Video Article

Isolation, Culture, and Functional Characterization of Adult Mouse Cardiomyoctyes

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Abstract

The use of primary cardiomyocytes (CMs) in culture has provided a powerful complement to murine models of heart disease in advancing our understanding of heart disease. In particular, the ability to study ion homeostasis, ion channel function, cellular excitability and excitation-contraction coupling and their alterations in diseased conditions and by disease-causing mutations have led to significant insights into cardiac diseases. Furthermore, the lack of an adequate immortalized cell line to mimic adult CMs, and the limitations of neonatal CMs (which lack many of the structural and functional biomechanics characteristic of adult CMs) in culture have hampered our understanding of the complex interplay between signaling pathways, ion channels and contractile properties in the adult heart strengthening the importance of studying adult isolated cardiomyocytes. Here, we present methods for the isolation, culture, manipulation of gene expression by adenoviral-expressed proteins, and subsequent functional analysis of cardiomyocytes from the adult mouse. The use of these techniques will help to develop mechanistic insight into signaling pathways that regulate cellular excitability, Ca^{2+} dynamics and contractility and provide a much more physiologically relevant characterization of cardiovascular disease.

Video Link

The video component of this article can be found at https://www.jove.com/video/50289/

Introduction

Murine models of cardiovascular disease have served as effective tools for elucidating fundamental disease mechanisms[1,2] as well as for identifying potential therapeutic targets[1,3]. In particular, the use of both murine models of acquired heart disease (such as pressure-overload)[4,5] and transgenic mouse models have advanced our understanding of heart disease[6-8]. The use of cell culture techniques to study signaling cascades[3,9,10] and alterations in individual proteins that underlie cellular excitability and excitation-contraction coupling in the heart[11-13] at the level of the single cell have complemented the *in vivo* mouse models. However, the lack of adequate cell lines that reflect adult CM structure and function has been a significant limitation. Investigators have sought to overcome this by studying individual proteins, such as ion channels, in heterologous expression systems[14], and while these studies have provided us with useful information in terms of ion channel biophysics or protein trafficking, inadequate representation of the native microenvironment of CMs is a significant limitation. Secondly, since most of these heterologous cells do not have a mature contractile apparatus, it has not been possible to study contractile function and the complex interplay between cellular excitability and contraction. For this reason, researchers have turned to primary cardiac cell cultures for many of their *in vitro* functional studies. Finally, isolated cardiomyocyte studies allow assessment of contractile function without the confounding factors of multicellular preparation including the effect of scar or fibrosis and fiber orientation.

Primary neonatal rat ventricular cardiomyocytes (NRVMs) are relatively easy to culture, can be infected with adenoviruses and lentiviruses to manipulate gene expression[15], and have therefore been used successfully[1], but have limitations of their own. Although they provide a physiologic microenvironment[1] and have been the workhorse of the signaling field, substantial differences between the morphology and subcellular organization of NRVMs and adult cardiomyoctyes make them an inadequate model for the investigation of ionic fluxes and excitation-contraction coupling in the adult heart. Most notably NRVMs lack a definitive t-tubular subsystem[4]. Since Ca^{2+} flux and dynamics are critically dependent on mature t-tubular and sarcoplasmic reticulum (SR) structure[6], Ca^{2+} dynamics and functional studies of the cardiac contractility in NRVMs are not an accurate reflection of these critical processes in adult cardiomyocytes. Further, some components of signaling pathways differ between neonatal and adult mice[9], thereby providing another limitation for studying disease processes and their impact on cellular excitability and contractility in NRVMs. Finally, the distribution of the contractile machinery leads to multidirectional and non-uniform cell shortening limiting the accuracy of the contractile measurements.

The use of isolated adult cardiomyocytes provides therefore a more accurate *in vitro* modeling system. The extraordinary growth of knowledge made possible by the genetic manipulation of mice underlines the significance of obtaining functional isolated cardiomyocytes from mice. In fact, the characterization of adult CMs isolated from mouse models has shed light on many biological and pathological events. Isolated CMs from transgenic mouse models have allowed for studies of the gain or loss of function of proteins on the contractile properties of single cells[2,16], and viability in disease models such as ischemia/reperfusion[17,18], thereby complementing information gained from *in vivo* studies on these mice. Use of isolated adult CMs from murine models of acquired heart disease[3,19,20] (such as transverse aortic constriction-induced pressure overload, that mimics hypertension or aortic valve stenosis) or exercise[5,21] (for modeling physiological hypertrophy) allows for examination of the interaction of signaling cascades implicated in these processes with cellular excitability and excitation-contraction coupling at the level of the single cell. Furthermore, the ability to manipulate gene expression using adenoviral-driven gene expression in adult CMs affords us the opportunity to dissect the components of complex signaling pathways.

From an electrophysiological perspective, whole-cell voltage and current clamp experiments on isolated adult CMs have been critical in elucidating the nature of ionic fluxes at baseline and in various disease states. Because of the complex structure of the cellular membrane and the differential protein scaffolding structures between adult CMs and NRVMs or heterologous cell lines, the ability to patch adult cells gives a much better representation of the effects of certain membrane proteins, structural proteins, and ion channel interacting partners on the electrophysiological components of the adult heart.

Despite such prominent advantages in studying adult murine cardiomyocytes, isolating and culturing adult mice cardiomyocytes has been challenging, urging the need for a systematic and accurate description of the methodology to isolate viable mouse cardiomyocytes and to maintain them in culture to allow further genetic manipulation using viral vectors. Previous studies have used either acutely isolated mouse adult CMs or cultured rat adult CMs. The latter are easier to culture than adult mouse CMs, and most experiments manipulating gene expression *in vitro* have used rat adult CMs. Few studies have successfully altered and investigated functional gene expression in mouse adult CMs, presenting a large limitation in the scope of experiments. Therefore, here we present in detail such methodologies, modified from previous investigations, for the isolation[7,8,22,] culture[3,10,15,23], adenoviral infection[11-13,15], and functional analysis of adult mouse ventricular cardiomyoctyes. This isolation protocol results in Ca^{2+}-tolerant, excitable cardiomyocytes that we have successfully cultured for up to 72 hr and transiently transfected with adenovirus. The functionality of these isolated cells can be assessed using the MMSYS imaging system[14,24] and patch clamp, which will also be discussed.

Protocol

1. Cardiomyocyte Isolation

Materials (Figure 1)

Microdissecting forceps
Tissue forceps
Delicate hemostatic forceps
Hemostatic forceps
Microdissecting, serrated, curved forceps
Operating scissors, straight
Operating scissors, curved
15 ml Falcon tubes (5)
60 mm Petri dish
Phosphate Buffered Saline (PBS)
Nylon Mesh - 400 µm pore size
Small funnel
Wax coated, braided silk 4-0, 19 mm, 7-10 cm long
Non-hypodermic needle, blunt end, 24 G or commercial animal-feeding needle (24 x 1 in, W/1-1/4)
Heparin sodium
Ketamine/xylazine mixture
Pasteur Pipettes
OptiVision Dissecting Goggles
IonOptix MMSYS system

Note: If culturing cells, see section 2 on cell culture before beginning this protocol.

Note: All mice were cared for in a barrier facility and sacrificed according to approved IACUC regulations, practices, and procedures.

*Note: Prior to beginning the procedure, the entire system should be preheated to ensure that all the elements of the Langendorff system (**Figure 2A**, **Figure 3**) are warmed to 37 °C to allow for proper and complete collagenase activity.*

1. Inject adult mice (~12 weeks) with 150 USP units heparin sodium into the abdominal space.
2. Anesthetize with an injection of a ketamine/xylazine mixture at a weight-dependent dose (**Table 1**).
    1. 80:12 mg/kg ketamine:xylazine recipe: 2.6 ml ketamine (100 mg/ml) + 0.4 ml xylazine (100 mg/ml) + 37 ml PBS. Final: ketamine = 6.5 mg/ml; xylazine = 1 mg/ml
3. Secure the sedated mouse to the operating pad, excise the heart, and place into a dish of room temperature PBS or 0.9% saline (**Figure 1J**). Physiologic saline allows the intact heart to contract for better extrusion of blood in the chamber and easy identification of the aorta prior to

step 1.6. Mice were checked to ensure that they are deeply anesthetized via loss of hind limb toe pinch reflex and slowing of respiratory rate prior to onset of thoracotomy.

4. Use OptiVision dissecting goggles (**Figure 1A**) to identify and expose the aorta. Removing the tissue around the aorta, although it does facilitate the visualization of the vessel, is not necessary for the optimization of the cell isolation if the aortic root is clearly visible.
5. Prime the pump with perfusion buffer (**Table 2**). Temperature should be held at 37 °C for the length of the procedure using a controlled, circulating water bath (**Figure 2D**).
6. Mount the heart onto the Langendorff Apparatus (**Figure 2A**). Using two micro-dissecting forceps (**Figures 1D-E**), prime the aorta around the non-hypodermic, blunt end needle (24 G) with silicone tube at the distal tip. Alternatively a commercial animal-feeding needle (24 x 1 in, W/1-1/4) can be used. The silicone tube on the 24 G needle or the animal feeding needle will allow for securing the suture over the groove. Place the aorta on the needle as a sock. (**Figure 2C**).
    1. Note: It is important to be sure that the end of the needle rests in the ascending aorta, ideally in the aortic root distal to the right innominate, but that it does not extend through the aortic valve into the left ventricle, as this will severely limit the ability of the buffers to effectively perfuse through the heart via the coronary arteries.
7. Secure the heart to the needle by tying a small length of suturing silk (7-10 cm long) (**Figure 1K**) around the top of the aorta, ensuring that the tie is at the level of the ascending aorta, below the right innominate artery, but is above the end of the needle.
8. Lower the heart into the interior of the conical glass (**Figure 2B**) to help maintain a suitable ambient temperature.
9. Perfuse the heart with perfusion buffer for 5 min at a rate of 1 ml/min and clamp the glass chamber outflow to allow the perfusate to collect in the conical glass and envelope the heart. The schematic for digesting the heart is shown in **Figure 3**.
    1. At this point you should see the volume of the heart increase. Additionally, blood in cardiac tissue will be replaced by perfusate, causing tissue pallor.
10. Release the outflow clamp to drain the perfusate. Stop the pump to move the tubing from the perfusion buffer container to the enzyme buffer container (**Figure 2E**). Restart the pump to begin to perfuse the heart with enzyme buffer (**Table 2**) at 1 ml/min. Clamp the outflow again to allow the enzyme buffer to envelope the heart.
    1. Here, as the enzyme is perfusing the heart and digesting the connective tissue, the heart will become paler and the structural integrity will diminish.
    2. To determine when to cut the ventricles from the digested heart, lift the heart out of the conical glass, gently squeeze the heart with forceps and collect a few drops of perfusate in a small Petri dish and check to see whether or not single cells are dropping.
    3. For the novice it is helpful to connect the perfusion line with a manometer. When the heart is getting digested the pressure will rapidly decline, as the connective tissue does not hold the resistance. The manometer is also useful for the novice to ensure that the position of the needle is above the aortic valve and not in the ventricle. Low pressure will indicate that the needle is in the ventricular chamber and high pressure indicated that the needle touches the valve.
11. Cut the ventricles from the heart into a dish full of transfer buffer (A) (**Table 2**) when the ventricular pressure begins to drop dramatically or when you are able to see single cells in the perfusate. This usually takes ~7-10 min.
12. Again using micro-dissecting forceps (**Figure 1C**), mince the ventricles into small pieces to dissociate. A successful digestion will leave almost no solid chunks of tissue after mincing, with most of the tissue becoming amorphous upon dissociation.
    1. To further dissociate the tissue, pipette the solution in/out using a plastic Pasteur pipette (**Figure 4**) from which the tip had been cut at an ~45° angle. This modification serves to increase the inner diametric space of the pipette tip thus decreasing the amount of shear stress on the cells as the tissue is triturated.
    2. Before dissecting the tissue with the forceps it is possible to separate the individual chambers and separately dissociate atrial, left ventricular or right ventricular cells.
13. Secure the squared mesh (**Figure 1M**) to a small funnel (**Figure 1L**) using clamps and place this filtering apparatus into a 15ml Falcon tube (**Figure 1I**).
14. Use the modified Pasteur pipette to remove the cell solution from the dish and filter the solution into the Falcon tube.
15. Allow the live cells to settle to the bottom of the tube (live cell sedimentation should take 2-4 min).
16. Aliquot 2.5ml of each of the four calcium solutions (**Table 3**) into 4 separate 15ml falcon tubes.
17. Again using a standard Pasteur pipette, carefully remove the cell pellet from the bottom of the tube and transfer the cells to the first of the calcium solutions.
18. Allow the cells to settle to the bottom of the tube and repeat step 1.17 in the subsequent calcium solutions until the cells are in the last solution.
19. Do not let the cells settle in the fourth calcium solution. Cap the tube and turn it on its side.

2. Cell Culture

1. Before the isolation, plate 1 ug/ml natural mouse laminin onto glass coverslips and incubate for at least 1 hr at 37 °C and 2% CO_2. Also allow your plating and culture media (**Table 4**) to warm in the incubator at 37 °C and 2% CO_2. This allows the dissolved CO_2 in the media to equilibrate.
    1. Do not remove the top from the media. Simply loosen the top to avoid contamination.
2. Allow the isolated cells to pellet at the bottom of the Falcon tube.
3. Remove the pellet using a standard plastic Pasteur pipette and resuspend in the appropriate amount of plating media.
4. Plate the cells on the laminin-coated coverslips in the appropriate sized dish and incubate in 37 °C and 2% CO_2 for at least 30-60 min to allow the cells to adhere to the coverslips.
5. **If transfecting with adenovirus, dilute the virus in an appropriate amount of plating media and replace the plating media in the dish with the virus-containing media. Let the virus incubate on the cells for 2 hr in 37 °C and 2% CO_2.**
6. Remove the plating media from the dish and replace with culture media.

7. Carefully change the culture media each day so as not to disturb the cells on the coverslips.

Using this procedure, cells have been successfully cultured for up to 72 hr. Images of cultured and GFP transfected cells can be found in **Figure 5**.

3. MMSYS System

1. Power the MMSYS system ensuring that the arc lamp is initiated first.
2. Connect the tubes from the pump to the appropriate inlet and outlets of the MMSYS chamber (**Figure 6**).
3. Prime the system with calcium buffer (B) (**Table 2**).
4. Place an appropriately sized glass coverslip into the chamber and fasten (**Figure 6E**). Most MMSYS chambers use either 22 mm or 25 mm square coverslips. Consult your MMSYS user manual to determine the appropriate coverslip for your chamber.
5. Transfer 500 µl of the cell solution to a small Eppendorf tube.
6. Add 0.5 µl Fura2-AM (stock solution of 1 µg/µl) to the small tube of cells and allow them to incubate in the dark at RT for 5-7 min. This allows the Fura2-AM to "load" into the cells through rapid passive diffusion through the cell membrane.
    1. Let the Fura-2 loaded cells pellet to the bottom of the tube, and wash the excess Fura with 500 µl of calcium buffer B.
7. Turn off the room lights.
8. Using a standard Pasteur pipette, drop 1-2 drops (depending of the cell concentration) of the "loaded" cell solution into the center of the chamber (**Figure 6C**) and allow the cells to settle onto the coverslip for 5 min.
    1. The density of the cells in the chamber should be such that single non-overlapping cells can be easily viewed in the MMSYS data acquisition platform.
9. Carefully begin to flow calcium buffer (B) through the chamber.
10. Increase the chamber temperature to 37 °C.
    1. IMPORTANT: Do not turn on the heating apparatus until the flow has been initiated. This can severely damage the feedback thermocoupler (**Figure 6A**).
11. Make sure that the chamber is connected to the MyoPacer via the connection wires (**Figure 6B**) and begin to pace the cells 5 Hz at 1.5x the pacing threshold for the cells.
12. Choose a cell that is beating at the correct frequency and move it into the framing aperture.
13. Adjust the camera and framing aperture dimensions so that the entire cell is in the center of the window, directed horizontally, with the sarcomeres apparent.
    1. For cell length adjust the red and green (right and left) photodiode indicators to the edge of the cell. Adjust the contrast to optimize the black/white contrast at the edges of the cell. Refer to the Ionoptix manual for further details.
14. Place the box in an area of the cell containing well-defined sarcomeres and adjust the focus to optimize the peak of the power spectrum (red). Also adjust the brightness of the microscope to optimize the blue smoothing window and black contrast information.
15. Adjust the green threshold bars to capture as much of the red power spectrum (peak) and as little background as possible.
16. Begin recording.
17. After enough data has been recorded, click "Pause" to temporarily stop recording and, ensuring that neither the focus nor dimensions of the viewing window are altered, move the microscope's stage to an area of the coverslip in which no cells or cellular material can be seen. Length of recording varies depending on the investigator's experimental protocol.
    1. Note: Clicking "Stop" will terminate the recording and no background will be able to be recorded for that cell.
18. Click "Resume" to record a background measurement.
19. After enough background has been recorded click "Stop" to terminate the recording.
20. Click "File >> Save" to save all the traces for that cell in a single .zpt file.
21. Repeat steps 3.12 - 3.20 until enough cells have been recorded.
22. Consult the IonOptix-MMSYS User Manual 12 for instructions on analysis of the recorded data. Characteristic recorded data of wild-type cardiomyocytes are shown in in **Figure 7**.

4. Patch Clamp

Patch clamping of different cell types have been well described previously in this journal[25-28]. We therefore focus on some critical parameters for successful patch clamping of adult cardiomyocytes isolated using our protocol described.

1. Using a pipette puller and borosilicate glass (with filament: O.D. 1.5 mm, I.D. 0.86 mm - 10 cm length) pull a pipette that exhibits ~2.0-3.0 MΩ when filled with pipette solution (**Table 5**).
2. Fire-polish the pipette tip gently using a Narishige or other vertical fire polisher. The resistance of the patch pipette should be 2-4 MO after fire polishing.
3. Turn on the computer, amplifier (Axopatch 200B), A-D converter (Digidata 1440A, Axon Instruments) and Micromanipulator (MP-285). For whole cell patch clamping, we start with standard parameters as previously described.
4. For cultured adult CMs, remove them from the incubator and gently wash the coverslip on which the CMs are plated with PBS at room temperature.
5. Gently remove the coverslip and place it in the perfusion chamber on the inverted microscope (Nikon TE 2000).
    1. Ensure that the inlet for the perfusion system and outlet (for suction) are just above the surface of the coverslip.
6. Start perfusing with the appropriate extra-cellular solution (**Table 5**).
7. For freshly dissociated adult CMs, place a collagen-coated coverslip in the perfusion system as above.

1. Since the cells will be in solution, gently replace the solution with extracellular buffer.

8. Using the modified Pasteur pipette (**Figure 4**), gently resuspend the cells in the extracellular buffer, take an aliquot and place it on the coverslip.
9. Let the cells attach for 10-15 min before starting the perfusion as in 4.4.
10. Back-fill the whole cell patch pipette with intra-cellular buffer using a Microfil (World Precision Instruments, 28 G) needle. Ensure that there are no air bubbles in the tip of the pipette; gently tap to allow the air bubbles to float to the surface of the solution.
11. Attach the filled patch pipette to the pipette-holder, ensuring that there is contact between the microelectrode and the internal solution.
    1. We use silver chloride electrodes, but take care of 'chloriding' the silver wires at least once a week by immersing overnight in household bleach.

12. Gently lower the pipette into the bath, ensuring that the bath electrode is immersed at all times in the bath/extra-cellular solution. Offset any liquid junction potentials at this time. Large junction potentials may be a sign of deteriorating silver chloride on the electrodes.
13. Healthy cylindrical adult CMs are identified in the microscope and centered in the field of vision. As previously described, a combination of moving the micromanipulator and adjusting the focus allows for close proximity of the patch pipette and the surface of the CM.
    1. Once they are in the same focal plane, we use a combination of visualization of the cell and test pulse (available in the Axopatch 200B).
    2. Once the resistance of the pipette decreases in half (suggesting that the pipette is in contact with the surface of the cell), and the cell continues to look cylindrical, we establish gigaohm seal using gentle suction.
    3. For CMs, we prefer mouth suction to establish negative pressure. If a gigaseal is successfully obtained, we again use gentle rhythmic mouth suction to 'break-in' to the cell to establish whole cell patch mode.

14. Typical resting potentials of healthy CMs are in the order of -85 to -90 mV. Once a gigaseal is obtained, measure the resting membrane potential using current clamp with I=0 pA.
    1. If the resting membrane potential is depolarized this can indicate a damaged cell or a poor seal.

15. Because the CMs are large cells with a lot of surface area, careful attention needs to be paid to capacitance compensation, especially when rapidly activating currents such as the sodium channel needs to be studied. If adequate capacitance compensation cannot be obtained using the built in compensation settings on the pulse generator, prepulses at sub-threshold voltage and opposite polarity may have to be applied and the resultant current subtracted from the recorded signal. Such a protocol is easily programmed in Axopatch.
16. Once whole cell patch mode has been established, wait 15 min to allow for equilibration and to ensure stability of the seal prior to beginning voltage or current clamp protocols. We use standard protocols that have been described previously in this and other journals and will not be elaborated upon.

Representative Results

The isolation of adult cardiomyoctyes results in rod-shaped, striated, and quiescent (not spontaneously beating) cells (**Figure 5A**). Dead cells will look rounded and no striations will be present. Quiescent cells can be cultured and transfected with adenovirus to manipulate gene expression (**Figures 5B** and **5C**). After 24 hr of culture, the morphology of the live cells does not change, they are still Ca^{2+}-tolerant, and they can be paced by field stimulation. With the proposed procedure, a yield of 80-90% viable cells can be achieved.

To measure the cell's contractility, the MMSYS system software takes the phase information from the microscope to report either the changes in sarcomere or cell length. Shown in (**Figure 7A**) is a representative contractility trace via sarcomere length measurement of a wild type C57BL6 mouse. For healthy adult mouse myocytes from these mice, the baseline sarcomere length is ~1.7-1.9 µm. The MMSYS system software is capable of simultaneously measuring contractility and calcium handling by measuring the ratio of the bound to the unbound forms of the calcium-activated dye Fura2-AM. While the ratio measures may vary slightly between setups, for healthy cells, the ratio is typically between 1.5-2.5 (**Figure 7B**). If the MMSYS system has been calibrated for calcium, this ratio can then be translated into an absolute measure of intracellular calcium concentration. In order to analyze the data that are generated from the contractility and calcium traces, the traces must be averaged to create a characteristic trace for that cell (**Figures 7C** and **7D**). Those characteristic traces are then subject to a monotransient data analysis algorithm from the MMSYS system software which then generates parameters for systolic and diastolic function. Further details are present in the Ionoptix manual.

Adult mouse cardiomyocytes were also subject to whole cell patch clamp experiments in which the action potential was recorded in current clamp mode (**Figure 8**).

Body Weight (g)	Ketamine/Xylazine (ml)
15	0.18
20	0.25
25	0.31
30	0.37
35	0.43
40	0.49
45	0.55
50	0.62

Table 1. Adult Mouse Weight-dependent Ketamine/Xylazine Dosage (80:12).

Solution	Recipe
Tyrode Buffer* (pH 7.4)	**1 L ddH_2O** 137 mM NaCl 4 mM KCl 1 mM MgCl 10 mM HEPES 0.33 mM NaH_2PO_4
Perfusion Buffer* (pH 7.4)	**500 ml Tyrode Buffer** 10 mM D-(+)-Glucose, minimum 5 mM Taurine 10 mM Butanedione Monoxime (BDM)
Enzyme Buffer*	**50 ml Perfusion Buffer** 0.024 g Collagenase D (Type IV) 0.018 g Collagenase B (Type II) 0.003 g Protease XIV from *Streptomyces griseus*
Transfer Buffer (A)	**45 ml Perfusion Buffer** 5 ml Bovine serum albumin (from 5 mg/ml stock solution)
Calcium Buffer (B)	**1 L Tyrode Buffer** 1.2 mM CaCl 5.5 mM D-(+)-Glucose, minimum

Table 2. Adult Cardiomyocyte Isolation Buffer Recipes.

[CaCl]	Transfer Buffer (A)	Calcium Buffer (B)
0.06 mM Ca^{2+}	9.5 ml	0.5 ml
0.24 mM Ca^{2+}	8.0 ml	2.0 ml
0.60 mM Ca^{2+}	5.0 ml	5.0 ml
1.20 mM Ca^{2+}	0 ml	10 ml

Table 3.Calcium Titration Solution Recipes.

Plating Medium	Culture Medium
• Minimum Essential Media (MEM) (with Hanks balanced salt solution (HBSS)) • 5% Bovine Calf Serum • 2 mM L-Glutamine • 100 U/ml Penicillin/Streptomycin • 10 mM Butanedione monoxime (BDM)	• Minimum Essential Media (MEM) (with Hanks balanced salt solution (HBSS)) • 0.1 mg/ml Bovine serum albumin (BSA) • Insulin/Transferrin/Sodium selenite media supplement (1:100) • 2 mM L-glutamine • 100 U/ml Penicillin/Streptomycin • 10 mM Butanedione monoxime (BDM)

Table 4. Plating and Culture Media Recipes.

Pipette Solution	Bath Solution
• 5 mM NaCl • 40 mM CsCl • 80 mM Glutamate • 5 mM Mg-ATP • 5 mM EGTA • 10 mM HEPES • 1.5 mM CaCl2 (free [Ca^{2+}] = 100 nM) pH 7.2 with CsOH, LJP = +5 mV	• 10 mM NaCl • 2 mM $MgCl_2$ • 5 mM CsCl • 125 mM TEA-Cl • 20 mM HEPES pH 7.4 with CsOH

Table 5. Patch Clamp Solutions (solutions shown are for measuring sodium currents).

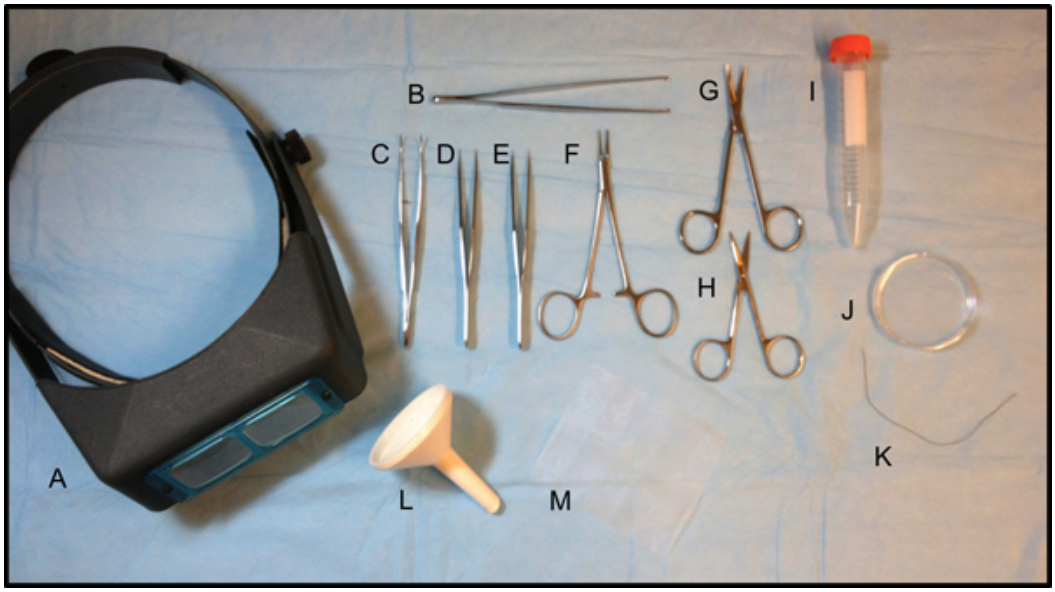

Figure 1. Adult cardiomyocyte isolation instruments. A) *OptiVisor* optical glass binocular magnifier. **B)** Tissue forceps, 5.5 in, 1x2 teeth - Roboz Scientific. **C)** Moloney forceps - 4.5 in (11.5 cm) long slight curve, serrated - Roboz Scientific. **D-E)** Dumont #3 Forceps, Dumostar, tip size 0.17 x 0.10 mm. **F)** Packer Mosquito Forceps 5 in Straight Flat - Roboz Scientific. **G)** Micro Dissecting Scissors 4.5 in Curved Sharp/Sharp - Roboz Scientific. **H)** Micro Dissecting Scissors 3.5 in Straight Sharp/Sharp 20 mm - Roboz Scientific. **I)** 15 ml Falcon tube - Thermo-Fisher Scientific. **J)** 60mm Petri dish containing PBS. **K)** Softsilk: Wax coated braided silk, 19 mm - Covidien. **L)** Plastic funnel, 6.0 cm diameter. **M)** Nylon mesh - 400µm pore size.

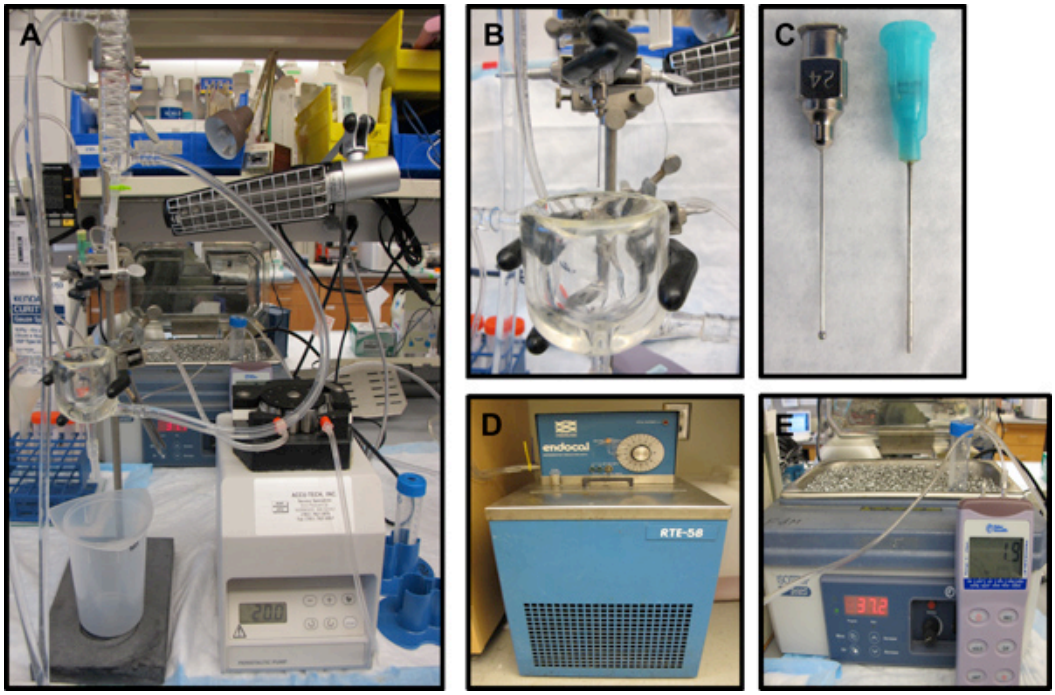

Figure 2. Langendorff Apparatus. A) The entire Langendorff perfusion system used for adult cardiomyocyte isolation. **B)** Enlarged view of the hollow, conical glass collection funnel used to warm the cannulated heart. **C)** Enlarged view of the non-hypodermic cannulation needle. (Reusable Feeding Needles (24 G, straight, round tip, 25 mm long; Fine Science Tools Inc. Item 18061-24) **D)** Circulating water bath. **E)** Water bath for incubating perfusion, enzyme and transfer buffers.

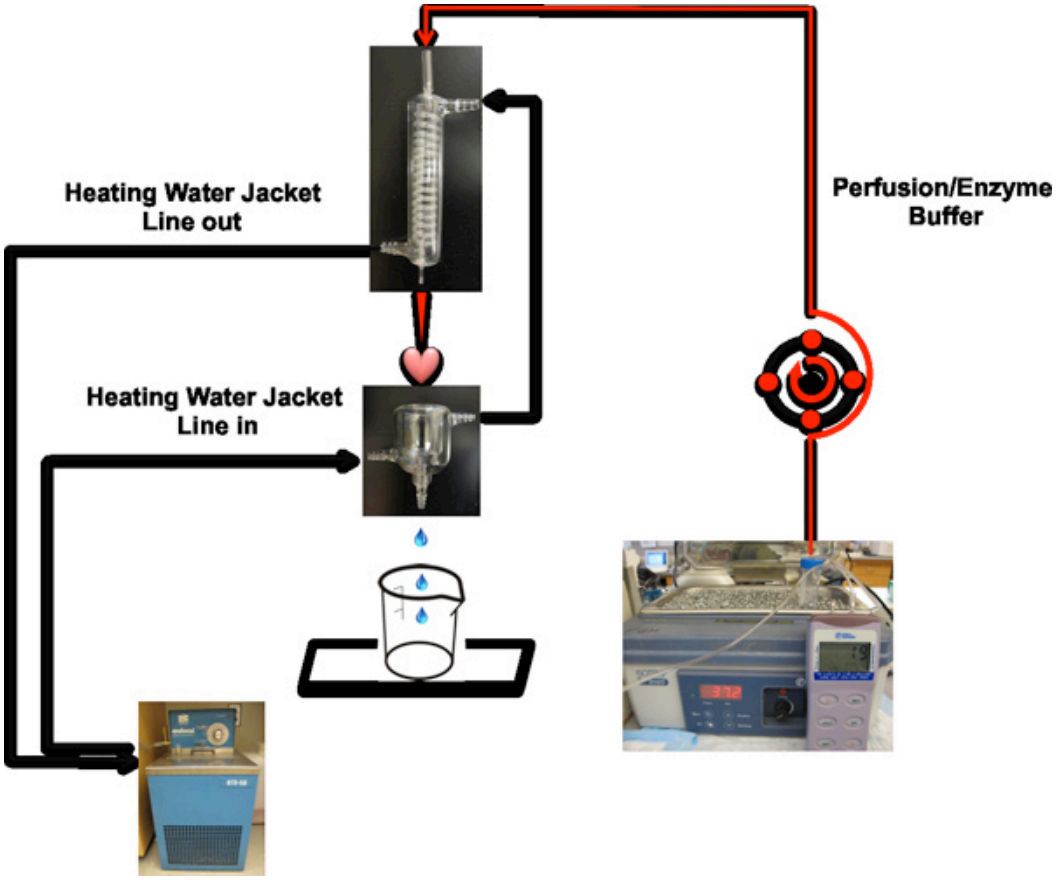

Figure 3. A detailed schematic of the perfusion system.

Figure 4. Modified Pasteur pipette. The pipette was modified by cutting of the tip at an approximately 45° angle. This allows for a greater surface area and creates less shear stress on the cells as they are being dissociated. The inset shows a closer view of the modified tip.

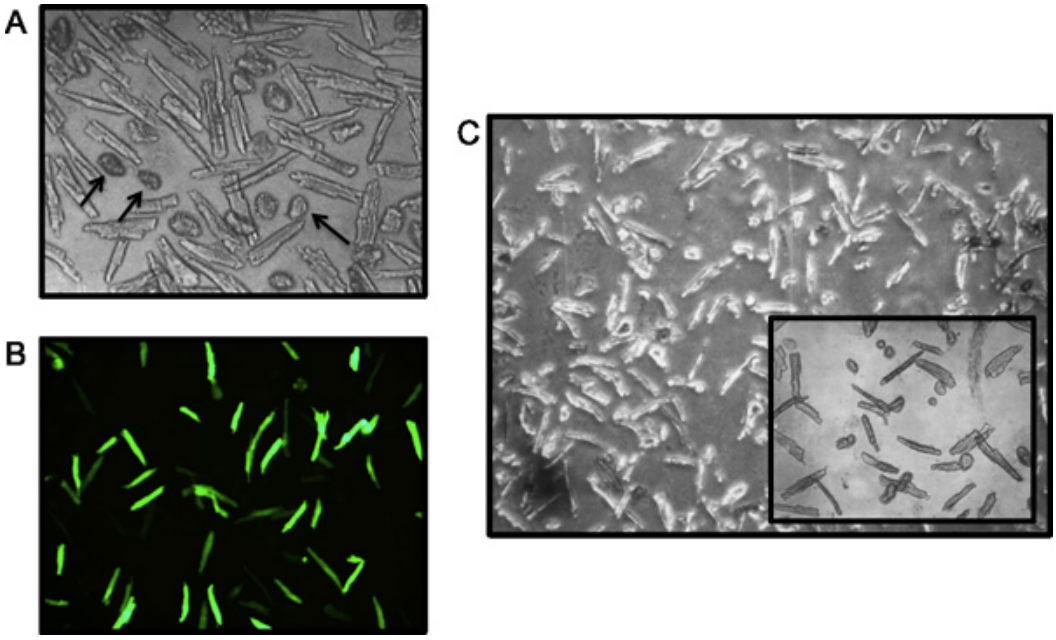

Figure 5. Cultured and GFP transfected cardiomyocytes. A) Freshly isolated adult cardiomyocytes from a C57BL6 mouse of 12 weeks. Arrows point to dead or dying cells. These cells are more rounded than the healthy, rod-shaped adult CMs. **B)** GFP-transfected adult mouse cardiomyoctyes after 24 hr of culture. **C)** Cultured adult mouse cardiomyocytes after 24 hr. Inset shows increased magnification of cultured myocytes.

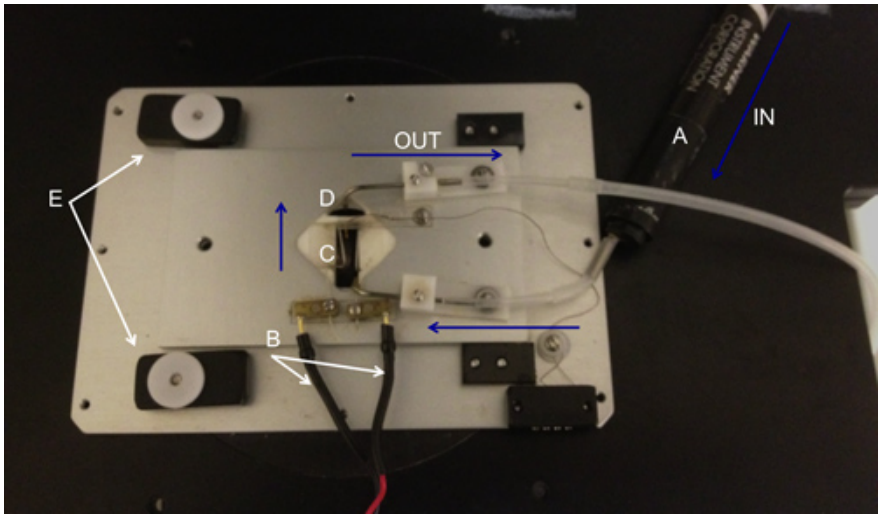

Figure 6. MMSYS system chamber. Blue arrows represent the flow of the perfusion buffer (Buffer B). **A)** Single, in-line solution heater - Warner Instrument Corporation. **B)** Connecting wires from the MMSYS system chamber to the MyoPacer field stimulator. **C)** Platinum electrodes for field stimulation located in the center of the chamber. **D)** Outflow reservoir. **E)** Chamber clamps in the open position.

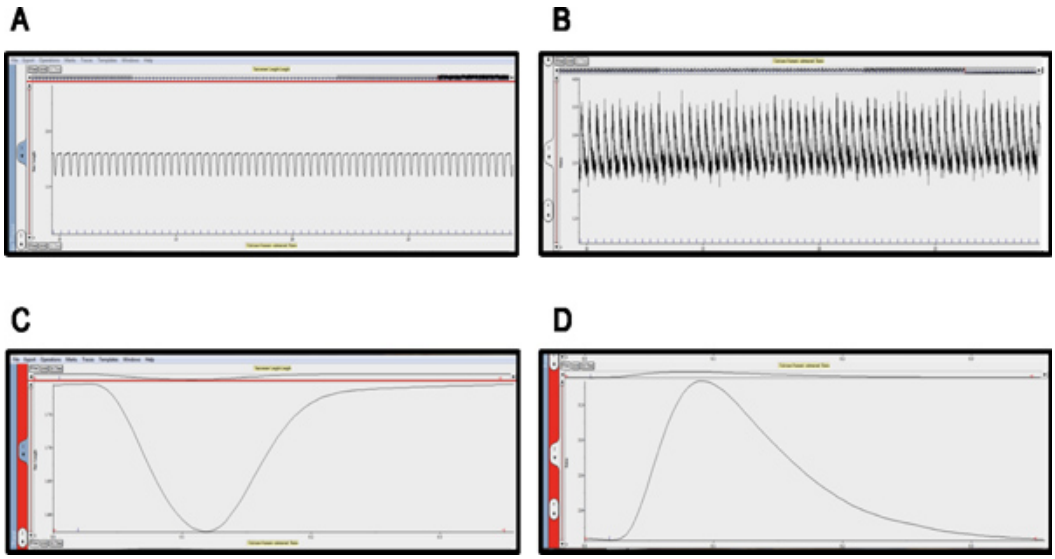

Figure 7. Representative MMSYS system data recorded for wild type C57BL6 mice obtained from Jackson Labs. A) MMSYS base contractility traces. **B)** Ratio of Ca^{2+}-bound to Ca^{2+}-unbound Fura2-AM recorded to generate base calcium traces. These traces correspond to the contractility measurements in A). **C)** Averaged base contractility traces compiled by the MMSYS system software into a characteristic contractility trace. **D)** Characteristic calcium trace compiled from the base traces in B). Analysis of the composite traces will allow the researcher to generate surrogate parameters for systolic and diastolic function. Click here to view larger figure.

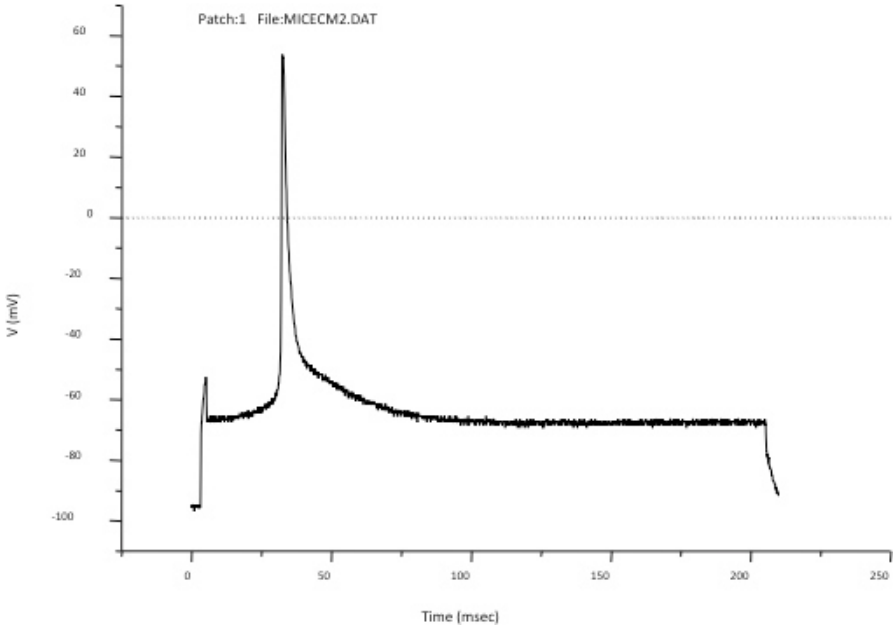

Figure 8. Patch clamp trace showing the action potential of a wild-type adult mouse cardiomyocyte.

Discussion

In this report, we have described the techniques necessary for successful isolation and culture of adult CMs from the mouse heart. Our technique allows for subsequent study of CM function and excitability using the methods described above. The critical parameter for studying functionality of adult CMs is the health and quality of the isolated CMs. As described above, our techniques allow for a high yield of functional cells that are amenable to manipulation of gene expression using adenoviral/lentiviral infections in culture, and analysis of cellular excitability and contractile function.

One of the most important parameters for the isolation of functional adult mouse CMs is precise and rapid cannulation of the heart onto the non-hypodermic needle. Care must be ensured that the aorta attached to the left ventricle is dissected to a sufficient length to allow for easier and more rapid cannulation of the heart. When cannulating the heart, it is also essential that the tip of the needle remains in the ascending aorta and does not extend past the aortic valve into the left ventricle to allow for efficient perfusion of the tissue via the coronary arteries. The next crucial step is securing the aorta to the stem of the needle using a silk suture. The suture should be tied on the ascending aorta, so that the

perfusion buffer flows retrogradely into the coronary arteries and left ventricular cavity but does not flow antegradely out through the distal end of the dissected aorta or the great vessels.

Some investigators find it helpful to set up a pressure gauge to assess the quality of the cannulation and subsequent perfusion of the enzyme. When using a pressure gauge, the initial left ventricular pressure should be ~80 - 100 cmH_2O, and upon perfusion with the enzyme, the pressure will decrease to ~40-50 cmH_2O. When the ventricular pressure reaches this point, it is most likely ready to be to be cut from the needle. To ensure that the ventricles are well digested, a few drops of the flow-through can be collected from beneath the heart into a Petri dish and checked to see if any live, quiescent cells are present.

The resulting isolated cells from this procedure lend themselves well to immunocytochemistry. They can be fixed using regular fixing methods[29] (*i.e.* formalin, paraformaldehyde, ice-cold methanol or acetone), but a higher level of permeabilization than is typically used for neonatal cardiomyoctyes is sometimes needed to image cytosolic proteins or protein structures (*i.e.* alpha-sarcomeric actin, beta myosin). Finally, the cells can be cultured and subsequently infected with adeno- or lentiviruses to manipulate gene expression, or used for functional analyses of the cellular contractile apparatus.

Culture of adult mouse CMs has long been described as challenging. Using the techniques described here, we can routinely culture these cells for up to 72 hr, although the yield of functional cells decreases markedly after 36 hr. When culturing the cells, it is imperative that the cells be plated at 37 °C in 2% CO_2. This is different from neonatal CMs, which prefer to be cultured at 10% CO_2 for the first 24 hr and then switched to 5% CO_2, or neonatal cardiac fibroblasts, which also prefer 5% CO_2. The plating and culture media can be equilibrated so that the dissolved CO_2 is 2%.

The cells are plated on laminin-coated coverslips so that they do not lift off of the glass when the media is changed. However, the cardiomyocytes do not attach very securely to the glass even with the laminin coating. It is therefore very important that caution be taken when changing the culture media, using sterile pipettes, and not a vacuum aspiration system. Once the cells are plated, they can be transfected with adenovirus by incubating the virus for no more than 2 hr. The timing depends on the titer of the virus and the protein being over-expressed, so we recommend conducting preliminary experiments to determine LD_{50} and ED_{50} for the virus. In most cases, we use an MOI of 20-100 for the adenovirus. Once the cells have been incubated with virus, the expression of the target gene(s) typically takes ~24-36 hr.

Isolated cells that are plated on coverslips of a suitable size for the MMSYS system chamber can also be analyzed for contractility and calcium handling using the MMSYS imaging system. These coverslips can be directly placed into the chamber, and the culture medium can be removed by turning on the perfusion system. If the cells are not being cultured and are being used for analysis directly after isolation, they can be added directly to the chamber once a coverslip has been placed. While it is not necessary to pre-coat the coverslips with laminin when analyzing freshly isolated cells, in our experience, laminin does help the cells better adhere to the coverslip. Using a slow flow rate for the MMSYS unit additionally ensures that cells remain attached to the coverslip. Finally, the optics of the microscope, particularly the objective lens must be scrupulously cleaned prior to each experiment, to allow for accurate visualization of sarcomeres needed for assessment of contractility.

The most crucial component of the MMSYS system is the single in-line fluid heater that heats the perfusion buffer (B) as it flows into the chamber. The thermogenic element of this piece of equipment is designed to exchange heat with passing fluid via convective heat transfer, so if the flow is absent, the exchanger will overheat and could irreparably damage the system. It is therefore imperative that the heater is turned on only after the flow is initiated.

Other methods of analyzing the contractile apparatus of adult cardiomyocytes have been previously described, including sophisticated methods using atomic force microscopy[30-32]. Some of these methods allow for more sophisticated measurement of localized contractile force and properties such as Young's modulus, making them more suited for measurement of irregularly shaped cells or cells without clearly defined sarcomeric systems such as CMs derived from induced pluripotent stem cells. These techniques can be just as easily used on the isolated adult CMs. The advantage of the MMSYS system is the relative ease of measuring contractility and the ability to measure a large number of cells in a short period of time in cells with clearly defined sarcomeres. Additionally, the soft-edge and sarcomere analysis systems that can measure the length of the cell or the length between sarcomeres respectively allow for two different (but related) methods to measure contractility. The data acquisition software combined with the advanced, user-friendly analysis software makes this system easy to learn and use. Finally, the ability to measure calcium dynamics simultaneously allows for correlation between contractility and calcium homeostasis, which is not feasible with atomic force microscopy. Also, because the data output is a ratiometric measure of a calcium-activated dye (Fura2-AM), with correct calibration, absolute measures of internal calcium concentrations can be collected.

Successful recording of ion channel signals from adult cardiomyocytes requires the cells be in optimal condition. Immediately following isolation and plating of the cells they are not adherent and will float in solution; at this stage they cannot be studied. Within a few hours they will adhere to a coated coverslip and it is at this point that they may be studied. In our experience patching within a few hours of plating yields the optimal result, as waiting too long adversely affects the cells. As described above, testing the resting membrane potential after establishing a gigaohm seal and breaking into the cell, as well as the ability to establish a gigaohm seal itself are two parameters that reflect how healthy the cells are at the time of patching. While it is easier to patch onto cells that are not contracting, this is not a prerequisite; adequate seals can be obtained onto beating cells as well. The protocols used at this point depend on the goals of the patch clamping study; using current clamp techniques, spontaneous or induced action potentials can be recorded. Alternatively, voltage clamping can be used to study specific ion channels. Given the panoply of channels in the membrane, such recordings are usually performed in the presence of ion channel blockers. Tetrodotoxin (TTX), nisoldipine, and cesium are often used to block sodium, calcium and potassium channels respectively, although a variety of medications have been used, and the details of their use have been published extensively. Most approaches involve either recording currents before and after the application of ion channel blockers and subtracting the currents, and/or blocking background channels with the appropriate blockers prior to recording.

Disclosures

The authors declare that they have no competing financial interests.

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