Video Article

Isolation and Cryopreservation of Neonatal Rat Cardiomyocytes

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Abstract

Cell culture has become increasingly important in cardiac research, but due to the limited proliferation of cardiomyocytes, culturing cardiomyocytes is difficult and time consuming. The most commonly used cells are neonatal rat cardiomyocytes (NRCMs), which require isolation every time cells are needed. The birth of the rats can be unpredictable. Cryopreservation is proposed to allow for cells to be stored until needed, yet freezing/thawing methods for primary cardiomyocytes are challenging due to the sensitivity of the cells. Using the proper cryoprotectant, dimethyl sulfoxide (DMSO), cryopreservation was achieved. By slowly extracting the DMSO while thawing the cells, cultures were obtained with viable NRCMs. NRCM phenotype was verified using immunocytochemistry staining for α-sarcomeric actinin. In addition, cells also showed spontaneous contraction after several days in culture. Cell viability after thawing was acceptable at 40-60%. In spite of this, the methods outlined allow one to easily cryopreserve and thaw NRCMs. This gives researchers a greater amount of flexibility in planning experiments as well as reducing the use of animals.

Video Link

The video component of this article can be found at http://www.jove.com/video/52726/

Introduction

Cell culture of cardiomyocytes is a critical tool in modern cardiac research. Neonatal rat cardiomyocytes (NRCMs) are commonly used since the isolation and culture is easier than that of adult rat cardiomyocytes[1]. The NRCM method still has several limitations including a long isolation procedure and limited cell proliferation in the dish. There are numerous protocols for the isolation of NRCMs with most generally requiring 4-48 hr of work[2–6]. In addition, the cells are frequently isolated from 1 to 2-day old rat pups[2,4–7]; the timing of the birth can be unpredictable and conflict with other work in the lab. The isolations can be inefficient and wasteful if only a small amount of cells are needed for experiments. Most efforts on improving the workflow focus on reducing the isolation time, yet this does not solve the problems of timing the birth of the pups.

As alternatives, many labs utilize cardiomyocytes derived from embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs). However, the reprogramming and/or differentiation process can be very time consuming and costly as well. There can be other problems when using these cells as *in vitro* myocyte models. Both ESC- and iPSC- derived cardiomyocytes have been shown to exhibit differences in electrophysiology from primary cardiomyocytes[8–10].

Dissociated NRCMs are capable of being stored for several days using refrigeration[11], yet this does allow for long term storage. Liquid nitrogen is typically used to preserve cells for a greater period of time, but requires a cryoprotectant such as dimethyl sulfoxide (DMSO). Previous research has shown that the ideal concentration between 5-10% DMSO in the freezing media allows for cryopreservation of NRCMs, yet even then the viability remains low[12]. Although DMSO helps protect the cells during freezing, it can be toxic to cells at concentrations above 1.5%[13]. Previous studies have shown that slowly removing DMSO from the cells, may improve cell viability[14].

We sought to improve the efficiency of NRCM cell-based assays by cryopreserving the cells following isolation. This allows for the cells to be thawed and used when necessary, reducing the frequency of isolations and consumption of animals. Using this method, we show that it is possible to cryopreserve NRCMs and thaw them for use at a later time. After thawing the cells maintain an acceptable viability and produce NRCM cultures that are positive for α-sarcomeric actinin (α-SA) and contract spontaneously.

Protocol

The following protocol is designed for the isolation of cardiomyocytes from one litter of neonatal rat pups (10-14 pups). If litter size is significantly different, the procedure may have to be scaled to compensate. The pups should be around 48±6 hr old. All procedures herein have been approved by the North Carolina State University Institutional Animal Care and Usage Committee (IACUC).

1. Cell Isolation Preparation

NOTE: Perform the following steps the day before isolation.

1. Autoclave the following: large scissors, small scissors, small sharp forceps, large forceps. Use a gravity cycle for 60 min at a minimum of 121 °C and a drying time of 30 min.
2. Place 40 ml of 0.1% trypsin solution in refrigerator to thaw O/N. Refrigerate Hank's Balanced Salts Solution (HBSS) if not already cold. Prepare a 20% fetal bovine serum (FBS) stock media solution.
    1. In 400 ml of Iscove's Modified Dulbecco's media (IMDM) add 100 ml FBS, 5 ml L-glutamine (200 mM), 2.5 ml gentamicin (10 mg ml^{-1}), and 0.9 ml 2-mercaptoethanol. Sterilize using vacuum filter. Store media at 4 $^{\circ}C$ for up to 3 weeks and this is scalable for smaller or larger volumes. Dilute 20% FBS stock media 1:1 with IMDM to produce 10% FBS NRCM media. Only make in small batches to prevent repeated heating and cooling of media.

2. Cell Isolation Day 1

1. Perform the following work in a culture hood to prevent contamination of the samples. For best timing start this work in the afternoon since there is an O/N incubation.
2. Prepare workspace inside a sterile culture hood. Place bench pad in culture hood. Fill two 250 ml beakers with 70% ethanol and place surgical tools in them. Place small bucket of ice in hood. Ensure that it is large enough to hold several tubes and a 150 mm dish. UV sterilize the hood for 10 min. Place 40 ml 0.1% trypsin on ice.
3. Retrieve neonatal rat pups from the mother. To keep the animals warm until use, place them in an insulated container such as an ice bucket wrapped in a bench pad to keep them warm.
4. Fill a 150 mm culture dish with 25 ml HBSS and place on ice. Unwrap 2-3 gauze pads and place in work area. Holding pup by the skin on the back of the neck, spray it with 70% ethanol and place in hood. NOTE: To best maintain sterility, this is can be done by another person who then places the cleaned pup in the hood.
5. Hold the pup by the skin on the back of the neck. Then using the large scissors, in one quick, strong cut euthanize by decapitation. Blot the blood with gauze. NOTE: Due to variances in IACUC requirements, it may be more appropriate to clean the pup with an ethanol wipe rather than spraying it. Be sure to check with the university IACUC as to their guidelines for this step.
6. Continue cutting down anterior side of chest through the ribcage. Squeeze the skin on the neck to help open the ribcage until the heart is visible. Using the small scissors, remove the heart and place in the HBSS culture dish on ice. Repeat for the other pups.
7. Remove any non-heart tissue from the samples and swirl the HBSS gently to wash the hearts. Transfer the heart tissue to another culture dish containing fresh HBSS and further wash the tissue. Cut the tissue further until the pieces are 1-3 mm^3, and then transfer to the trypsin solution. Place trypsin solution on rocker in 4 $^{\circ}C$ refrigerator O/N.

Cell Isolation Day 2

NOTE: While aspirating in the following steps, use a pipette rather than a vacuum line. The tissue moves easily, and a vacuum line will clog or remove tissue still containing cells. It is best to use a pipette for aspiration instead. If tissue enters the pipette, pipette back out to remove it.

1. Make three aliquots of 10% NRCM Media: one with 25 ml and two with 15 ml. Place one 25 ml tube of media in 37 °C water bath. Add 4 ml HBSS to 5 different 15 ml conical tubes and place all on ice. Label each 15 ml tube #1-5.
2. Add 40 ml HBSS to a 50 ml conical tube. Add 10 ml of HBSS to collagenase, pipette to suspend, and combine the solutions to create 50 ml of 1 mg ml^{-1} collagenase solution. Sterilize using 0.22 µm vacuum filter, and store at RT.
3. Retrieve heart tissue/trypsin tube. Ensure that trypsin is clear, and the tissue appears fluffy. Allow the tissue to settle to corner of tube and aspirate as much liquid as possible.
4. Add 25 ml warm 10% FBS NRCM media to the tube and swirl by hand. Cap and rotate in 37 °C water bath for 2 min. Aspirate liquid again. The tissue should float at this step; if so, aspirate from the bottom of the tube.
5. Add 10 ml collagenase. Rotate in water bath for 2 min or until the solution is cloudy. Quickly aspirate the liquid. Add 10 ml fresh collagenase to tube containing the tissue and rotate in water bath for 2 min. Pipette to mix, transfer solution to tube #1, and place on ice. It may not be possible to remove all of the liquid. Repeat steps 3.2-3.6 for the other three 15 ml conical tubes. Transfer any remaining liquid to tube # 5.
6. Centrifuge 15 ml tubes at 410 rcf for 8 min. While centrifuging, place 15 ml 10% FBS NRCM media tube in water bath. Place 40 µm cell strainer on top of a 50 ml conical tube and wet with 2 ml cold HBSS.
7. Aspirate supernatant from spun down cells. Add 6 ml cold HBSS to each tube and resuspend. Pipette cell suspensions through the strainer into 50 ml conical to remove any tissue. Rinse sides of each 15 ml tube with 3 ml cold HBSS and add to strainer. Centrifuge cells at 410 rcf for 10 min. Aspirate supernatant. Resuspend cell pellet in 10 ml 10% FBS NRCM media and transfer to T-75 flask. Rinse conical with 5ml media and add to flask.
8. Incubate for 1 h at 37 °C and 5% CO_2. Place last 15 ml 10% FBS NRCM media tube into water bath. Transfer contents of T-75 to a new T-175 flask, rinse T-75 with 15 ml media and add to T-175. Incubate for 1 hr.

4. Cryopreservation

1. Place freezing media on ice. Transfer cell media from T-175 to 50 ml conical tube, and collect 10 µl of cell suspension for cell counting using Trypan blue. Centrifuge at 410 rcf for 5 min. While centrifuging, count cells using a cell counter.
2. Aspirate culture media, and resuspend cells in freezing media at a concentration of 2-4 million cells per ml. Place 1 ml of cell suspension in cryogenic vial, then place vials a controlled cooling rate freezing container, and place in -80 °C freezer O/N. Within 1-2 days, transfer vials in liquid nitrogen.

5. Thawing Cells

1. Coat dish in fibronectin by dissolving 250 µl of fibronectin (stock concentration 1 mg ml^{-1}) into 9.75 ml water (final concentration 0.025 mg ml^{-1}). Add enough solution to cover culture plate and incubate at 37°C for at least 30 min.
    1. In four 15 ml conical tubes, make the DMSO/media mixtures as follows by adding DMSO to 20% Media. Add (5%) 9.5 ml media with 500 µl DMSO. Add (2.5%) 9.75 ml media with 250 µl DMSO. Add 10 ml media (make two of these).
2. Heat the aforementioned media mixtures in 37 °C water bath. Remove cells from liquid nitrogen and place on dry ice. When media mixtures are warm, thaw cells in 37 °C water bath until just thawed. Do not leave cells in water bath for too long as this may cause damage to the cells.
3. Transfer contents of cryogenic vial to 5% DMSO/media tube. Centrifuge at 410 rcf for 8 min. Aspirate supernatant leaving cell pellet. Add contents of 2.5% DMSO/media tube to cell pellet and resuspend. Centrifuge at 410 rcf for 8 min. Aspirate supernatant leaving cell pellet.
4. Add contents of one 0% DMSO/media tube to cell pellet and resuspend. Centrifuge at 410 rcf for 8 min. Aspirate supernatant leaving cell pellet.
5. Add contents of second 0% DMSO/media to cell pellet and resuspend. Take small sample for cell counting. Centrifuge at 410 rcf for 8 min. While centrifuging count cells in a haemocytometer using Trypan Blue to label dead cells. Record cell amount, as well as viability.
6. Aspirate supernatant leaving cell pellet. Resuspend in 10% NRCM media (optional: add 100 µM bromodeoxyuridine (BrdU)) and plate cells on fibronectin-coated dishes.

6. Cell Culture

1. Plate cells at about 100k cells per cm^2. Adjust the plating density based on the intended use of the culture. Prepare 2% NRCM media by diluting 20% Media in IMDM in a 1:10 ratio. The following is an outline of a typical culture.
2. On day 1, rinse dead cells away with warm IMDM, add 10% NRCM (optional 100 µM BrdU). On day 2, rinse dead cells away with warm IMDM, add 10% NRCM. Day 3—add 2% NRCM. On day 4 add 2% NRCM. On day 5 ensure that the cells are confluent and beating. Maintain cells in culture for around one week. At that point fibroblasts may begin to significantly outgrow the NRCMs.

Representative Results

Following the isolation of the neonatal rat cardiomyocytes, the cells are frozen down to liquid nitrogen, and can be stored for at least several months. Typically upon thawing, the viability determined by Trypan Blue analysis will be between 40-60%. Though this is lower than other cell types, with proper seeding and culture the cells will proliferate (**Figure 1**). In order to verify contractility, the cells can be imaged using a phase contrast microscopy. The cells should begin to spontaneously contract within about three days (**Figure 2**). For immunocytochemistry (ICC) assays, cells were plated in 4-well culture slides with 200,000 cells per well. In order to show that the cells in culture are NRCMs the cells were labeled with α-SA and imaged using fluorescence microscopy. There are numerous α-SA positive cells indicating NRCMs in the culture (**Figure 4**). Other experiments have been performed including culturing NRCMs with human cardiac stem cell-derived exosomes to test regenerative properties of exosomes (**Figure 5**).

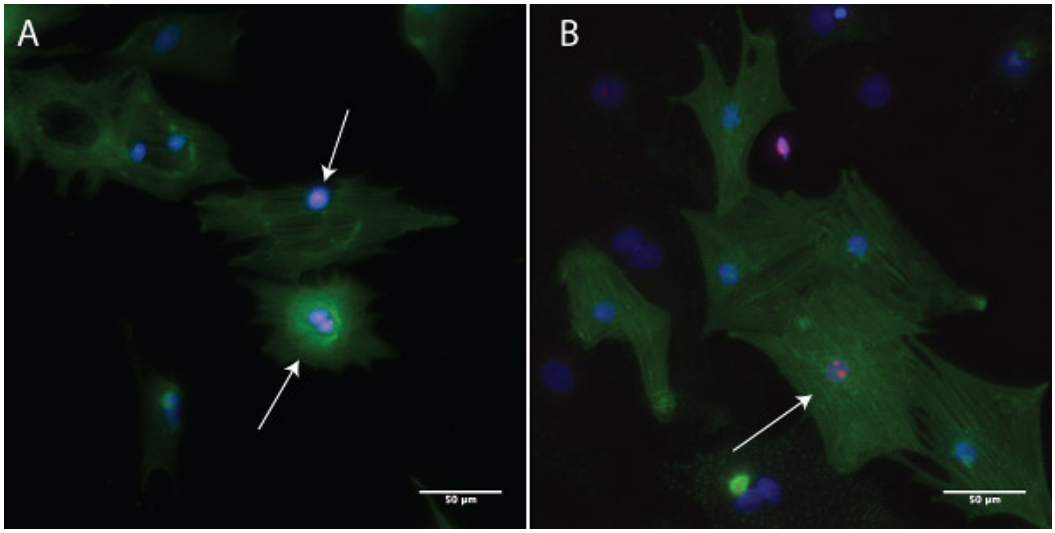

Figure 1. NRCM Proliferation *In Vitro.* Fluorescence microscopy image of NRCM with staining for α-SA (green), Ki67 (red), and DAPI (blue) for both **(A)** freshly isolated NRCMs and **(B)** cryopreserved then thawed NRCMs. Arrows indicates proliferating cells (ki67-positive). Please click here to view a larger version of this figure.

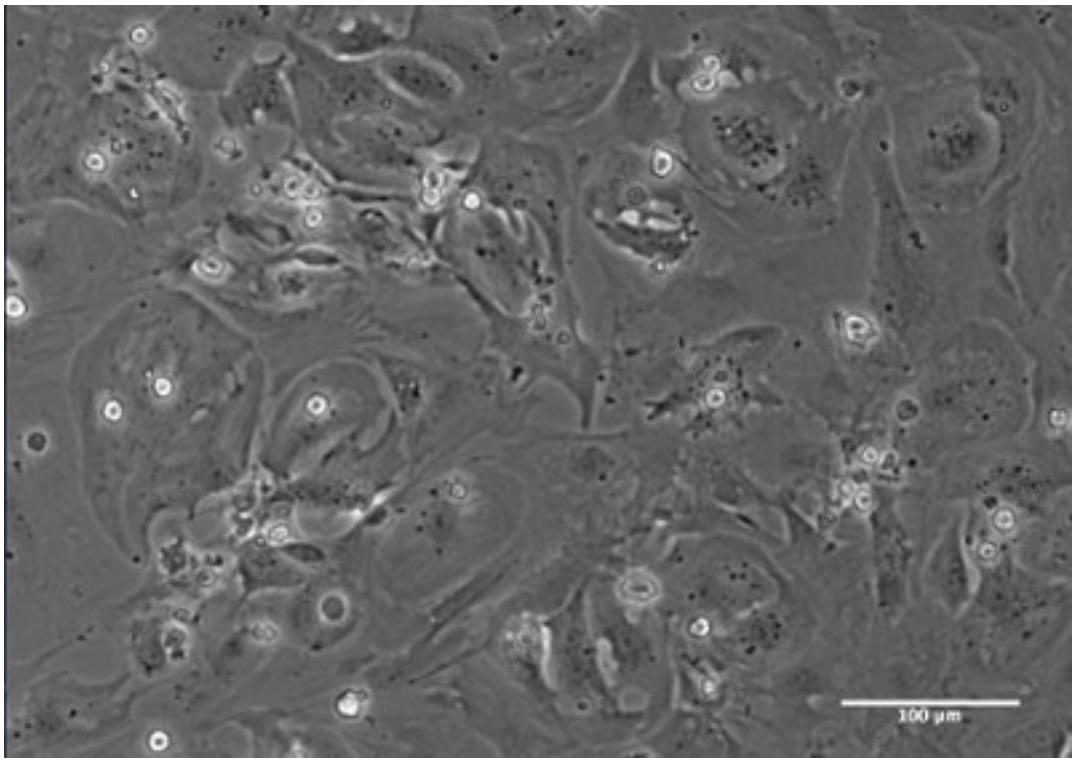

Figure 2. Contraction of Cryopreserved and Thawed NRCM *In Vitro.* NRCMs spontaneously contracting *in vitro*. Unlike freshly isolated NRCMs, it may take a few days before contractility is recovered. Video were recorded five days after thawing. Please click here to view this video.

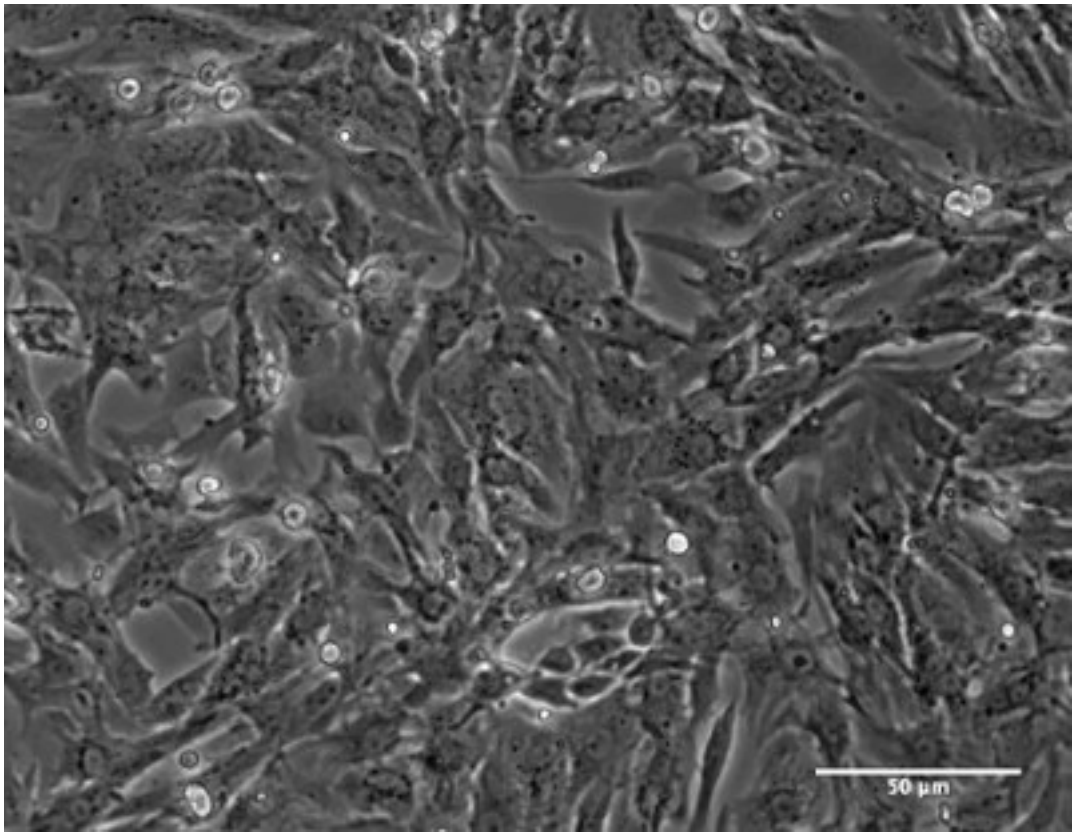

Figure 3. Contraction of Freshly Isolated NRCM *In Vitro.* Freshly isolated NRCMs spontaneously contracting *in vitro*. These cells will begin to exhibit contractility much sooner. Video were recorded 3 days after isolation. Please click here to view this video.

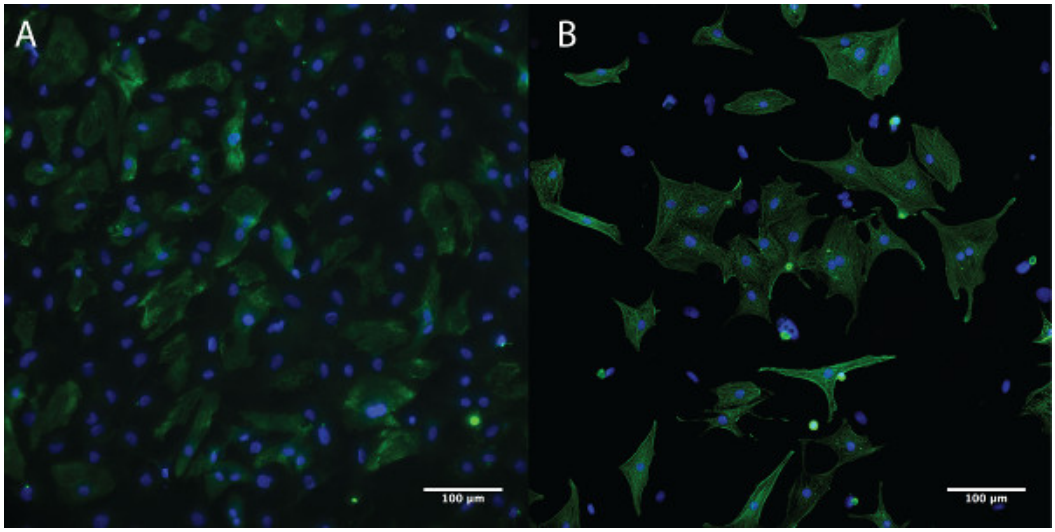

Figure 4. Purity of NRCM Culture. Fluorescence microscope images of both **(A)** freshly isolated NRCMs in culture and **(B)** cryopreserved and thawed NRCMs after 5 days in culture. NRCMs show positive staining for α-SA (green) whereas fibroblasts can be seen where nuclei (blue) are not surrounded by α-SA. Please click here to view a larger version of this figure.

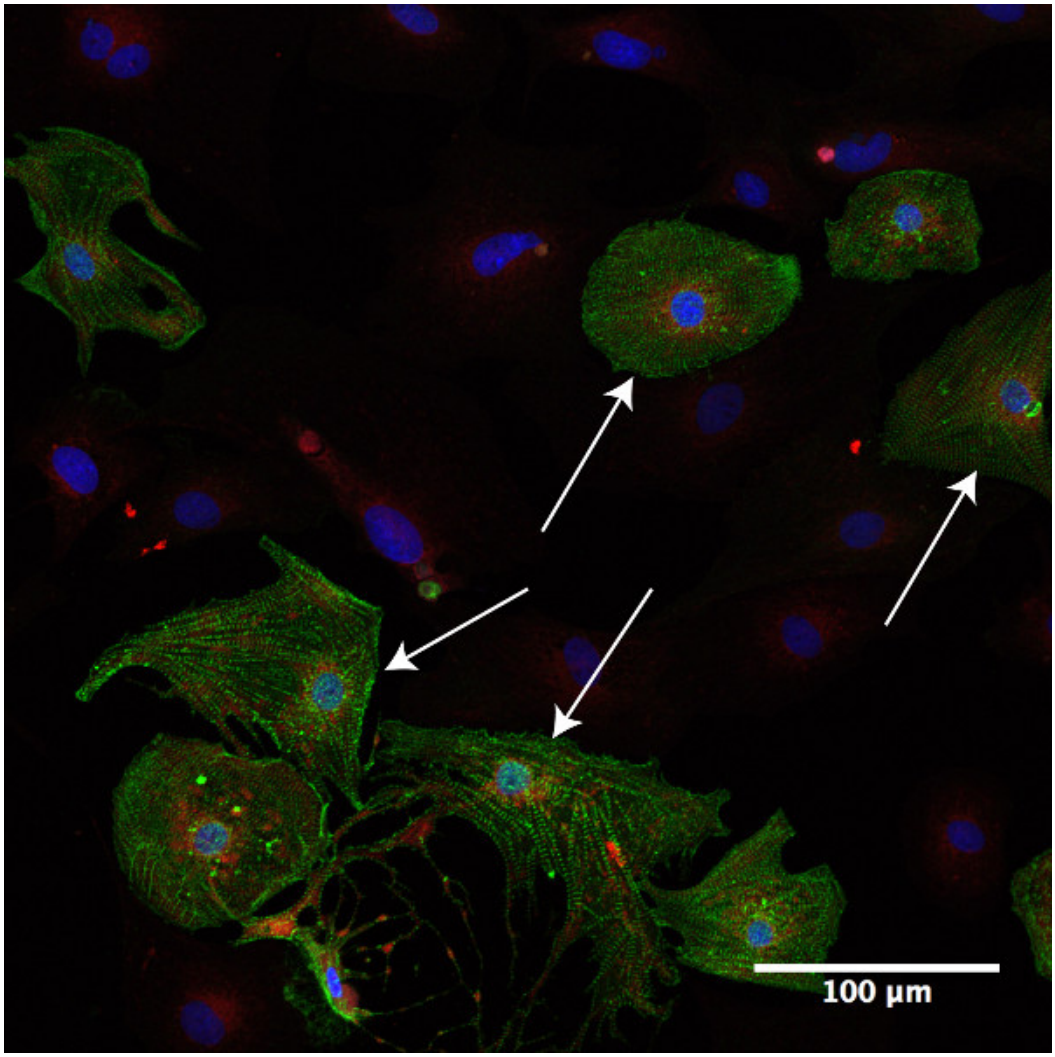

Figure 5. Use of Thawed NRCM for Cell-Based Assay. Confocal microscopy image of NRCMs, highlighted by α-SA (green), incubated with DiI-labeled exosomes (red) from cardiac stem cells. Cell nuclei labeled with DAPI (blue). In order to create an oxidative stress model, NRCMs were treated with 100 µM hydrogen peroxide for 18 hr, prior to being incubated with exosomes for 4 hr to reverse the oxidative stress. Arrows indicate regions of high exosome concentrations in some cells. Please click here to view a larger version of this figure.

Discussion

This protocol allows for the NRCMs to be isolated, cryopreserved, and thawed. Thawing the cells is a crucial portion of the procedure. A series of DMSO dilutions is used to slowly remove DMSO from the cells[14]. It is important that the extraction of DMSO be performed quickly as the cells are particularly sensitive to dying immediately after thawing. If more or less cells are required for thawing, the volumes of the DMSO solutions can be scaled as needed.

One challenge to NRCM isolation and culture is the rapid proliferation of fibroblasts. This protocol uses preplating which has been shown to reduce the number of fibroblasts[15]. Preplating works by using uncoated flasks to which the fibroblasts attach much faster than cardiomyocytes. The sequence of media changes can be altered to reduce fibroblast growth. Fibroblasts will outgrow NRCMs in 10% NRCM media, but fibroblast growth is significantly slower in 2% NRCM media. There are other methods such as Percoll gradients to remove fibroblast prior to plating[16,17] or the addition of BrdU to slow fibroblast growth[3]. Excessive fibroblast growth can be hard to detect using phase microscopy, but is easily shown using NRCM specific staining such as α-SA (**Figure 3**). If there remains excessive fibroblast growth, the amount of FBS in the media can be decreased to 2% FBS on day 2. If necessary, plating can be done in 5% FBS media, though this may result in increased cell death.

This procedure is limited and no longer beneficial if using cell cultures that require large amounts of cells[18,19]. In cases where the amount of cells needed approaches the half amount of cells possible to harvest from a litter, this procedure will require more animals since approximately half of the cells are lost due to cryopreservation. The benefits of cryopreservation are greater when using fewer cells for assays such as immunostaining.

This protocol illustrates the methods for the isolation, cryopreservation, and subsequent of NRCMs. There are few alternatives, such as buying frozen cells from vendors, to using this method but buying cells may be cost prohibitive to some labs. Our methods only require materials and

reagents commonly used in cell culture. In addition, utilizing these methods can greatly increase the efficiency and flexibility of lab work while reducing the use of lab animals. The methods described produce viable cultures that can be used for subsequent *in vitro* studies.

Disclosures

The authors have nothing to disclose.

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