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Methods in Cardiomyocyte Isolation, Culture, and Gene Transfer

William E. Louch[a,b], **Katherine A. Sheehan**[c], and **Beata M. Wolska**[c,d]

[a]Institute for Experimental Medical Research, Oslo University Hospital Ullevaal, Oslo, Norway

[b]Centre for Heart Failure Research, Faculty of Medicine, University of Oslo, Oslo, Norway

[c]Department of Physiology and Biophysics, University of Illinois at Chicago, Chicago, IL 60612, USA

[d]Department of Medicine, Section of Cardiology, Center for Cardiovascular Research, University of Illinois at Chicago, Chicago, IL 60612, USA

Abstract

Since techniques for cardiomyocyte isolation were first developed nearly 35 years ago, experiments on single myocytes have yielded great insight into their cellular and sub-cellular physiology. These studies have employed a broad range of techniques including electrophysiology, calcium imaging, cell mechanics, immunohistochemistry and protein biochemistry. More recently, techniques for cardiomyocyte culture have gained additional importance with the advent of gene transfer technology. While such studies require a high quality cardiomyocyte population, successful cell isolation and maintenance during culture remain challenging. In this review, we describe methods for the isolation of adult and neonatal ventricular myocytes from rat and mouse heart. This discussion outlines general principles for the beginner, but also provides detailed specific protocols and advice for common caveats. We additionally review methods for short-term myocyte culture, with particular attention given to the importance of substrate and media selection, and describe time-dependent alterations in myocyte physiology that should be anticipated. Gene transfer techniques for neonatal and adult cardiomyocytes are also reviewed, including methods for transfection (liposome, electroporation) and viral-based gene delivery.

Keywords

methods; cardiomyocyte; isolation; cell culture; gene transfer; transfection; transduction

1. Introduction

Animal models have long been employed as tools to expand our knowledge of cardiac physiology and disease. While such models were initially developed for the study of surgical

Correspondence: William E. Louch, Institute for Experimental Medical Research, 4.etg. Kirurgisk Bygning, Oslo University Hospital Ullevaal, 0407 Oslo, Norway, Phone: +47 23 016 831; Fax: +47 23 016 799; w.e.louch@medisin.uio.no. Beata M. Wolska, Department of Medicine, Section of Cardiology (M/C 715), University of Illinois at Chicago, 840 S. Wood St., Chicago, IL 60612-7342, USA, Phone: 1-312-413-0240; Fax: 312-996-5062; bwolska@uic.edu.

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and pharmacological interventions in the intact heart, later advancements lead to the development of intact muscle preparations which allow more direct experimental manipulation of the muscle. However, these preparations do not permit easy access to the middle of the muscle for treatment or recording. Furthermore, electrophysiological examination of intact cardiac muscle is hindered by the large size of these preparations, which does not allow adequate control of membrane potential during voltage-clamp experiments. This limitation was a major driving force to develop techniques for isolation of single cardiac myocytes. Usage of isolated cardiac myocytes has a number of additional advantages such as the ability to select cells from different areas of the heart including the atria, left and right ventricle, the conductive system or a specific region of the heart following myocardial infarction. As well, while imaging techniques are often limited in thick tissue, isolated cells are well-suited for experiments aimed at visualizing cellular structure and the precise localization of intracellular molecules. Isolated cardiomyocytes are also routinely used for studies examining intracellular Ca^{2+} homseostasis, cellular mechanics, and protein biochemistry, and can be easily infected or transfected for gene transfer studies. Thus, isolated cardiomyocytes can be utilized in an array of experimental settings to provide valuable insight into cellular and sub-cellular physiology. By combining these data with experimental findings made *in vivo* and in intact tissue, animal models can be employed to examine cardiac function in a hierarchical manner.

While immortalized cardiac cell lines such as HL-1 cells are commercially available [1], acutely isolated cells are more physiologically relevant both structurally and functionally to the living organism. Cardiomyocyte isolation techniques are therefore of critical importance, as are methods for cell culture which aim to preserve as closely as possible the *in vivo* integrity and function of the myocytes. In this review we will discuss basic methods used for cell isolation, short-term culture, and gene transfer of adult and neonatal ventricular myocytes from rats and mice. This discussion will provide general guidelines as well as detailed specific protocols used in our laboratories (see appendices), highlighting possible caveats and factors to consider at crucial steps during these procedures.

2. Cardiomyocyte isolation

2.1 Isolation from adult hearts

Isolation of high quality cardiac myocytes is perhaps the single most important factor for successful experimentation with either fresh or cultured cells. However, although myocyte isolation has been conducted for almost 40 years [2], there remains no single universal method that can be easily employed to produce a large yield of high quality, viable cells without some adjustments. The protocols used by different laboratories vary and may depend not only on the species from which cells are isolated, but also on the type of experiment that is planned. Even laboratories that routinely isolate cells may suddenly and unpredictably experience difficulties with the isolation. Some of the most common causes for these difficulties are poor water quality, improperly cleaned or contaminated glassware and instruments, incorrect pH or temperature of solutions, new lots of chemicals (especially collagenase, protease or albumin), air bubbles in the perfusate, and inattention to detail in handling the heart. Here we provide important information regarding the most prominent factors to attend to during adult cardiomyocyte isolation.

2.1.1. Preparation of the Langendorff apparatus—Some laboratories have successfully isolated cardiomyocytes by simple enzymatic digestion of the left ventricle in a petri dish with dicing and agitation of the tissue. However, most believe that high quality cardiomyocytes can be more reproducibly isolated by the Langendorff method, using retrograde perfusion through the aorta with enzyme-containing solutions. The basic apparatus for this protocol is shown in Figure 1. Isolation of cardiomyocytes from small

animals can be conducted using either constant pressure perfusion, where solutions are gravity fed, or with constant flow perfusion by pumping the perfusate. There is no general agreement on which method gives better results and both are widely used. In our laboratories we have successfully employed both methods for isolation of cardiomyocytes from rat and mouse [3–7]. In a basic Langendorff apparatus, tubing leading from the solution reservoirs leads to a cannula, on which the heart is mounted via the aorta (Figure 2). The size of the cannula must be carefully chosen based on the size of the aorta. The typical size of cannula used for mice is between 22 and 16 gauge (0.6–1.3 mm) and for rats from 14 to 8 gauge (1.6 –3.2 mm) depending on the age and sex of animals. Both glass and metal cannulas (commercially available or home made from needles) may be employed [3, 8]. A groove near the bottom of the cannula may aid in securing the aorta if a ligature is to be used.

In many laboratories, the temperature of the perfusate is regulated via a heating coil that surrounds the perfusion tubing (Figure 1). While there is considerable variability in the temperature that is used during cell isolation, our laboratories [3–7] as well as many others use 36–37°C [9, 10]. However, some investigators prefer to use room temperature and longer time of perfusion (personal communication). The temperature is either reported at the end of cannula or on the surface of the heart.

Prior to dissection and cannulation of the heart, the perfusion system should be filled with solution and cleared of any air bubbles, as it is critical to prevent bubbles from entering the aorta during perfusion. A drip chamber may also be included in the Langendorff apparatus (Figure 1) to catch any bubbles that appear when changing between solutions.

2.1.2. Preparation of the animals—Animals that are used for cell isolation must be handled gently to minimize stress that may affect the neuro-humoral state of the cells. Importantly, animals should be injected with heparin (400–5000 u/kg b.w.) 20–30 minutes before the anesthetization [3, 11–13] to prevent blood clotting and possible myocardial infarction. Animals are anesthetized using either injectable (pentobarbital, ketamine/xylazyne) or inhalable (isoflurane) anesthetics [3, 13, 14]. It has been reported that the use of inhalable anesthetics for small animals such as mice and rats avoids the risk of myocardial ischemia since the onset of anesthesia is rapid with minimal effects on respiration [15].

2.1.3. Dissection of the heart and cannulation—When the animal has been successfully anaesthetized, the chest cavity is opened and the heart rapidly excised. If care is taken during the excision, the amount of tissue surrounding the heart can be minimized allowing greater ease in finding and cleaning the aorta. We suggest placing curved forceps under the heart, and then pulling gently upwards and towards the tail of the animal. It is then possible to cut the aorta relatively cleanly just below the thymus and to cut under the heart to exclude the lungs. It is vital to leave as much of the ascending aorta as possible to facilitate cannulation. Following excision, the heart is rapidly immersed in cold nominally Ca^{2+}-free solution (see Appendices 1 and 2) and the area surrounding the aorta is cleaned of excess tissue using microdissecting forceps and scissors. If the aortic arch is present it must be shortened below the level of the branches to prevent solution loss during perfusion.

As illustrated in Figure 2, the aorta is then positioned onto the cannula using fine-tipped forceps and secured with a silk suture (size 4-0). This method ensures that the coronary arteries, which are distal to the aortic valve, are filled with the enzyme solution. The solution penetrates deep into the tissue through the vessel branches, promoting uniform digestion. Positioning of the cannula too deep so it passes the aortic valve and enters into the left ventricle prevents proper perfusion through the coronary arteries and will not result in good

quality cells. To make cannulation easier, especially if mice or small rats are used, use of a dissecting microscope or magnifying glasses is recommended. Connecting the cannulated heart to the Langendorff apparatus can be accomplished in one of two ways. In some laboratories, the cannula remains fixed to the Langendorff apparatus, and prior to heart excision the cannula tip is submersed in the nominally Ca^{2+}-free solution. With this setup, the heart can be mounted directly, perfusion started, and adequacy of perfusion noted as blood is washed from the heart and the color of the coronary arteries changes. In other laboratories, the cannula is initially detached from the Langendorff and affixed to a one-way Luer valve and syringe filled with Ca^{2+} free perfusion solution (see Appendix 1). Once the aorta is cannulated and sutured, putting pressure on the syringe fills the coronary arteries with solution and confirms the adequacy of perfusion. The cannula is then carefully removed from the syringe and fastened to the Langendorff setup, with care taken to avoid introducing air bubbles.

2.1.4. General method of perfusion—Most protocols for cell isolation start the perfusion with a zero or low Ca^{2+} solution, to disrupt intercellular connections at the intercalated discs [16], followed by enzyme digestion to break down the extracellular matrix. However, the time of perfusion should be limited to a few minutes, normally 3–5, to avoid the phenomenon of 'Ca^{2+} paradox' [17, 18]. The enzymes used for digestion are dissolved in low Ca^{2+} solution, although some Ca^{2+} may be necessary to allow for adequate enzyme activity. Perfusion with constant pressure has the advantage that it allows better monitoring of the adequacy of cannulation. It also allows observation of the changes in flow rate that normally occur during the isolation procedure due to vasodilation and digestion of the intercellular matrix. However, a sudden and dramatic increase in flow rate may mean that that solution is leaking into the left ventricle. At this point, the Langendorff portion of the isolation protocol is complete as there is no longer adequate perfusion through the coronary arteries. The end-point of the isolation procedure in a constant flow protocol is established using different criteria. The time of perfusion with enzyme solution is either experimentally established and used consistently for each species, modified based on the heart weight of each animal, or determined by palpation. After perfusion with the enzyme solution the heart should be soft to the touch, flaccid and pale. It is generally true that isolation of cells from older animals requires a longer digestion time and/or higher enzyme concentration because of the larger size of the heart and differences in the extracellular matrix. Enzyme solution that has perfused the heart can be recycled to the solution reservoir either manually or by pumping. Such recycling aids in successful isolation likely by exposing the entire tissue to the same gradual decline in enzyme activity (personal communication). Collected enzyme solution may also be held in a heated organ bath so that the heart is submerged, as this may additionally facilitate digestion of the heart from the outside.

2.1.5. Choosing the enzyme—Proper enzyme selection is perhaps the most difficult but also the most critical determinant for successful isolation. For adult mouse and rat cardiac myocyte isolation different types of collagenases may be employed alone or with other enzymes added such as proteases, pancreatin etc. [3, 6, 7, 9, 10, 19, 20]. Collagenase used for tissue digestion is always a crude preparation containing other proteases. For example, collagenase type II from Worthington is a popular choice for cardiomyocyte isolation as it contains greater clostripain activity than other types. Other commonly used collagenases include collagenases B and D from Roche [12, 21]. Importantly, collagenase activity varies significantly between lots, requiring that newly acquired batches be tested for isolation at a range of concentrations [3, 22]. To reduce variability between batches of enzyme, Belndzyme and Liberaze TM (Roche, see appendices) may be substituted as the activity of

these enzymes is better standardized. Attention should, however, be paid to time-dependent alterations in enzyme activity which may occur following purchase.

2.1.6. Trituration—After perfusion with enzyme solution is completed, the heart is taken down from the cannula and, depending on the investigators' goals for experiments, the ventricles and/or atria are dissected and put into the proper solution. The preparation is cut into small pieces and gently triturated with a plastic transfer pipette. It is important to use pipettes with a relatively large opening and no sharp edges to minimize mechanical stress and cell tearing during trituration.

2.1.7. Sedimentation of cells: gravity vs. centrifugation and restoration of Ca^{2+}—To enrich the cell suspension with viable cardiac myocytes and to remove large, undigested chunks of tissue, dispersed myocytes are filtered though a mesh cell collector. For isolation of adult cardiac myocytes, the size of the mesh holes is usually 200–500 μm. Cells are then allowed to sediment either by gravity or gentle centrifugation. This process allows separation of the rod shaped cardiac myocytes from other cell types present in the heart and from rounded dead myocytes, which float. The sedimentation is normally repeated several times, while Ca^{2+} concentration is gradually increased in several steps [3, 6]. This allows the cells to gradually return to normal cytosolic Ca^{2+} levels without becoming Ca^{2+} overloaded and depolarized. To further increase the proportion of viable cells to dead, short term culture [23] or Percoll gradients may be used.

2.1.8. Advantages and disadvantages of using BDM—BDM (butanedione monoxime), which prevents strong cross-bridge formation by inhibition of the myosin ATPase, has been used in high concentrations to protect the myocardium from damage due to terminal contracture during dissection [24], to protect hearts from ischemia-reperfusion injury [25], and to maintain paralysis in cardioplegic solutions [26]. Recently, BDM has been extensively used in the isolation and culture of mouse myocytes [9, 27], with significant improvement in the number and morphology of isolated cells [9, 28]. However investigators should be aware of the potential side effects of including this agent, which acts as a non-specific phosphatase, in the isolation solutions and especially in culture media. We [3] and others (personal communications) have observed that prolonged perfusion with BDM may result in cells with better morphology, but after removing BDM the cells either enter a state of terminal contracture and die, are not excitable, or have significantly altered cellular electrical properties [29, 30]. Therefore, the time and concentration when BDM is present should be limited and it should be washed out well prior to experiments.

2.2 Isolation of neonatal cardiomyocytes

For experiments in which differentiated cardiomyocytes are not required, neonatal cells may provide a simpler experimental system. In comparison with adult cardiomyocytes, neonatal cells have the advantage of being easily cultured and are suitable candidates for non-viral gene transfer, as will be described in the following sections. Isolation of neonatal cardiomyocytes is also technically more straight-forward than cell isolation from adult hearts, as it does not require the rather difficult procedure of aorta cannulation and perfusion. Instead, a simpler two-step procedure can be employed, consisting of enzyme digestion and mechanical agitation of the ventricular tissue followed by purification of the cardiomyocyte population.

2.2.1. Enzymatic digestion—Neonatal cardiomyocytes are generally isolated from mice or rats that are 1–3 days old [31–33]. A number of hearts can be digested simultaneously to increase the myocyte yield. As detailed in Appendix 3, animals are decapitated, hearts removed, atria excised, and the ventricles then minced in ADS buffer. The solution of

ventricular tissue is then transferred to a spinner bottle, and an enzyme solution added. We recommend the use of collagenase type II enzyme from Worthington. The bottle is then spun at low speed for 20 minutes, at which point the enzyme solution containing cardiomyocytes and other cell types is removed from the tissue chunks and set aside. A new enzyme solution is then added to the tissue, and the procedure repeated 8 times.

2.2.2. Purification of cardiomyocytes—The collected enzyme-solution is next centrifuged, the supernatant discarded, and the cardiomyocyte fraction re-suspended in ADS containing fetal calf serum. Further purification of cardiomyocytes can be attained using percoll gradients and centrifugation [34]. Cells are then plated and often cultured using methods described below. For further details on neonatal cell isolation please consult the protocol in Appendix 3, which has been modified from protocols developed by Professors Kai Wollert, Masahiko Hoshijima, and Ken Chien [35, 36].

3. Cardiomyocyte culture methods

Recent advances in molecular biological techniques have brought about increased interest in reliable and reproducible culture methods that preserve as closely as possible the *in vivo* integrity and function of the myocytes. Here we discuss the specific features of the culture methods needed to ensure long term survival and *in vivo* properties of the cultured cells, including aseptic methods, the substrate for cell plating, the density of cell plating, and the composition and pH of media and buffers. We focus on methods for mouse and rat single adult myocyte culture since in particular the availability of transgenic mice has advanced the need for cultured, viable myocytes.

3.1. Goals of primary cell culture

The overall goal of primary adult cardiomyocyte cell culture is to produce a homogeneous population of cells that can be maintained over a longer time period than acutely isolated cells, allowing more lengthy experimental treatment and measurement of parameters, in as near to *in vivo* conditions as possible. One additional advantage of culturing is the opportunity for the myocytes to recover from the stresses and damage that occur during the enzymatic digestion and physical disruption of the isolation procedure [37]. For the culture to be successful there are several important features of the initial conditions that must be considered. The initial quality of the isolation must be as high as possible, yielding a high percentage of rod-shaped myocytes that are quiescent in normal Ca^{2+} conditions [37]. The cells must be Ca^{2+} tolerant to avoid sarcoplasmic reticulum (SR) Ca^{2+} overload and terminal contracture. The pH of the final isolation solution should be around 7.3 to 7.4. Myocyte health can be assessed by exclusion of a dye such as trypan blue, but this is not completely reliable since cells may take up the dye but still recover from the stresses of isolation and survive well in culture [38]. The most dependable measure for cell condition is a square-edged rod-shaped appearance with clear striations, and contractile response to electrical stimulation [23].

3.2. Types of culture method

Two general types of method for culture of Ca^{2+} tolerant myocytes have been described based on the use of serum and on cell attachment [23]. When Ca^{2+} tolerant myocyte culture systems were initially developed, the cells were suspended in a serum-supplemented medium and did not initially attach to the substrate. Spontaneously contracting myocytes assumed a rounded morphology that gradually returned to a more typical shape concurrent with slow cell attachment to the substrate. The shift in myocyte morphology caused this to be called *the redifferentiated* method [1, 37, 39–41]. Later, the *rapid attachment* method promoted rapid cell attachment using a serum-free medium and coating of the culture

substrate with a material to aid adherence [23]. Since rapid attachment results in better retention of *in vivo* myocyte morphology and functionality and ease of use in experiments, we will focus on this method here. Adult myocytes cultured by rapid attachment are generally quiescent and remain viable for up to 14 days [42, 43]. A major advantage of not using serum is that proliferation of non-myocyte cells such as fibroblasts is inhibited, improving the cellular homogeneity of the culture.

Rapid attachment is also routinely used for neonatal cardiomyocyte culture, although serum is generally included for an initial period of time (approx 24 hrs) while cells attach. The solution may then be replaced with one that is serum-free if required by the experimental protocol. While neonatal cells can be maintained in culture for quite extended periods, many report that best results are attained when cells are used in experiments within a week of isolation.

3.3. Substrates and media

The choice of culture substrate is critical for the type of experiment that is performed, as well as for the health and function of the myocytes. Substrates not only simply facilitate attachment, but also engage components of the extracellular matrix [42]. Recent advances in the field of tissue engineering have brought about a vast increase in materials and methods for 3-dimensional cell culture and manipulation of myocytes and fibroblasts, although beyond the scope of this work (for review see Curtis and Russell [44, 45]. For 2-dimensional culture a wide variety of glass coverslips and plastic culture dishes are commercially available. Glass coverslips that fit into small culture dishes are useful for culturing myocytes that are later transferred to a microscope perfusion chamber for electrophysiology or fluorescence experiments. The cells remain adherent to the glass, which can easily be moved about using forceps. Cells to be used for biochemical treatment and analysis are generally cultured in plastic dishes, which are sterile, inexpensive, and commercially available in a range of sizes and number of wells per dish. Some brands are manufactured to facilitate cell attachment. An advantage of not using coverslips in this case is that cells cover the entire surface area of the dish and maximize the amount of protein that can be harvested.

Substrate agents used to coat the coverslips or dishes must be selected with care to avoid unwanted changes in cell function. Commonly used culture substrate coatings include laminin, fibronectin, poly-d-lysine or synthetic peptides (Sigma, BDBioSciences, Invitrogen, Gibco) [46–49]. As laminin is a component of the extracellular matrix (ECM), its use as a culture substrate produces strong myocyte adherence by binding of β1-integrin surface receptors, which affects several cell functions [50–52]. Of note, integrin receptor signaling that may be induced by laminin binding is implicated in hypertrophic signaling [53, 54] and in developmental proliferation of myocytes [52]. Atrial myocytes plated on laminin exhibited significantly reduced NO-dependent rebound in $I_{Ca,L}$ following acetylcholine (Ach) withdrawal and enhanced β_2 along with reduced β_1 signaling [55, 56]. Some researchers favor gel substrates to promote a more normal cell morphology and function. These include agarose, collagen, or synthetic gels [57, 58].

The main considerations for choice of medium are buffering, ionic composition and nutritional supplements. The basic choice for culture of adult cardiac myocytes is sodium bicarbonate buffered Medium 199 (Cellgro M-199, Mediatech) [59], which maintains a pH of 7.3 to 7.4 in the standard incubator atmosphere of 5% CO_2. Commercially available M-199 has the composition (mM): $CaCl_2$, 1.8; NaCl, 116; Na acetate, 0.6; $NaHPO_4$, 1; KCl, 5.3; $MgSO_4$, 0.8 plus all amino acids except glutamine, and vitamins. The basic media is commonly supplemented to make ‘CCT’ (Creatine-Carnitine-Taurine) with (mM): creatine, 5; L-carnitine, 2; taurine, 5; Na_2HCO_3, 10; HEPES, 10 ([60]). For neonatal cells, basic media may be substituted with Dulbeco’s Modified Eagle Medium (DMEM) [31]. The pH

of the media is adjusted to 7.3–7.4 with NaOH, 100 I.U./mi penicillin and 100 μg/ml streptomycin added, filter sterilized (0.2 μm filter bottle-top filter, Corning) and stored at 4 °C for up to 2 weeks.

3.4. General methods

It is critically important to maintain asceptic conditions during all steps of culturing and isolation. A dedicated tissue culture room or part of a room is ideal, but at minimum a laminar flow hood equipped with vacuum, a water bath and an incubator that can keep cultures at 37°C and an atmosphere of 95% O_2 −5% CO_2 are required. All steps should be performed in the laminar flow hood, wearing lab gloves (latex or nitrile). All items brought into the hood should be sprayed with 70% ethanol (EtOH) before each entry. Fresh, sterile media is warmed to 37°C in the water bath prior to plating the cells. Prepare the dishes to be used by coating with the substrate chosen; generally laminin or fibronectin (see Appendix 4 for detailed methods). If glass coverslips are used, wipe with 70% EtOH and let air dry before coating. Coated dishes should rest in the incubator at least one hour before plating myocytes. Unused dishes may be stored by aspirating the coating solution, drying in the hood, and storing at 4 °C. Freshly isolated myocytes to be plated are brought into the laminar flow hood and counted with a hemacytometer designed for myocytes (Fisher cat. # 02-761-10; 0.1 mm depth). The remaining cells are allowed to settle, the solution aspirated, and media added to make the desired final concentration. For adult cells, Schafer et al have suggested a final dilution of 140,000 cells per 35 mm dish [61]. Studies in neonatal cells generally employ a similar plating density [31]. Plating at higher density often results in cell death or a hypertrophic growth response due to cell-to-cell contact [62]. Excessive coating agent (laminin, gelatin etc) is aspirated from the culture dishes, myocytes plated in media, and returned to the incubator for attachment (adult: 1–4 hr, neonatal: 24 hr). After attachment, non-attached, rounded cells are washed away using warmed media and the media replaced with fresh. If adenoviral infection is used, the virus is added at the time the media is replaced. Media is changed daily until the cells are used for experiments, typically electrophysiology, calcium fluorescence, or lysis for biochemical analysis.

3.5. Changes of adult myocytes over time in culture

With successful isolation, plating, and culture under sterile conditions, myocytes can be maintained in these conditions for up to 2 weeks. However, there is significant loss of total cell number (50–70% in one week [63, 64]) after even one day in culture, and cellular morphology begins to change in adaptation to culture conditions. The ends of the myocytes become rounded, the cells shorten, striations become indistinct, and t-tubule density is reduced [65, 66] (Figure 3), though there is recent evidence that this may be species-dependent [67]. In rat, intercalated discs are internalized as the cell ends become rounded [40]. Not only cell morphology, but also electrical and contractile properties are altered in adult cardiac myocytes in proportion to time in culture and with species [68]. Thus care must be taken in experiments involving alterations in gene and protein expression or long-term drug treatment to compare the results to controls cultured under matching conditions.

The functional changes that occur during culture in adult myocytes are well documented. The resting membrane potential can significantly depolarize after only one day in culture and continue to decrease over the next several days in association with decreased K^+ current density [40, 65]. Importantly, reductions in inwards rectifier and transient outwards K^+ currents can promote action potential (AP) prolongation, which can increase susceptibility to early after-depolarizations (EADs) [65, 69]. Diastolic Ca^{2+} levels in rat myocytes are relatively unaffected by culturing but peak systolic Ca^{2+} significantly decreases over the first 4 days. The loss of t-tubule organization and relative area is greatest during the first three days of culture, inducing reduced membrane capacitance and spatial disorganization of

SR Ca^{2+} release [65, 66, 70, 71]. Peak L-type Ca^{2+} current decreases, and channel localization becomes disrupted during culture, exacerbating the loss of synchronous SR Ca^{2+} release [65, 66, 68, 70]. After 4 days in culture, Na^{+}/Ca^{2+} exchanger (NCX) in rat myocytes appears to still be present in the sarcolemmal membrane, but it is unresponsive to stimulation by isoproterenol. In regards to intracellular signaling, cultured adult rat cardiomyocytes have a greater dependence on the inositol-3-phosphate receptor (IP3R) pathway of Ca^{2+} release, similar to neonatal myocytes, than do freshly isolated cells [72]. There are likely additional shifts in signaling pathways during culture that remain to be documented. When considering using a cultured cell experimental model, it is generally worthwhile to test the untreated myocytes for culture-related functional changes in the parameter to be measured before committing to a treatment protocol.

A number of methods are used to attempt to delay changes that occur during culture. Electrical pacing in culture maintains cell morphology and contractility, and experimentally can be used to determine biochemical changes directly related to the beating frequency [73, 74]. Treatment with drugs such as adrenergic agonists may be effective in maintaining cell contractile function but also stimulate hypertrophic and apoptotic pathways [75]. None of these can completely inhibit or reverse the adaptation of myocytes to culture conditions over time, thus shorter duration of culture and attention to correct control experiments are critical in obtaining physiologically relevant data from cultured myocytes.

4. Gene Transfer

In recent years, studies in isolated cardiomyocytes have increasingly employed various gene transfer technologies. These techniques are successfully utilized to investigate the mechanisms regulating myocyte structure and function, and targeted genes have included those involved with cardiomyocyte Ca^{2+} homeostasis, the contractile apparatus, the cytoskeleton, and signaling pathways. Gene delivery to cardiomyocytes is currently of interest as a treatment approach in cardiovascular disease, and targeted genetic alteration strategies established in single myocytes are now being investigated for their efficacy *in vivo*. For example, early work showing that viral-based transfer of the sarcoplasmic reticulum Ca^{2+} ATPase (SERCA) gene improved contractility in failing cardiomyocytes [76], has lead to an ongoing clinical trial investigating the therapeutic benefit of this technique in heart failure patients [77].

Successful gene transfer requires that the DNA encoding a protein of interest reach the nucleus of the host cardiomyocyte. This can be accomplished by *transfection*, a process by which DNA is administered in plasmid form (so-called “naked DNA”). Alternatively, DNA can be incorporated into the genome of a virus, which is then inserted into the host cell by a process known as *transduction*. While transduction allows for very efficient gene transfer [78, 79], virus work can be time-consuming, and there is evidence that viruses disrupt the physiology of the cell [80]. On the other hand, transfection techniques are generally non-toxic to cells, simple, and cheap, but result in much lower efficiency gene transfer and only transient expression of the gene. Thus, as will be described in the following sections, either technique may be appropriate for cardiomyocyte work depending on the experimental needs. Importantly, both transfection and transduction require that a relatively pure cardiomyocyte population be isolated and that cell integrity is optimally maintained during cell culture, using the methods described above.

4.1. Transfection

Adult cardiomyocytes that are fully differentiated are generally reported to be poor candidates for non-viral gene transfer. However, greater success has been achieved with transfection of neonatal cardiomyocytes using approaches such as liposomal reagents,

electroporation, and optical transfection. The calcium-phosphate co-precipitation method has also been applied in neonatal [81] and embryonic [82] cardiomyocytes. However, the very low transfection efficiency obtained by this technique (typically 1–2%) makes it impractical for most applications in cardiomyocytes, and the reader is directed elsewhere for a description of this method [81]. Direct needle microinjection may also be employed in cardiomyocytes (described in [83, 84]) when only a very small number of transfected cells is required.

4.1.1. Liposome transfection—Liposomes are readily able to fuse with cell membranes, since both consist of a phospholipid bilayer. Liposome transfection or "lipofection" exploits this capability as a means to introduce DNA into host cells. By this technique, a cationic lipid is used to form an aggregate with negatively charged DNA. These complexes have a net positive charge, and as they fuse with the anionic cell surface, DNA is delivered by endocytosis [85]. The low toxicity and relative simplicity of this technique has made it a popular method for gene delivery in non-cardiac cells, although it has also been employed in neonatal [32, 33, 86] and embryonic [87] cardiomyocytes. Transfection rates up to 15% have been reported using lipofection in primary muscle cells [31], although greater success is possible using cell lines such as HL-1 immortalized cardiomyocytes [88].

A broad range of liposomal reagents is commercially available. Popular examples include Lipofectamine 2000 (Invitrogen), Metafectene (Biontex), GenePORTER (Genlantis), and DreamFect (OXBiosciences). These products are typically mixtures of synthetic cationic lipids with other "helper lipids" such as DOPE (dioleoyl phosphatidyl-ethanolamine), which de-stabilizes the endosome following endocytosis, allowing release of the DNA into the cytoplasm [89]. Although specific manufacturer's instructions vary the basic method for lipofection is generally straight, as outlined in Appendix 6.

4.1.2. Electroporation and nucleofection—An alternative transfection strategy is electroporation, also known as electrotransfer or electropermeabilization. With this technique, brief electric pulses are employed to increase the permeability of the cell membrane, and allow uptake of large molecules such as plasmid DNA [31, 90], small interfering RNA (siRNA), and antibodies [84, 91]. Recent work has suggested that this technique may be fine-tuned for application to adult cardiomyocytes [92]. However, at present electroporation has most successfully been employed in neonatal [31, 90, 93] and embryonic [84, 91] cardiomyocytes. Djurovic *et al* have reported transfection rates of 7.5% with this technique [31]. However, greater transfection success rates have been obtained with "nucleofection", which combines electroporation with solutions that stabilize the cells in the electric field [93–95]. Gresch *et al* observed that 37% of cardiomyocytes were transfected by nucleofection with plasmid DNA [94], and Frias *et al* reported that nucleofection with siRNA reduced target gene expression by an impressive 70% [93].

The electroporation technique is well liked by many as it is simple to conduct and affordable, although it does require the purchase of an electroporator. Several varieties are commercially available which are suitable for use with cardiomyocytes, including the ECM 630 (BTX, San Diego, USA), the GIBCO-BRL (Gaithersburg, MD), and the Nucleofector device (Amaxa). The precise voltage, resistance, and capacitance settings on the electroporator are recommended by the manufacturer, but can be independently optimized for highest transfection levels. For example, Djurovic *et al* have reported that optimal settings for neonatal cardiomyocytes with a nucleofector (Amaxa) are: voltage = 360 V, resistance = 725 Ohm, and capacitance = 50 μF [31]. A basic protocol for electroporation is outlined in Appendix 6.

4.1.3. Optical transfection—Optical transfection employs *optoporation*, whereby focused laser light generates a transient pore in the cell membrane through which DNA may enter. An advantage of this technique is that cells can be individually selected through the microscope, which makes it feasible even in an impure cell population. Although first described by Tsukakoshi et al. in 1984 [96], optical transfection has not yet been widely applied in cardiomyocytes. However, Nikolskaya *et al* have shown that neonatal cardiomyocytes can be transfected by this technique with 5% efficiency, and that this can be conducted with the argon laser (488 nm) of a standard confocal microscope [97]. The laser is typically focused on the cell membrane, and laser absorption is believed to result in a local rise in temperature and a reversible increase in membrane permeability [98]. Phenol red, which is a common component of cell culture medium or can be added, is believed to aid in the absorption of light at this wavelength (488 nm). The membrane dye FM 1–43 (Invitrogen) may further enhance laser light absorption [97]. Since it only fluoresces when incorporated into the cell membrane, this agent may additionally serve to indicate successful membrane poration [97]. A suggested optical transfection protocol is outlined in Appendix 6. If low cell viability is observed, laser power and exposure time can be reduced. Myocytes may additionally be pre-treated with agents known to protect against apoptotic damage and promote membrane resealing (ie. cyclosporin, ROS scavengers, surfactants) [97].

4.2. Transduction

In comparison with the transfection methods described above, viral-based techniques enable much more efficient and stable gene transfer. Thus, this method of gene delivery is thought to hold great therapeutic promise for targeting genetic expression in the heart. Viral-based techniques have also been shown to be reliable for *in vitro* use, and are well suited for efficient gene delivery to either neonatal or adult cardiomyocytes. These *in vitro* techniques are outlined below, while the reader is directed elsewhere for a review of *in vivo* methods in cardiac gene delivery [99].

Three families of viruses have been employed for transduction of cardiomyocytes; adenovirus, adeno-associated virus, and retrovirus. Before selecting a virus for *in vitro* use, it is important to consider the advantages and disadvantages of each type as discussed below.

4.2.1. Adenovirus transduction—*Adenoviruses*, which are non-enveloped and contain linear double-stranded DNA, can be easily manipulated and will accept large inserts up to 35 kilobases in size [100]. Adenoviruses can infect host cells with very high efficiency and are capable of generating high levels of gene expression [78]. A multitude of studies have employed adenoviruses for gene transfer in neonatal and adult cardiomyocytes (for examples, see [101–104], and infection efficiency has been reported to be as high as 100% for both cell types [103]. Adenoviral techniques result in relatively brief (2 weeks [105])) gene expression since the transgene is not integrated in the host genome. While this brief expression time limits the use of adenoviruses for many therapeutic applications, this is normally not of concern on the time-scale of *in vitro* work. Importantly, adenoviruses typically elicit a significant inflammatory and immunogenic response, but these can generally be controlled for by parallel experiments with empty vector. Alternatively, the inflammatory response can be reduced by the use of next generation or “gutless” adenoviruses that do not express the virus gene product [106].

Methods in construction, propagation, titration, and purification of recombinant adenoviruses are beyond the scope of this review, but thoroughly described elsewhere [107]. Once viral stocks are prepared, the appropriate virus concentration should be calculated based on known cell numbers and the desired multiplicity of infection (MOI), ie. the number

of virus particles per cell. Efficient infection of adult cardiomyocytes can generally be attained with an MOI of 100 [108–110]. Similar MOI values can be employed in neonatal cells [108, 111], although efficient infection may also be possible at lower virus concentration (MOI = 10–20 in [112, 113]). Alternatively, a dose response curve can be conducted with a range of MOI to determine optimal values [103]. Efficiency of gene transfer is typically assessed by monitoring reporter genes such as green fluorescence protein (GFP) or β-galactosidase (LacZ) that are included in both the control vector and as a fusion gene with the insert in the test vector. A basic protocol for adenoviral infection is described in Appendix 6.

4.2.2. Adeno-associated virus transduction—*Adeno-associated viruses (AAV)* are non-enveloped, single-stranded DNA viruses with defective replication. They can readily infect cardiac cells resulting in stable gene expression for up to one year [114], and since they are not known to cause any human disease, very little immune response is triggered [100]. For these reasons, AAV are considered excellent candidates for use in gene therapy in humans [106]. AAV has also been employed for gene transfer in isolated cardiomyocytes [31, 115, 116], although to a much lesser extent than adenovirus. However, AAV is well suited to studies employing a combination of *in vitro* and *in vivo* gene transfer experiments, as it may be desired to use the same vector in both cases [115, 116]. One drawback of AAV is that the vector capacity is limited to 4–5 kilobases, making it unsuitable for transfer of very large genes [106]. As with adenovirus, AAV transduction is generally reported to be highly efficient (90% [31, 116]), although a relatively high MOI may be required (10,000 in [31, 116]). Techniques for AAV transduction are otherwise similar to those for adenovirus (Appendix 6).

4.2.3. Retrovirus/Lentivirus transduction—*Retroviruses* are RNA viruses that require reverse-transcription into DNA by the host cell. These viruses generally require host cell division so that the DNA may then be integrated in the nucleus. However, *lentiviruses*, which are a genus of the retrovirus family, can multiply in non-dividing cells [117], making them suitable for use in adult [80, 118] as well as neonatal [80, 117–119] cardiomyocytes. Importantly, lentiviruses do not induce inflammatory and immune responses, and can induce long duration expression by incorporating the transgene into the host genome [100]. These viruses can accommodate a relatively large (up to 18 kb [120]) insert, which makes them better suited for the transfer of large genes than AAV. Unfortunately, traditional lentiviral gene transfer is hindered by poor nuclear translocation [121], which likely explains why these viruses have not been more widely employed in isolated cardiomyocyte experiments. However, this problem has been largely overcome with the advent of “advanced generation” lentiviruses, which are based on a modified HIV genome [80, 117, 118, 121]. In these specialized lentiviruses, greatly improved gene transfer efficiency is reported with infection rates of 70% in adult cardiomyocytes and virtually 100% in neonatal cardiomyocytes [80, 119]. Lentiviral transduction techniques are similar to those for adenovirus and AAV.

5. Conclusion

Isolated cardiomyocytes from small rodents are an invaluable tool in the study of cellular level function and regulation of electrophysiology, intracellular calcium fluxes, contractile mechanics and protein expression. The ability to culture myocytes, even for short times, further benefits researchers by permitting the incorporation of transgenes into cells and facilitating longer-term treatments. Combined with the recent availability of transgenic animals, the array of techniques for manipulating cellular physiology in the investigation of molecular mechanisms regulating function has vastly expanded.

Although myocytes have been isolated and cultured for a number of years by standard techniques, it remains somewhat of an art to consistently produce high-quality cells. Knowledge of the described reagents and protocols, along with common caveats and critical points in the procedures will enable researchers to more easily establish cell-based research strategies, and advance the pursuit of therapeutics for human disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Research Highlights

- Successful experimentation with single cardiomyocytes requires quality cell isolation
- Gene transfer additionally requires maintenance of cell quality during culture
- Techniques for cardiomyocyte isolation, culture, and gene transfer are reviewed

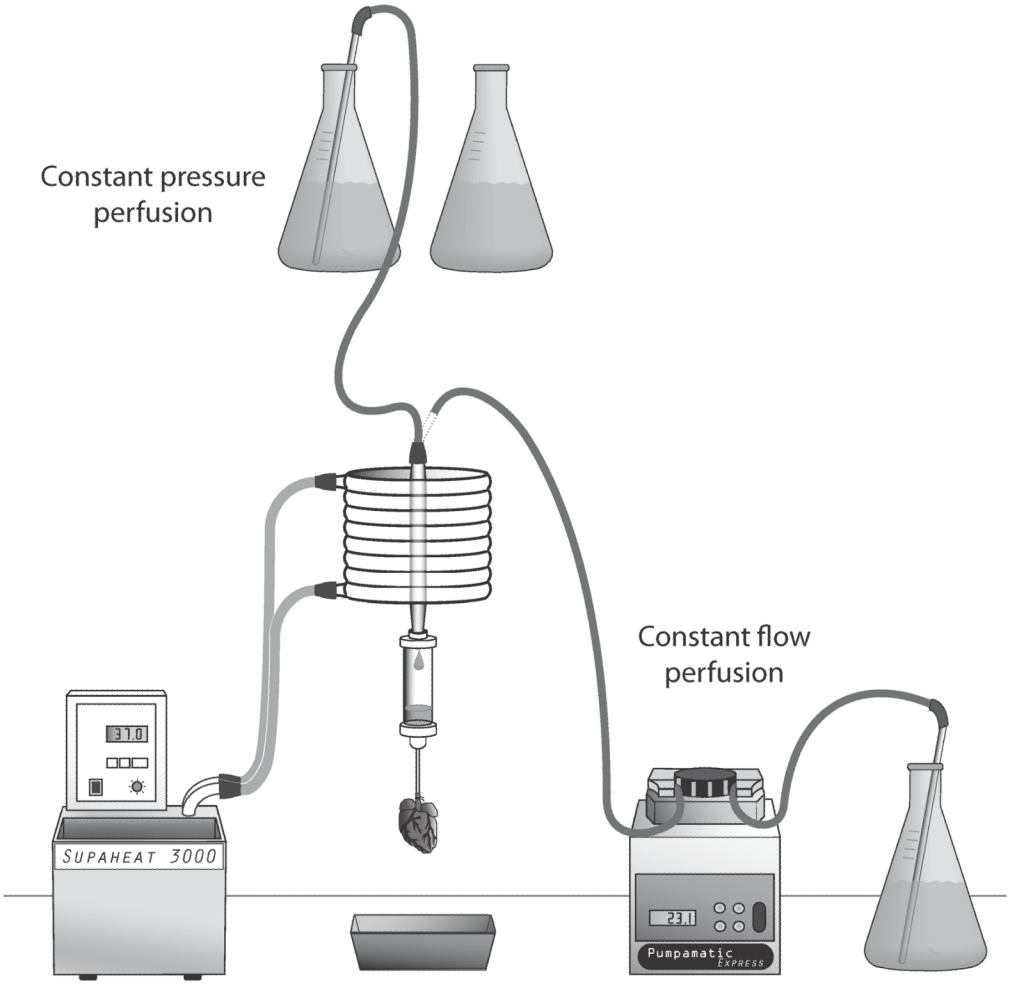

Figure 1. Apparatus for ventricular cardiomyocyte isolation

Myocytes can be reliably isolated using a Langendorff setup, with cannulation of the aorta and retrograde perfusion of the heart with enzyme-containing solutions. One isolation method is to use constant pressure perfusion, where solutions are suspended above the apparatus and gravity fed to the heart. Alternatively, constant flow perfusion can be employed by pumping the perfusate (shown at right, connection by dotted lines). By both methods, the perfusate may be heated, often to 37°C, by inclusion of a heating coil connected to a water bath (shown at left). Inclusion of a drip chamber can be included to prevent air bubbles from reaching the heart. The heart is typically fixed to the cannula with a silk suture.

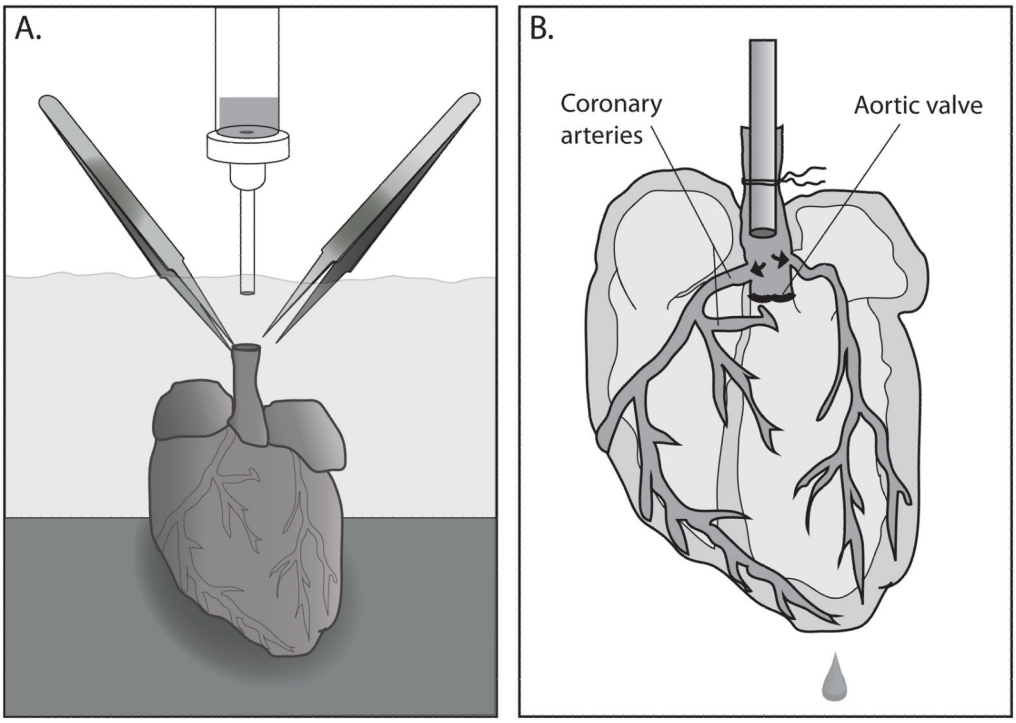

Figure 2. Method for cannulation of the aorta and perfusion of the coronary arteries

A) The heart and tip of the cannula are immersed in low-Ca^{2+} solution. Using fine-tipped forceps to grip the sides of the aorta, the heart is then cannulated and secured by silk thread tied around the aorta. **B)** Following proper cannulation, the aortic valve closes when perfusion is initiated and the perfusate is forced through the coronary arteries. Placing the cannula too deep in the aorta and through the aortic valve will prevent adequate perfusion of the coronaries and cardiomyocyte isolation will not be successful.

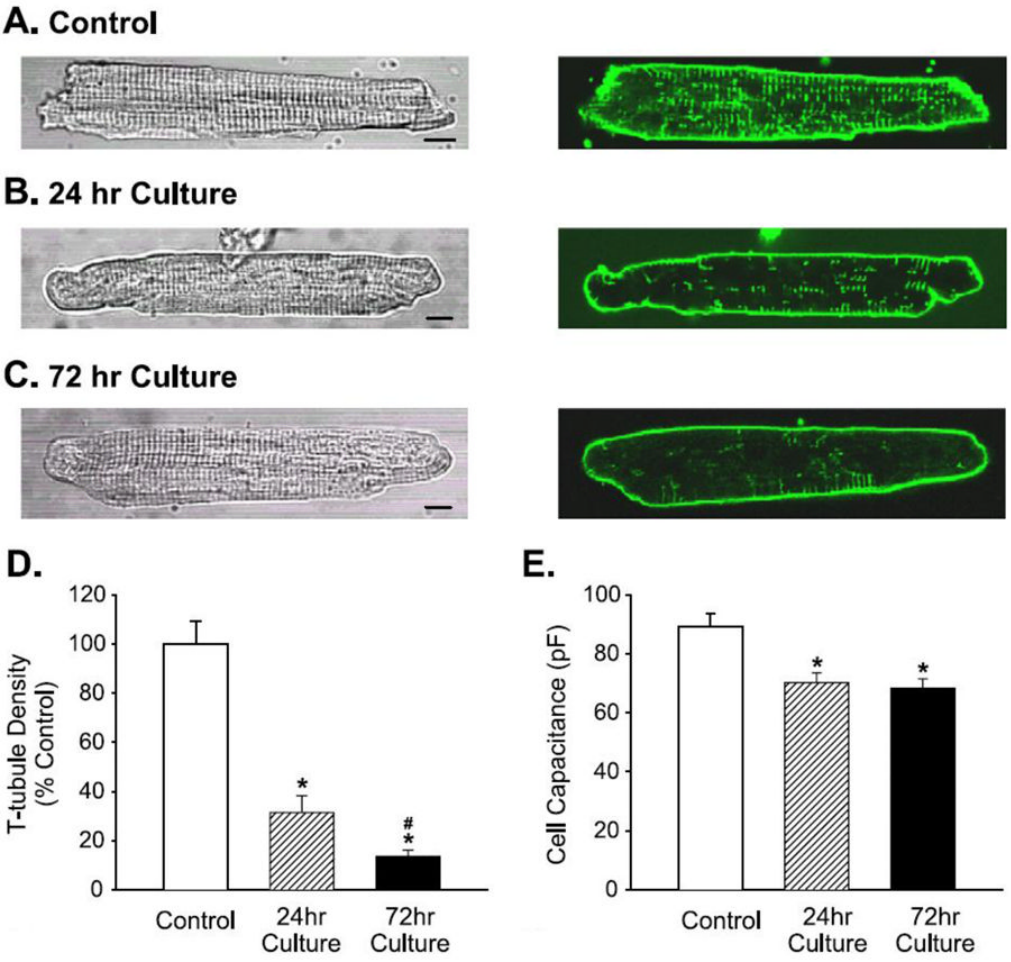

Figure 3. Typical alterations in cardiomyocyte morphology during cell culture

(A–C) Light images **(left panels)** show that myocytes become rounded at the ends with time in culture. T-tubule density is also rapidly and progressively reduced as indicated by confocal cross-sectional images of di-8-ANEPPS stained cells **(A–C, right panels)** and mean density measurements **(D)**. T-tubule loss results in a reduction in cell surface area, reflected as a measured decrease in cell capacitance **(E)**. * denotes $P<0.05$ vs. control, # denotes $P<0.05$ vs 24 hr culture. Scale bar = 10 μm. Reprinted from [65] by permission.