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A rapid and efficient method for the isolation of postnatal murine cardiac myocyte and fibroblast cells.

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Abstract

The capacity to isolate and study single cardiomyocytes has dramatically enhanced our understanding of the fundamental mechanisms of the heart. Currently, two primary methods for the isolation of cardiomyocytes are employed; i) The neonatal isolation protocol and, ii) the Langendorff isolation method. A major limiting feature of both procedures is the inability to isolate cardiomyocytes between 3 days and 3 weeks post-birth. Herein we report the establishment and validation of a new method for the rapid and efficient isolation of mouse cardiomyocytes, regardless of age. This novel procedure utilizes whole heart perfusion of a trypsin/collagenase Krebs-based buffer through the left ventricle at a high flow rate. Cardiomyocytes can be isolated in significantly less time with a simple, syringe pump-based apparatus. Typically, we can digest 10-15 hearts per hour. Altogether, we have established an efficient and reproducible method for the rapid isolation of fresh cardiomyocytes from postnatal mouse hearts of any age.

Keywords: Cardiomyocyte; isolation; cardiac; perfusion; neonatal; fibroblast

Introduction

Although the medical community has made great strides in reducing the morbidity and mortality from heart disease, it remains one of the leading causes of death worldwide (Benjamin et al., 2017). Both basic and translational researchers in the cardiac field continue to develop and refine their methods and techniques to improve our understanding and treatment of heart disease (Ackers-Johnson et al. 2016; Gao et al. 2010). Historically, two rodent cardiomyocyte (CM) isolation methods have played a critical role in advancing our general knowledge of CM biology; i) the Langendorff isolated perfused adult heart model and ii) neonatal ventricle CM isolation. Oscar Langendorff, in 1897, was the first to describe the retrograde perfusion of the mammalian heart, a method that was derived from the pioneering work by Carl Ludwig and Eli Cyon of the development of the isolated frog heart (Bell, Mocanu, & Yellon 2011; Skrzypiec-Spring, Grotthus, Szelag, & Schulz 2007; H.-G. Zimmer 1998). During the 1980's the Langendorff method was further developed (H. G. Zimmer 2000) and is now the standard robust isolation method to study adult CMs, however, it can be a significant technical challenge that involves cannulation of the ascending aorta followed by retrograde perfusion of the left ventricle and coronary arteries with a Krebs/collagenase buffer (Bell et al. 2011). Neonatal CMs can also be isolated from very young mice (< 3 days old) using a multiple-day protocol that involves tryptic digestion (Vidyasekar, Shyamsunder, Santhakumar, Arun, & Verma 2015). These two methods are widely used for the isolation of CMs. However, these methods both have several limitations. Most importantly, these two current methods do not provide an approach that can be used to easily isolate CMs from any post-natal heart, regardless of age. Here we report a novel cardiomyocyte isolation strategy that allows for the isolation of cardiomyocytes from murine postnatal heart regardless of age that we have successfully utilized in two recent studies (Abdul-Ghani et al. 2017; Jiang et al. 2015).

We are specifically interested in studying the perinatal heart, that being the period in the heart during late embryonic development and neonatal development (E19 to P14), and so studying isolated CMs from these time points is critical. Isolating cells from early time points (E19 to P3) may be accomplished using the previously described neonatal method, however to conduct similar CM analysis across an aging cohort there is no consistent method for retrieving variable-aged CMs. Our goal was to develop a new approach that utilizes the beneficial aspects of both the Langendorff (collagenase perfusion) and the neonatal (collagenase/trypsin) method of CM isolation to create a universal procedure that can be used to isolate fresh CMs as well as non-cardiomyocytes from mouse hearts of any age.

Materials and Methods

All the mice were studied according to protocols approved by the Institutional Animal Care Committee in accordance with the Canadian Council on Animal Care's Guide to the Care and Use of Experimental Animals and the Animals for Research Act.

All steps were performed in a laminar flow biosafety level 2 cabinet and sterile technique was used to prevent contamination. A diagram of the perfusion apparatus is shown in Figure 1A. Perfusion tubes were cleaned with 70% ethanol for 5-10 minutes followed by several rinses with distilled water. The tubes were then filled with Perfusion Buffer (recipe in appendix A) before beginning the isolation. Injection of heparin into mice is not required.

Mice were anesthetized by injecting Avertin (0.4mg/g) intraperitoneally. Mice were then secured in surgical position using medical tape. Next, the thoracic cavity was opened to expose the heart. A syringe pump was then used to begin pumping pre-warmed (37°C) Perfusion Buffer through the needle. A heat exchanger should be used to continue warming the solution as it is pumped in the heart (Figure 1A). For neonatal mice, we empirically determined that a 30g needle with a flow rate of 2mL/min was optimal for perfusion of the neonatal heart. While in fully

grown adult mice we determined that a 26g needle with a flow rate of 4mL/min was optimal. Each of these factors (syringe size, needle gauge, and flow rate) must be adjusted based on mouse size or age. To ensure proper coronary artery perfusion, it is essential to achieve a flow rate from the needle that produces a strong, continuous flow as pictured in Figure 1B. After the perfusion buffer was flowing, the needle was inserted into the left ventricle through the apex of the heart while the right atrial appendage was severed. The mice were then perfused for 2-5 minutes, depending on mouse age (2 minutes for early neonates, 5 minutes for adults).

After perfusion, the heart was carefully removed and placed into a 15mL tube containing Stop Buffer (recipe in appendix A) and incubated at 37°C for 10 minutes. The heart + Stop Buffer was then poured into a 60mm petri dish, and the heart was quickly minced into small (1mm x 1mm) pieces. To promote dissociation of cells from the extracellular matrix (ECM), gentle trituration (10-20 times) of tissue with a wide-bore pipette (smooth edged to limit sheering) was performed. A 100µm nylon mesh filter (Fisher 352360) placed in a 50mL conical tube was used to filter out large debris. Cells were then centrifuged at 50xG for 5 minutes to pellet CMs. When performing the isolation from adult mice (>10 weeks), tubes can be left in the bio-hood for 10-15 minutes, and CMs will settle to the bottom of the tube. At this point, the supernatant can be removed and centrifuged again at 500xG for 5 minutes to pellet cardiac fibroblasts, which can also be cultured for use in experiments. Alternatively, the supernatant containing non-cardiomyocytes can be plated on appropriately treated cell culture plates to allow cells to attach.

The CMs were then re-suspended in media, and a pre-plating step was performed to remove any non-CMs in the pellet. After 2 hours of pre-plating, CMs were moved to a gelatin-coated ExCellness (01.100.100.00) plate and incubated overnight. When media is changed daily, CMs can live in culture for up to 24-48 hours. Alternatively, after CMs have been pelleted, they can be resuspended in 10% neutral buffered formalin and fixed for 15 minutes in suspension.

Cardiomyocytes can then be gently spun down onto glass slides with a cytopsin (Fisher A78300003) and be used immediately for staining and immunofluorescence. Alternatively, the freshly isolated CMs and non-CMs can be labelled in suspension for analysis flow cytometry.

Results

The primary limitation for any CM isolation method is to establish the conditions by which a digestion buffer could be delivered efficiently to the coronary arteries, to break down the extracellular matrix and release intact cells from the heart. Typically, whole body fixation is accomplished by delivering fixative via the left ventricle, using a syringe or pump, such that the fixative can permeate throughout the circulatory system (Gage, Kipke, & Shain 2012).

We used a syringe pump with a 30-gauge needle to deliver digestion buffer at a high flow rate (2ml/min) with constant pressure (Figure 1A). We were able to successfully perfuse the heart and isolated CMs in a reproducible manner from adult hearts using this approach. Using the method described, we perfused a 3-day old and a 10-day old pup via the left ventricle for 2 minutes. The heart was excised and placed in a pre-warmed Krebs buffer containing BSA and incubated for 10 minutes at 37°C. After 10 minutes, the heart was cut into several small fragments and then gently triturated. Single cells were separated from large tissue pieces using a 100micron filter. The filtrate was then gently centrifuged (50g). The pellet was gently disrupted, and the cell suspension was spun onto a glass slide using a cytopsin apparatus. The isolated CMs were stained with phalloidin (Fisher A12379)/ propidium iodide (Fisher P1304MP) or lamin A protein and visualized under a fluorescence microscope (Figure 1C – 1F).

To demonstrate the utility of this new method, we measured the cellular dimensions of CMs isolated from the hearts of mice that were chronically stimulated with phenylephrine (PE). PE is commonly used to induce pathological cardiac hypertrophy, and a hallmark of this organ growth

is an increase in cardiac cell size (Maillet, van Berlo, & Molkentin 2013). Osmotic pumps were implanted into two groups of mice, with one group being infused with saline while the other group was infused with PE for a two-week period. Using our new method for CM isolation, we have successfully isolated cells from 18 adult mouse hearts in one day. The cells were stained with a fluorescently labeled phalloidin and DAPI and were subsequently visualized with a fluorescent microscope (Figure 2A). As shown in Figure 2, CMs isolated from mice treated with PE exhibited increased cell width ($p < 0.001$) and area ($p < 0.001$) when compared to PBS treated mice, indicating that the PE treatment on mice caused concentric CM growth leading to pathological hypertrophy.

Discussion

The significant advantage of the method we have developed and described is that CMs can be isolated from mice of any age, and processed for alterations in morphology and structure. Another advantage of this method compared to others is the duration of the isolation procedure. It is not unreasonable to expect to isolate cells from 5-7 adult hearts in an hour when working alone, not including preparation of buffers nor time spent culturing cells. This number is substantially increased when working in a pair (10-15 per hour), or when isolating from neonatal hearts as the perfusion time is reduced. Langendorff based CM isolations typically take 40-60mins per adult heart and require a specialized perfusion apparatus. Another beneficial outcome of this method is that during perfusion, all blood is evacuated from the vasculature, ensuring no erythrocyte contamination in the isolated cells without performing a red blood cell lysis step. Previous studies using perfusion in other tissues have noted that elimination of erythrocyte contamination is essential for producing high-quality images and isolated cells (Assmus et al., 2010; Chow et al. 2005; Toyota et al. 2002).

We have successfully cultured these freshly isolated cardiomyocytes overnight and have not cultured them for long periods of time. The overnight cultures retain their rod-shaped phenotype. Long term culturing of rodent CMs has been problematic as adult rat CM cultured cells do not retain their phenotype overtime whereas adult guinea pig ventricular myocytes retain their phenotype (HORACKOVA & BYCZKO 1997; Horackova & Mapplebeck, 1989).

In summation, we have now established an efficient and reproducible method for rapid isolation of fresh CMs from postnatal mouse hearts of any age. We envision that this method will have broad utility, allowing rapid and efficient isolation of all cell types that comprise the post-natal heart, including CMs, endothelial cells and fibroblasts. We also believe that the method can be readily scaled and adapted for the isolation of CMs from larger animal models.

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Figures

Figure 1. Cardiomyocyte isolation perfusion apparatus and flow rate. A) Isolation apparatus includes a syringe mounted on a syringe pump, a heat exchanger, and a tube connecting the syringe to a needle through the heat exchanger. B) Flow rate on the syringe pump should be adjusted such that a strong, steady flow is achieved. For neonatal mice, a 2mL/min of perfusion buffer flowing through a 30-gauge needle is sufficient. For adults, a flow rate of 4mL/min through a 26-gauge needle is sufficient. Cardiomyocytes isolated from C) 3 day old heart and D) 14 day old heart. Cells were stained with phalloidin (green) and propidium iodide (red). E, F) Cardiomyocyte isolated from a 3 month old heart stained with phalloidin (red) and Lamin A/C (green). Bar represents 10µm.

Figure 2. Isolation of cardiomyocytes (CMs) for cell measurements upon phenylephrine (PE) treated hearts *in vivo*. A and B) Representative images of PBS treated (A) and PE treated (B) CMs isolated using the perfusion-based method (n=6 mice per time point). C and D) CMs treated with PBS or PE were measured (n>100 cells measured per group) and average length/width and cell area are displayed. PE-treated cells are significantly wider and larger in area compared to PBS-treated. Student's t-test was used to test for significant differences.

Appendix A: Required Materials

Recipes:

Avertin: 20mg/mL solution in PBS

Solution A: 126mM NaCl, 4.4mM KCl, 1mM MgCl₂, 4mM NaHCO₃, 30mM 2,3-butanedione monoxime, 10mM HEPES, 11mM glucose, 0.5mM EDTA. pH to 7.4, filter sterilize, and store at 4°C for up to a month.

Perfusion Buffer: Solution A + 0.09% Collagenase Type 1, 0.125% trypsin, and 25µM CaCl₂.

Made fresh on day of perfusion.

Stop Buffer: Solution A + 100µM CaCl₂ and 2% BSA. Made fresh on day of perfusion.

Formalin: 10% neutral buffer formalin in PBS

Cell culture media: M-199 (Fisher 11150059) with 10% FBS and 1x antibiotic/antimycotic.

Other required items:

50mL centrifuge tubes (Fisher 352070)

100µm nylon cell strainer (Fisher 352360)

60mm cell culture plates (Fisher 430589)

Excellness 100kPa 100mm plate (ExCellness 01.100.100.00)

Surgical tools (scissors and forceps)

Perfusion Apparatus with Heat Exchanger

1mL & 60mL syringes

30g & 26g needles

Figure 1

Figure 2