4 °C with gentle agitation. Following predigestion (**Figure 1G**), we transferred the tissue fragments into freshly made digestion medium, and incubated the tissue fragments for 20 min at 37 °C with gentle agitation. The resulting cell suspension (**Figure 1H**) was filtered through a cell strainer (**Figure 1G**). Following centrifugation, the pelleted cells were resuspended in 8 ml plating medium and preplated into a 10 cm cell culture dish (**Figure 2A**), and cultured for 2 hr to allow for attachment of cardiac fibroblasts and endothelial cells (**Figures 2B, 2C**). Cells that did not rapidly attach were resuspended into the plating medium. 10 µl of the cell suspension containing cardiomyocytes was pipetteted into an eppendorf tube and stained with a trypan blue solution in a 1:1 ratio. Cells were counted using an automated cell counter, resulting in approximately 5.1 x 10^5 living cells/ml plating medium. After counting, cells were plated into four 30 mm collagen coated cell culture dishes (2 ml per dish), resulting in approximately 1 x 10^6 plated cells per dish (1.4 x 10^5 cells/cm^2; **Figure 2D**). 18 hr after plating, cardiomyocytes were attached to the collagen coated cell culture dishes (with approximately 70% confluency) and started to contract spontaneously (**Figure 2E, Supplemental Movie S2**). Subsequently, cardiomyocytes were either used for liposomal transfection (see **Table 1**) or the cells were directly transferred from the plating into the maintenance medium (**Figure 2F**) and cultured for another 3 days. Following culture, cardiomyocytes were briefly washed in 1x PBS, fixed for 5 min in 4% PFA/PBS and processed for immunofluorescence as described in one of our recent publications [22]. Representative results depicting immunologically stained untransfected and transfected cardiomyocyte cultures are shown in **Figures 2G** and **2H**, respectively. The cultured neonatal cardiomyocytes display the expected cytoarchitecture, as evident from the visualization of the myofibrils (red signal in **Figure 2G**) and the intercalated disk-like structures (green signal in **Figure 2G**). They are also amenable to transient transfections, as seen in **Figure 2H** (green signal is the transfected protein; red signal counterstaining of myofibrils using an alpha-sarcomeric actinin antibody).

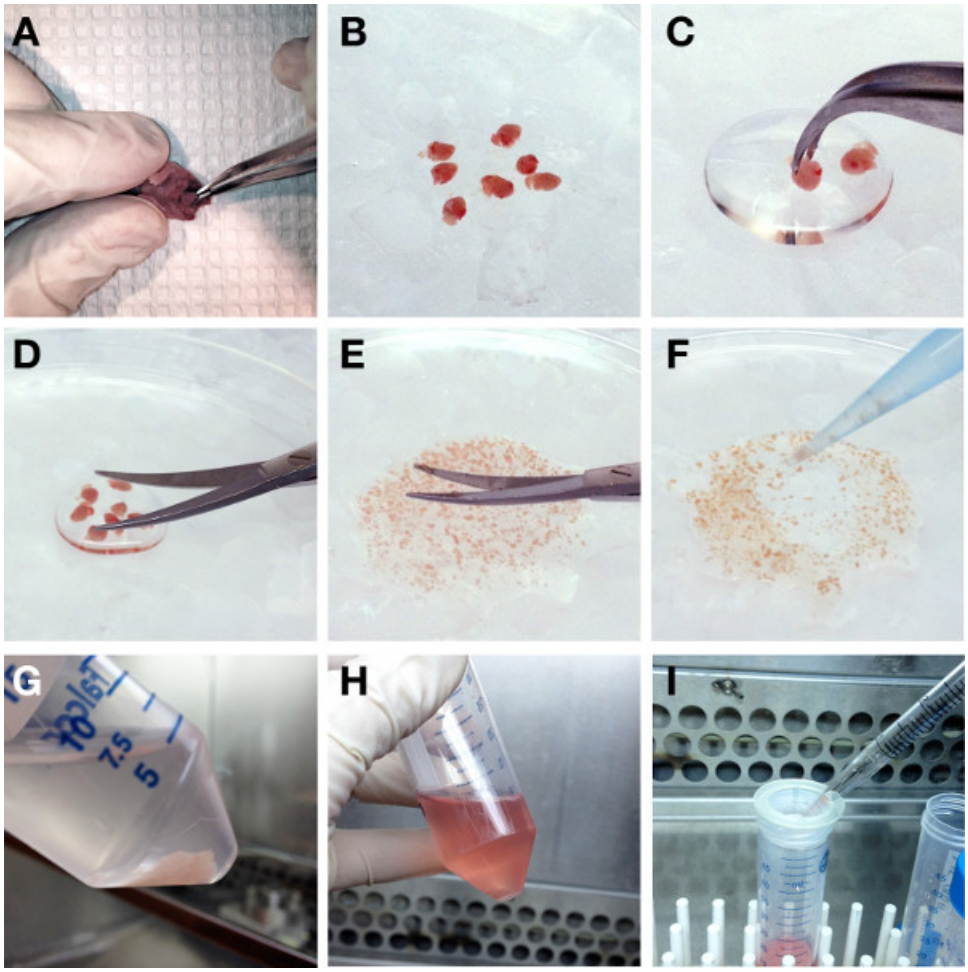

Figure 1. Isolation of cardiac tissue from neonatal mice. **A)** The heart is carefully removed from the thorax using curved forceps (see also **Supplemental Movie S1**). **B)** Hearts isolated from 8 neonates are washed in 1x PBS (without Ca^{2+}, Mg^{2+}) supplemented with 20 mM BDM on ice. **C)** Hearts are transferred into a drop of isolation medium. **D-E)** Mincing of neonatal hearts using curved scissors. **F)** Transfer of minced cardiac tissue using a pre-wetted pipette. **G)** Predigested cardiac tissue after over-night incubation at 4 °C. **H)** Cardiomyocytes in suspension after 20 min of digestion and trituration. Undigested cardiac tissue accumulates at the bottom of the tube. **I)** The suspension of isolated cardiomyocytes is filtered through a cell strainer.

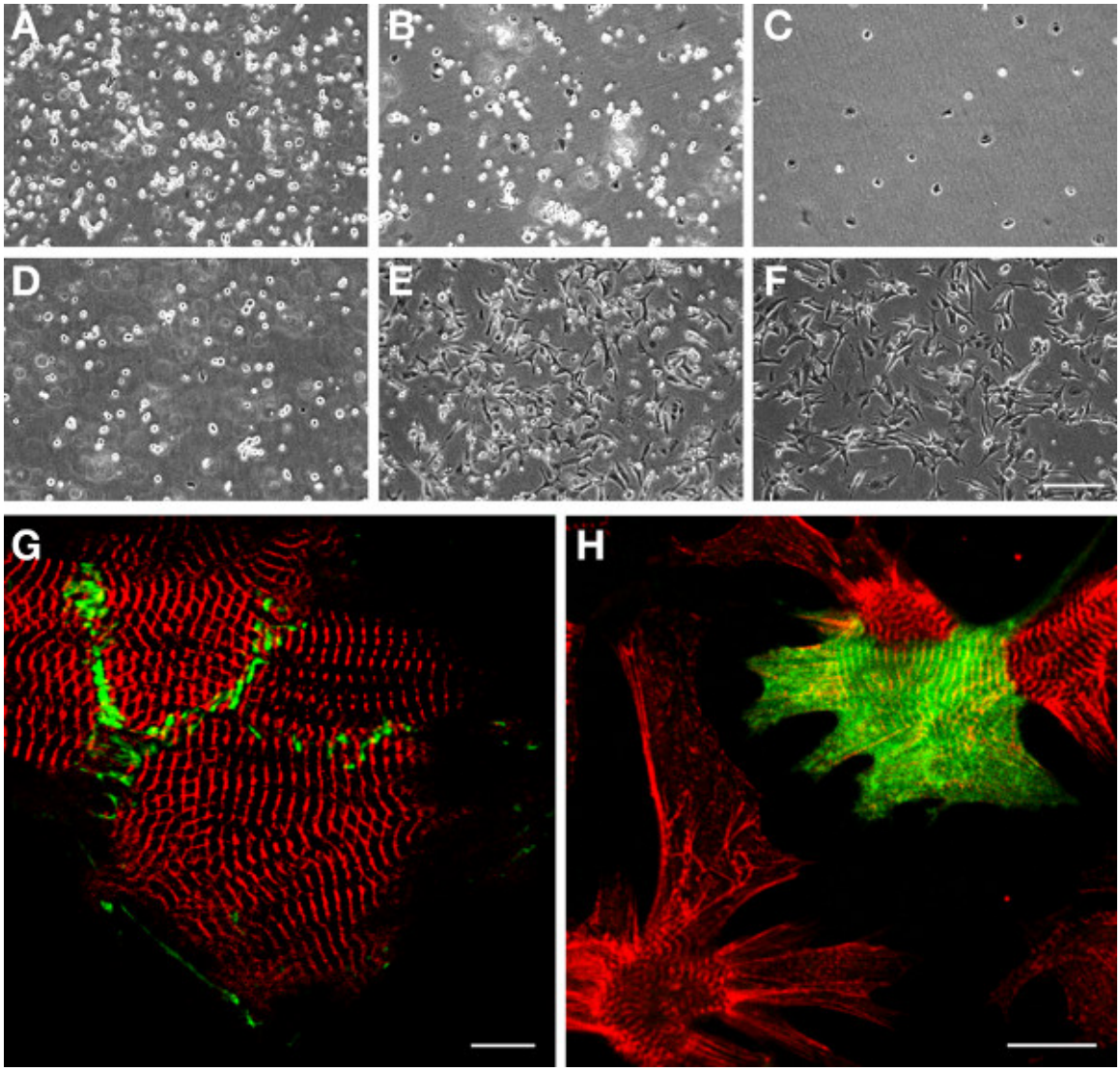

Figure 2. Culture of neonatal mouse cardiomyocytes and representative immunofluorescence images. **A)** Plating of isolated cells at the beginning of the pre-plating step. **B)** Cardiac fibroblasts and endothelial cells start to adhere to the uncoated cell culture dish after 1-3 hr of culture during the pre-plating step. **C)** After resuspension of non-adherent cardiomyocytes, remaining cardiac fibroblasts and endothelial cells can be further cultured, if desired. **D)** Plating of isolated and purified neonatal mouse cardiomyocytes in collagen coated cell-culture dishes. **E)** After 18 hr in culture, cardiomyocytes adhere to the coated dishes, and optimally start spontaneous contractions (see also **Supplemental Movie S2**). **F)** Replacing the plating medium with maintenance medium removes dead cells from the culture. A)-F) Scalebar 0.2 mm. **G)** Representative immunofluorescence image of neonatal mouse cardiomyocytes, stained with antibodies against myomesin (Developmental Studies Hybridoma Bank, Iowa, mMaC myomesin B4, in red) and beta-catenin (Sigma, Cat.-No.: C2206, in green). Scalebar 10 µm. **H)** Representative immunofluorescence image of transfected neonatal mouse cardiomyocytes with the expressed GFP-tagged protein in green (GFP-titin-N2B fragment), and alpha-actinin (Sigma, Cat.-No.A-7811) in red. Scalebar 20 µm. A method for the transfection and immunological staining used in 2G) and 2H) can be found in **Table 1**, and is summarized in one of our recent publications [22].

Supplemental Movie S1. Click here to view supplemental movie.

Supplemental Movie S2. Click here to view supplemental movie.

Pre-plating	Pre-plating of cells after isolation greatly removes cardiac fibroblasts and endothelial cells from the whole cell mixture. A single pre-plating step removes approximately 50-80% of non-cardiomyocytes from the cell-population. Repetition of pre-plating more than twice, as well as of pre-plating times longer than 3 hr is not recommended, due to additional loss of cardiac myocytes. Substitution of pre-plating with percoll-gradient [23,24] may improve the purity of the cardiomyocyte cell population.
Coating and cell culture substrate	Coating of cell-culture dishes with extracellular matrix (ECM) components like collagen, fibronectin, laminin, complex ECM mixtures (*i.e.* matrigel [25,26]) or artificial substrates (*i.e.* silicone polymer [27],

	organosilane [28]) positively influences cardiomyocyte adherence, cell viability and cell spreading during the initial plating step and for the subsequent culture period. Particularly the culture of cardiomyocytes on glass substrates requires the coating of the surface with ECM components to achieve proper adherence of cardiomyocytes. We tested coating of cell culture treated plastic and glass substrates with a variety of ECM components, including 0.1% collagen solution (Sigma C-8919), 3 mg/ml collagen type 1 solution (Advanced Biomatrix, PureCol), 1% gelatin solution (Sigma G9391; dissolved in H_2O, autoclaved), laminin solutions (Sigma L4544), or complex ECM mixtures derived from pig hearts [29] or cardiac fibroblasts [30]. Cell culture dishes were incubated with the ECM solutions for a minimum of 1 hr to over night in the cell culture incubator, and after removal of ECM dried in the laminar flow cell-culture hood. ECM solutions may be reused many times (10-20 times) if sterility and ECM protein integrity during prolonged storage is maintained (*e.g.* storage at 4 °C or frozen). Caution, due to the acidic nature of some of the buffers used to dissolve ECM proteins, washing of culture dishes with 1x PBS after coating may be required before plating of the cells.
Plating technique and density	Depending on subsequent assays, optimal cell concentrations for plating should result in near-confluent cardiomyocyte monolayers. Plating of cardiomyocytes at high concentrations result in multilayered cardiomyocyte aggregates and low homogeneity, whereas low concentrations reduces cell viability and results in single, isolated cardiomyocytes devoid of specialized cell-cell contacts (intercalated disk-like structures). When pipetting cells for plating bear in mind that cardiomyocytes are rather large and heavy, and tend to concentrate on the bottom of the falcon tube. Resuspension of cells in between plating steps ensures even distribution of cells throughout your dishes. When plating cells move dishes in the shape of a figure "8" to avoid concentration of the cells to the center of the dish. The optimal confluency of neonatal cardiomyocytes designated for liposomal transfection should be around 70-80% the day after plating.
Serum	The usage of cell culture tested fetal (or newborn) bovine serum and horse serum in the plating medium is necessary for cell viability and adherence of cardiomyocytes during the plating step. The quality of the used serum may be one of the critical steps during the plating/culture procedure. Supplementation of the maintenance medium with low amounts of serum is optional, however may greatly improve duration of culture time. *Caution: the addition of serum may significantly alter biochemical signaling pathways in cultured cardiomyocytes and may be judged with respect to experimental parameters, hypothesis and subsequent interpretation of the results. Serum-free cultures of cardiomyocytes have been described [10,31], and may prevent skewing of results due to unknown growth factors present in the serum.*
Proliferation inhibitors	Terminally differentiated cardiomyocytes do not typically undergo cell-division. However, the addition of proliferation inhibitors, such as 10 µM AraC (Cytosine-B-D-arabino-furanoside hydrochloride; Sigma C6645) to the maintenance medium is highly recommended to prevent proliferation of cardiac fibroblast and endothelial cells. Even with the addition of pre-plating steps to reduce the population of cardiac fibroblasts and endothelial cells, the remaining fibroblasts will undergo cell proliferation and significantly change the cultured cell-population over time. *Caution: depending on genetic factors and the isolation time-point, embryonic and neonatal mouse cardiomyocytes may still have proliferation potential [23,32,33]. Usage of AraC should be judged depending on experimental parameters, hypothesis and interpretation of results.*
Addition of chronotropic agents	Supplementation of the maintenance medium with agents [34], such as phenylephrine (0.1 mM, preferentially for neonatal rat cardiomyocytes; Sigma P6126), or isoproterenol (1 µM, preferentially for neonatal mouse cardiomyocytes; Sigma I6501) greatly increases sarcomerogenesis, spreading of cardiomyocytes on the plate as well as spontaneous beating of cells. However, the addition of these agents should be

	weighed with respect to desired assay parameters (*e.g.* addition may skew contractile behavior and biochemical parameters).
Transfection	Liposomal transfection of DNA/RNA into neonatal mouse cardiomyocytes is greatly dependent on the transfection time-point, transfection reagent, culture density, DNA/RNA concentration and used DNA construct. We tested a variety of liposomal transfection reagents and found ESCORT III (Sigma) and lipofectamine 2000 (Invitrogen) applicable. Transfect cells 24 hr after plating by replacing the plating medium 2 hr prior to transfection with antibiotics free maintenance medium. For transfection of neonatal cardiomyocytes cultured in 30 mm dishes, mix 1-2 µg DNA with 200 µl DMEM in a sterile eppendorf tube, and add 4 µl of Escort III to the diluted DNA mixture. Gently mix and incubate for a minimum of 5 min in the sterile hood to allow DNA/liposome complex formation. Replace the antibiotics-free maintenance medium with 800 µl fresh antibiotics-free maintenance medium and add DNA/liposome mixture to the cells. Incubate cells in cell culture incubator and replace with fresh standard maintenance medium after 24 hr. A representative result for successfully transfected neonatal mouse cardiomyocytes is shown in **Figure 2H**. Transfection using calcium precipitation of DNA is also possible. Transfection of neonatal mouse cardiomyocytes is more difficult compared to neonatal rat cardiomyocytes, with typical transfection efficiencies ranging from 1-20%. Viral vectors are required to achieve higher efficiencies [35-37] (*e.g.*: adenovirus, adeno-associated virus, lentivirus). Electroporation of neonatal cardiomyocytes is in our hands unsuitable for the delivery of DNA/RNA, however several reports show success using electroporation of embryonic and neonatal cardiomyocytes [38,39]. In a recent article [38], Djurovic *et al.* summarize several transfection methods, success rates as well as their advantages and disadvantages.
Passaging Freezing/Storage	Passaging of cardiomyocytes is not recommended [1]. Freezing of isolated neonatal cardiomyocytes for prolonged storage is extremely difficult and not recommendable due to low efficiency and low recovery rates. Several companies offer specialized freezing medium for human cardiomyocyte primary cultures (*e.g.* celprogen, cat. no. M36044-15FM) that may also be suitable for the cryopreservation of neonatal mouse cardiomyocytes, however conditions may need to be optimized. The successful passaging and cryopreservation of atrial cardiomyocytes has been described in a transgenic mouse model [32].

Table 1. Optimization of plating and culture conditions, liposomal transfection.

No viable or adherent cells 1 day after plating	- reduce enzyme concentration and/or digestion time - change enzyme mixture - change serum for plating medium - change ECM/coating medium and/or type of cell culture dishes - add 20 mM BDM to isolation/digestion media - check for contamination of cells
Low cell yield	- increase digestion time and/or enzyme concentration - increase centrifugation time/speed during isolation procedure - increase trituration period
Confluency of cells in culture is too low/high; uneven plating	- count cells during isolation procedure and adjust number of cells/dish during plating - adjust plating concentration - carefully resuspend cell pellet after centrifugation and before plating by repeated pipetting (day 2 step 13), make sure no visible cell-clumps remain
No beating cells	- add chronotropic agents to maintenance medium (*e.g.*: phenylephrine, isoproterenol) - replace medium - check for high number of cardiac fibroblasts/endothelial cells
High number of fibroblasts	- add additional preplating step during isolation procedure - add proliferation inhibitor to maintenance medium (**Table 1**)
Many dead cells	- replace the cell culture medium - reduce enzyme concentration and/or digestion time during isolation procedure

	- check cell culture incubator (CO_2, temperature, humidity) - check for contamination
Contamination	- adhere to sterile cell culture working conditions [21] - replace medium, use new penicillin / streptomycin solution - surface sterilize neonatal mice with 75% ethanol solution

Table 2. Troubleshooting. Many factors can affect the successful isolation and culture of primary cells. Please consult manuals such as "Culture of Animal Cells" by Ian Freshney [21], as general introductions into basic cell culture techniques. Possible causes for problems in the isolation and culture procedur of neonatal mouse cardiomocytes that we encountered most often are listed in this table.

Discussion

The use of animal models to study cardiac diseases has become standard in cardiovascular research. Closer biochemical characterization of these models (*i.e.* studying direct responses of cardiac cells to biochemical or biomechanical stimuli) typically requires the isolation of heart tissues or cardiomyocytes. Studies investigating physiological responses of the heart *ex vivo* (*e.g.* to acetylcholine [40], or in ischemia-reperfusion scenarios [41]) generally utilize langendorff-perfused whole hearts [42,43], whilst explant cultures [44,45] of cardiac tissue fragments allow for investigations of cardiomyocytes in a three-dimensional tissue environment. However, culturing explant tissues may hinder diffusion of nutrients, potentially generating a necrotic cluster within the core of the tissue. Moreover, cardiomyocytes are immobile within the tissue, therefore migration of cells out of explants is only observed for non-cardiomyocytes, like fibroblasts or putative cardiac progenitor cells [44]. Investigating the developmental, cell-biological, biochemical and biophysical behavior of cardiomyocytes required therefore the establishment of isolation and culturing procedures. Early protocols for the isolation and culture of mammalian cardiomyocytes utilize neonatal rats [1,2]. However, the rise of the mouse as a model system for genetic studies (*i.e.* genetically modified knock-in or knock-out mice) necessitated the adaptation of the established isolation and culturing methodologies. Unfortunately, the isolation of neonatal mouse cardiomyocytes is particularly challenging compared with that of the more traditionally used neonatal rat cardiomyocytes. The presented protocol describes a reliable and robust method optimized for the isolation and culture of neonatal mouse cardiomyocytes. We describe the steps necessary for initial tissue extraction, for the dissociation and purification of cardiomyocytes from cardiac fibroblasts and endothelial cells, and for the plating and culture conditions. While the protocol offers a basic approach to the isolation and culture of neonatal mouse cardiomyocytes in standard conditions, it can be easily adapted for the isolation and culture of neonatal rat cardiomyocytes, as well as for specific subsequent assay types: *e.g.* culture of the isolated cells on flexible membranes (*e.g.* Bioflex culture plates) to investigate biomechanical properties, culture in glass bottom dishes (MatTek) for live cell imaging, or culture of cardiomyocytes in xCELLigence cardio e-plates (Acea, Roche, Cat.-No.: 06417051001) to study the effects of drugs on contractile functions. Furthermore, we offer options for the optimization of the isolation and culture procedure and discuss sources for possible problems and their solutions. Hence, this protocol should assist researchers examining an up-to-date, highly reproducible and affordable method for preparing neonatal mouse cardiomyocyte cultures.

Disclosures

None.

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