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TITLE:

Isolation of High Quality Murine Atrial and Ventricular Myocytes for Simultaneous Measurements of Ca^{2+} Transients and L-Type Calcium Current

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KEYWORDS:

arrhythmia, murine myocyte isolation, Langendorff-perfusion, L-type calcium current, calcium transient, patch clamp

SUMMARY:

Mouse models allow studying key mechanisms of arrhythmogenesis. For this purpose, high quality cardiomyocytes are necessary to perform patch-clamp measurements. Here, a method to isolate murine atrial and ventricular myocytes via retrograde enzyme-based Langendorff perfusion, which allows simultaneous measurements of calcium-transients and L-type calcium current, is described.

ABSTRACT:

Mouse models play a crucial role in arrhythmia research and allow studying key mechanisms of arrhythmogenesis including altered ion channel function and calcium handling. For this purpose, atrial or ventricular cardiomyocytes of high quality are necessary to perform patch-clamp measurements or to explore calcium handling abnormalities. However, the limited yield of high-quality cardiomyocytes obtained by current isolation protocols does not allow both

measurements in the same mouse. This article describes a method to isolate high-quality murine atrial and ventricular myocytes via retrograde enzyme-based Langendorff perfusion, for subsequent simultaneous measurements of calcium transients and L-type calcium current from one animal. Mouse hearts are obtained, and the aorta is rapidly cannulated to remove blood. Hearts are then initially perfused with a calcium-free solution (37 °C) to dissociate the tissue at the level of intercalated discs and afterwards with an enzyme solution containing little calcium to disrupt extracellular matrix (37 °C). The digested heart is subsequently dissected into atria and ventricles. Tissue samples are chopped into small pieces and dissolved by carefully pipetting up and down. The enzymatic digestion is stopped, and cells are stepwise reintroduced to physiologic calcium concentrations. After loading with a fluorescent Ca^{2+}-indicator, isolated cardiomyocytes are prepared for simultaneous measurement of calcium currents and transients. Additionally, isolation pitfalls are discussed and patch-clamp protocols and representative traces of L-type calcium currents with simultaneous calcium transient measurements in atrial and ventricular murine myocytes isolated as described above are provided.

INTRODUCTION

Cardiac arrhythmias are common and one of the current major healthcare challenges since they affect millions of people worldwide. Arrhythmias are associated with high morbidity and mortality[1,2] and represent the underlying cause for the majority of sudden cardiac deaths[3]. Up to date treatment options have improved patient survival but are still mainly symptomatic treatments rather than targeting the underlying mechanisms. Thus, these treatments have limited efficacy and may frequently cause severe side effects[4-6]. An improvement of current treatment options requires insight into the underlying pathophysiology, creating the need for suitable models to study. Small animal models - and specifically mouse models - play a crucial role in arrhythmia research as they allow to study key mechanisms of arrhythmogenesis, for example the genetic impact on cellular electrophysiology, ion channel function or calcium handling[7,8].

For this purpose, isolated atrial and ventricular cardiomyocytes of sufficient quantity and viability are required. A broad spectrum of different isolation approaches to obtain atrial and ventricular myocytes has been previously described[9-13] and some groups have presented data from simultaneous measurements of L-type current and calcium current induced calcium transients from either atrial[14] or ventricular[15] murine cardiomyocytes. However, to our best knowledge there is no data available of atrial as well as ventricular measurements from one animal. Researchers focus on a broad variety of topics ranging from electrophysiology to proteomics, functional studies as cell contractility or protein interactions, mitochondrial function, or genetics – all in need of isolated cardiomyocytes. Many of the published protocols thus have not been specifically developed for patch clamp studies, leading to limited yields and insufficient cell quality for patch clamp studies. Thus, simultaneous patch clamp and calcium transient measurements of atrial and ventricular cells isolated from one animal cannot be performed with established protocols.

Isolation of murine – especially atrial – myocytes for patch clamp experiments remains challenging. This article provides a simple and fast method for the isolation of high-quality murine atrial and ventricular myocytes via retrograde enzyme based Langendorff perfusion, which subsequently allows simultaneous measurements of both net membrane current and current induced calcium transients from one animal. This article elaborates a protocol for the

isolation of atrial and ventricular myocytes derived from wild type mice and mice carrying genetic mutations. This protocol can be used for male and female mice alike. The myocyte isolation, images, and representative results described below were obtained from wild type C57Bl/6 mice at the age of 6 (± 1) months. Nevertheless, this protocol has successfully been used for mice at various ages ranging from 2 to 24 months with different genotypes. **Figure 1** shows the isolation setup and a close-up of a cannulated heart during enzyme perfusion.

PROTOCOL

All animal procedures were approved by the Lower Saxony Animal Review Board (LAVES, AZ-18/2900) and were conducted in accordance with all institutional, national, and international guidelines for animal welfare.

1. Prearrangements

1.1 Prepare 1 L of 10x perfusion buffer (**Table 1**), 500 mL of 1x perfusion buffer (**Table 2**), 50 mL of digestion buffer (**Table 3**), 10 mL of stop buffer (**Table 4**), 1 L of Tyrode solution (**Table 5**), 10 mL of each calcium step solution (Tyrode solution with glucose and respective amount of calcium as indicated), 1 L of 4-AP solution (**Table 5**), 100 mL of pipette solution (**Table 6**) and 5 mL of pluronic acid (**Table 7**) according to the provided recipes.

NOTE: Bath solutions (Tyrode and 4-AP solution) can be prepared (without glucose) in advance and stored at +4 °C, glucose is added on the experimental day. Pipette solution can be stored at -20 °C, calcium indicator is added on the experimental day and solution then stored on ice until further use. 10x perfusion buffer can be stored at room temperature, 1x perfusion buffer, digestion buffer and stopping buffer should be freshly prepared on experimental day.

1.2. Turn on the water bath and roller pump.

1.3. Prefill Langendorff apparatus with perfusion buffer; make sure it is air-free.

1.4. Prepare aortic cannula by fixing it under the dissection microscope, connect with a 1 mL syringe filled with perfusion buffer and clear air by rinsing the cannula.

NOTE: It is crucial to avoid any air inside the perfusion system, as this will directly affect coronary perfusion and thus digestion effectiveness. A bubble trap might be added to the setup if necessary, to safely avoid any air trapping.

1.5. Prepare Petri dishes with enough perfusion buffer for organ collection and microscopy (buffer should securely cover the entire organ, a few millilitres – depending on the used Petri dish size – should be enough).

1.6. Prepare 3 Petri dishes with digestion buffer for tissue dissociation and microscopy, buffer should cover the organ for dissection under the microscope within the respective Petri dish, amount depends on the used Petri dish size. For the dissociation use 3 mL for ventricular tissue and 1.5 mL for atrial tissue.

2. Organ harvest

2.1. Inject mouse with 0.1 mL of heparin (1,000 U/mL) i.p. using a 1 mL syringe with a 27 G cannula and wait for 5-10 min.

2.2. Place the mouse into an induction chamber along with a small tissue soaked in approximately 500 µL of isoflurane. The animal should not be in contact with the tissue. To avoid that, one can use a plastic biopsy-embedding cassette to cover the tissue. Once the animal is fully anesthetized, check for toe pinch reflex and as soon as it is not present anymore, quickly euthanize the mouse by cervical dislocation.

2.3. Place the mouse on a platform on its back (e.g., on Styrofoam covered with a paper towel) and fix the paws down with cannulas to hold it in place.

2.4. Remove fur and skin covering the chest and part of the abdomen with a clear cut from jugulum towards symphysis and open the abdomen right under the xiphoid without injuring any organ structure using scissors. Lift the sternum with surgical forceps and cut the diaphragm with scissors along the edge of the ribs, then cut the ribs in medial axillary line and remove the rib cage to expose the heart.

2.5. Carefully remove the pericardium using blunt forceps and quickly remove the heart by lifting it with blunt forceps from below and by cutting the large vessels with one single cut using scissors.

2.6. Put the heart into room temperature perfusion buffer and cannulate the aorta with a blunt end needle under the microscope as quickly as possible.

NOTE: Remove any lung tissue and fatty tissue attached to the organ without losing too much time on it. While cannulating, make sure that the end of the needle does not extend through the aortic valve, as this will impair results by preventing buffers from entering the coronary arteries.

2.7. Tie the heart with a piece of suturing silk firmly to the needle and disconnect from syringe.

NOTE: The entire procedure from obtaining the heart (the moment when the large vessels are cut) until suturing the aorta to the needle should take as little time as possible. It is recommended not taking longer than 90-180 s from removing the heart until start of perfusion.

3. Enzymatic digestion

3.1. After aortic cannulation, immediately connect the cannulated heart to the Langendorff apparatus avoiding any air entering the system.

NOTE: It can help to have a hanging drop of perfusion buffer at the bottom of the Langendorff apparatus as well as a drop of perfusion buffer sitting on the top of the needle in order to avoid any air entering the system.

3.2. Perfuse the heart with perfusion buffer for 1 min at a temperature of exactly 37 °C and a perfusion rate of exactly 4 mL/min.

NOTE: In order to have a temperature of 37 °C at the tip of the perfusion needle, water bath temperature has to be set slightly above at approximately 40 °C. This should be tested regularly by measuring the temperature at the perfusion tip.

3.3. Switch perfusion to digestion buffer and perfuse for exactly 9 min at a temperature of exactly 37 °C and a perfusion rate of exactly 4 mL/min.

3.4. Transfer the digested heart to a Petri dish with enough digestion buffer to keep it fully covered. Then carefully dissect the atria and ventricles under the microscope.

3.5. Transfer the atria into a Petri dish with 1.5 mL of digestion buffer and the ventricles into another Petri-dish with 3 mL of digestion buffer.

3.6. Atrial dissection

3.6.1 Carefully, but without loss of time, pull the atria apart into tiny pieces using blunt forceps.

3.6.2 Dissolve the tissue by carefully pipetting up and down using a 1,000 µL pipette tip, which has previously been cut to widen the tip opening.

3.6.3 Transfer the solution to a 15 mL centrifuge tube and add an equivalent amount of stop buffer (1.5 mL) by carefully pipetting down at the side of the tube to end the reaction.

3.6.4 Carefully pass all 3 mL of cell/tissue-solutions through a 200 µm nylon mesh to remove remaining larger tissue pieces that have not been fully digested.

NOTE: A successful digestion will leave almost no solid chunks.

3.7. Ventricular dissection

3.7.1 Quickly chop the ventricular tissue into tiny pieces using dissection scissors and pipette up and down to dissolve. Use another 1,000 µL pipette tip for pipetting up and down, it may be shortened to widen the opening.

3.7.2 Transfer cell/tissue-solution into a 15 mL centrifuge tube and add an equivalent amount of stop buffer (3 mL) by carefully pipetting down at the side of the tube to end the reaction.

3.7.3 Carefully pass all 6 mL of cell/tissue-solution through a 200 µm nylon mesh to remove larger tissue pieces that have not been fully digested.

NOTE: A successful digestion will leave almost no solid chunks.

3.8. Leave both tubes (atrial and ventricular cell suspension) on the bench at room temperature for 6 min to settle.

3.9. Centrifuge both 15 mL tubes at 5 x *g* for 2 min.

4. Calcium reintroduction

NOTE: The following steps are identical for both atrial and ventricular cells (unless otherwise mentioned) and are performed at room temperature.

4.1. Discard the supernatant using a plastic Pasteur pipette and carefully resuspend pellet in 10 mL of calcium free Tyrode solution.

4.2. Leave cells for 8 min for sedimentation.

4.3. Centrifuge at 5 x *g* for 1 min (only atrial cells, sedimentation is enough for ventricular cells).

4.4. Discard supernatant and carefully resuspend pellet in 10 mL of Tyrode solution with 100 µM calcium concentration.

4.5. Leave cells for 8 min for sedimentation.

4.6. Centrifuge at 5 x *g* for 1 min (only atrial cells, sedimentation is enough for ventricular cells).

4.7. Discard the supernatant and carefully resuspend pellet in 10 mL of Tyrode solution with 400 µM calcium concentration.

4.8. Leave cells for 8 min for sedimentation.

4.9. Centrifuge at 5 x *g* for 1 min (only atrial cells, sedimentation is enough for ventricular cells).

4.10. Discard the supernatant and carefully resuspend the pellet in 1 mL (atrial)/ 5 mL (ventricular) of Tyrode solution with 1 mM calcium concentration.

5. Loading of myocytes with fluorescent calcium-indicator Fluo-3 AM

NOTE: Due to the light sensitivity of the fluorescent calcium indicator, the following steps should be executed protected from light (e.g., by covering tubes with aluminium foil).

5.1. Prepare Fluo-3 AM stock solution by adding 44 µL of 20% pluronic F-127 in anhydrous DMSO to 50 µg of Fluo-3AM (can be stored at -20 °C protected from light).

5.2. Add 10 µL of Fluo-3 AM stock solution to 1 mL of cell suspension and incubate for 10 min at room temperature protected from light.

5.3. Centrifuge at 5 x *g* for 1 min.

5.4. Discard the supernatant using a plastic Pasteur pipette and resuspend the pellet in a reasonable amount of bath solution to obtain a good working concentration (1-5 mL of bath solution depending on the cell density).

5.5. Leave for 30 min for de-esterification before starting with experiments.

6. Simultaneous patch-clamp and epifluorescent Ca^{2+}-transient measurements as previously described[16]

NOTE: Patch clamp measurements are not the topic of this article, the interested reader may be referred to major publications providing in depth descriptions of this method[17-22]. Nevertheless, for a better overall understanding, a summary on a protocol to measure L-type calcium currents along with current induced calcium transients is provided.

6.1. Transfer myocytes into a cell chamber and superfuse with bath solution at 37 °C.

6.2. Block potassium currents by adding 4-aminopyridine and barium chloride to the bath solution as indicated in **Table 5**.

6.3. Ensure that borosilicate microelectrodes have a tip resistance of 2-5 MΩ filled with pipette solution (**Table 6**).

6.4. Setup measurements to allow for simultaneous recording of both electrical signals and epifluorescence at the same time. Voltage clamp mode is used to measure L-type Ca^{2+}-current with a protocol holding the cell at -80 mV and a 600 ms ramp-pulse to -40 mV to inactivate the fast Na^{+}-current, followed by a 100 ms test-pulse to +10 mV at 0.5 Hz (**Figure 2**).

6.5. Use excitation at 488 nm, emitted light at <520 nm to detected and convert to $[Ca^{2+}]_i$ assuming

$$[\boldsymbol{Ca^{2+}}]_{\boldsymbol{i}} = \boldsymbol{k_d}\left(\frac{\boldsymbol{F}}{\boldsymbol{F_{max}} - \boldsymbol{F}}\right)$$

Where k_d = dissociation constant of Fluo-3 (864 nM), F = Fluo-3 fluorescence; F_{max} = Ca^{2+}-saturated fluorescence obtained at the end of each experiment [19].

REPRESENTATIVE RESULTS

Isolation yield is determined after calcium reintroduction by pipetting 10 µL of cell suspension onto a microscope slide. More than 100 viable, rod-shaped, non-contracting cells/10 µL for atrial cell isolation and more than 1,000 viable, rod-shaped, non-contracting cells/10 µL for ventricular cell isolation are considered as sufficient yield and are commonly obtained using this protocol. Atrial cells obtained with this protocol showed a variety of different cell types containing cells of the cardiac conduction system, especially the sinus node, as well as different myocytes from the right and left atrium as well as the atrial appendages. Since these regions are not dissected separately, various cell morphologies are the result. All atrial cells are small, with cell capacitances ranging from approximately 35 pF– 100 pF, some are more filiform than others. **Figure 3** panel A shows a typical cell from the atrial working myocardium loaded with calcium sensitive dye, ready for measurements obtained with this protocol. Ventricular myocytes are more rod-shaped and larger with cell capacitances ranging from 100

to around 400 pF, **Figure 3** panel B shows a typical ventricular cell from the working myocardium loaded with calcium sensitive dye, ready for measurements obtained with this protocol. **Figure 4** shows representative examples of L-type calcium current measurements with simultaneous cytosolic calcium transients from one atrial myocyte (panel C and D) and one ventricular myocyte (panel E and F).

FIGURE AND TABLE LEGENDS:

Figure 1: Langendorff-apparatus. (**A**) Photograph of the isolation setup with view of the custom designed heat exchanger and jacketed heart chamber. (**B**) Close-up of jacketed heart chamber with cannulated heart in place.

Figure 2: Stimulation protocol. voltage-clamp protocol (0.5 Hz) used to measure total net membrane current (I_M), predominantly reflecting L-type Ca^{2+} current and Ca^{2+} current induced Ca^{2+} transient.

Figure 3: Isolated murine cardiomyocytes. (**A**) An example of an atrial cardiomyocyte loaded with Fluo-3, ready for patch-clamp experiments. (**B**) An example of a ventricular cardiomyocyte loaded with Fluo-3, ready for patch-clamp experiments.

Figure 4: Representative recordings. (**A**) Voltage-clamp protocol (0.5 Hz). (**B**) Total net membrane current (I_M), predominantly reflecting L-type Ca^{2+} current in a murine atrial myocyte. (**C**) L-type Ca^{2+} current triggered Ca^{2+} transient in a murine atrial myocyte. (**D**) voltage-clamp protocol (0.5 Hz). (**E**) Total net membrane current (I_M), predominantly reflecting L-type Ca^{2+} current in a murine ventricular myocyte. (**F**) L-type Ca^{2+} current triggered Ca^{2+} transient in a murine ventricular myocyte.

Table 1: 10x perfusion buffer (1,000 mL).

Table 2: 1x perfusion buffer (500 mL).

Table 3: Digestion buffer (50 mL).

Table 4: Stop buffer (10 mL).

Table 5: Bath solutions.

Table 6: Pipette solution.

Table 7: Cell staining reagents.

Table 8: Common problems and how to solve them.

DISCUSSION

This article provides an easy and functional way to obtain high quality atrial and ventricular myocytes from the same mouse for patch-clamp studies with simultaneous calcium transient recordings. The quality of the obtained data highly depends on the quality of the cell isolation. As mentioned above, many methods to isolate murine cardiomyocytes have been described

previously[9-12]. The isolated cells are used for various analyses ranging from patch clamp experiments to contractility studies, morphological studies, proteomics, mitochondrial function research and many more. Each individual purpose requires isolated cells optimized differently. Due to this, none of the protocols led to high quality isolations suitable for patch-clamp experiments with simultaneous measurement of calcium handling properties of atrial and ventricular myocytes from one mouse. To change this, the adaption of numerous protocols described before resulted in the development of this protocol. Some steps are mechanically challenging, some are to be done in a specific fashion and some are simply crucial to isolation quality. These steps will be discussed below.

Yield and quality of cell isolation decrease with animal age, possibly due to increased tissue fibrosis[11,23,24] making individual adjustments of digestion time and enzyme amount necessary. The older an animal, the more fibrotic tissue is to be expected. The indicated digestion times and enzyme amounts are a good starting point for normal sized, healthy mice at the age of 6 months, but will need adaption to individual needs. It is suggested to apply more enzyme and longer digestion times when using older mice or transgenic mice favouring fibrosis. As previous work has emphasized, rapid and air-free cannulation as well as immediate perfusion of the heart are absolutely critical for good cell quality[11]. It is recommended not taking longer than 90-180 seconds from obtaining the heart and putting it into room temperature perfusion buffer to start of perfusion – the faster, the better. It is helpful to set up all the needed equipment in one room to avoid long distances. If further shortening of the ischemic interval is desired, it is possible to use another method of narcosis and subsequent euthanization. Fully anesthetize the animal as described above, apply a sufficient amount of fentanyl (or an equivalent analgesic medication in accordance to your guidelines for animal welfare) i.p. and move the animal to a platform with an isoflurane vaporizer and an anaesthesia mask suitable for mice, providing constant isoflurane and oxygen flow. Afterwards proceed as described above. Since the animal has a normal circulation until the large vessels are disconnected with a single cut, this method will reduce the no-flow time significantly and can thus improve isolation quality if needed.

After harvesting the heart, it is critical to place the aortic cannula at the right depth to secure coronary perfusion. It is, therefore, necessary to cut the aorta at the aortic arch or at the descendent aorta when removing the heart to leave at least 5 mm of the aorta for cannulation allowing to tie the suturing silk distal from the coronary arteries but before the first aortic side-branches.

During the isolation process, multiple cell transfers are necessary and pipetting with small pipette tips will cause high shear stress to the cells. Possible solutions to reduce shear stress are pipetting slowly and carefully, as well as cutting pipette tips by some millimetres in a 45° angle to widen the tip opening. Further reduction of shear stress can be achieved by melting the cut pipette tips to soften the edges. Isolation yield of the ventricular isolation usually is so high that shear stress reduction is not obligatory. Depending on one's individual goal, it can even be more useful to expose ventricular cells to shear stress to select for the 'fittest' cells. However, for the atrial isolation this procedure is critical, as atrial cells are particularly vulnerable after digestion. Whenever adding solutions, it is recommended to avoid pipetting directly onto the cells, but rather trying to pipet slowly at the tube wall.

The choice of collagenase will significantly affect isolation results. Since there are not only differences in enzyme preparations but also a relevant batch-to-batch variation in enzyme activity, titration of collagenase amount and digestion time are necessary when starting to work with a new batch of enzyme or a new strain of mice. However, the amount and time indicated in the tables above mark a good starting point for individual optimization[9]. Most companies provide small enzyme samples to test individual batches, which makes batch selection much more convenient.

Calcium transients with typical amplitudes and normal monophasic decay can be obtained if cells are isolated without the use of EGTA[25] as previously described by Voigt et al.[9,16]. This protocol, therefore, uses low calcium concentrations and avoids EGTA during isolation process. To protect cells from the Ca^{2+} paradox phenomenon[26], calcium is reintroduced stepwise and slowly until a final concentration of 1 mM. Due to higher intracellular sodium concentrations, rodent cardiomyocytes are especially prone to calcium overload and slow and stepwise reintroduction is crucial[27]. The need of calcium free solutions represents one of the major limitations of all Langendorff-based rodent cardiomyocyte isolations. Calcium reintroduction always leads to a significant loss of viable cells. Slow and stepwise reintroduction as described here nevertheless, leads to sufficient yield with good quality to obtain measurements from each animal reliably.

It is important to assess cell yield and quality right after calcium reintroduction. Although final evaluation takes place while patching the isolated cells, one can already judge cell quantity and quality by having a look under the microscope prior to loading cells with calcium sensitive dye. Good quality cells are clearly rod-shaped, have a homogenous membrane and are non-contracting. Contraction is a sign of low cell quality as it indicates a loss of membrane integrity and might be caused by overdigestion or application of high shear stress. Another sign of overdigestion is – besides many cell shadows in relation to viable cells – an overly nice-looking membrane that has been cleared of many surface proteins and is extremely vulnerable when one tries to approach the cell with the pipette tip for seal formation. Exemplary healthy, good quality cells are shown in **Figure 3.** (Panel A: atrial myocyte, Panel B: ventricular myocyte). Isolations with high yields can lead to cells immune to seal formation and vice versa. In the end, the ultimate quality test regardless of cell looks and yield, is the answer to a simple question: 'Is it possible to perform the desired measurements with high quality results in the isolated cells of each isolation?'. The protocol provided above makes a positive answer possible.

Many isolation protocols use the uncoupling agent Blebbistatin to obtain higher yields and better-quality cells. This protocol purposely avoids Blebbistatin, taking into consideration the published evidence of its influence on cardiac electrophysiology[28,29] in optical mapping experiments. The use of uncoupling agents in electrophysiologic studies must be treated with caution and should be limited to circumstances where uncoupling is mandatory.

Cells obtained with this protocol can be used for approximately six hours after loading with Fluo-3 with atrial cells being more vulnerable to time. Consequently, it is recommended to study atrial cells before ventricular cells if the same researcher processes them.

One limitation of this protocol – as true for most Langendorff-based isolation protocols – is that it is technically difficult. Clearly, mouse hearts are small, and all technical aspects require

a lot of exercise. This means that an experienced researcher will obtain better results. Some protocols use a constant pressure to perfuse the heart. This has the advantage that due to digestion and consecutive loss of tissue resistance the perfusion will accelerate, and this helps to define the optimal digestion time, nevertheless the acceleration can harm the tissue und decrease cell quality significantly. In a constant flow approach as used here, there are no clear benchmarks for digestion times and it can often be hard to stop the digestion at an optimal time, which marks a relevant limitation. Another limitation is that in this protocol isolated cells were only tested for suitability to the experiments described above. It is likely that these cells can be used for different analysis as well, but this still must be proven. A first step into that direction was usage of this cells to visualize action potentials by loading them with VF2.1CI, a recently derived voltage sensitivity dye with superior kinetics to previously used voltage sensitive dyes according to a protocol published by Seibertz et al.[30]. To help with troubleshooting, **Table 8** shows common isolation problems and how to approach them.

Taken together, the described isolation protocol offers a working approach to the simultaneous isolation of murine atrial and ventricular cardiomyocytes, enabling the researcher to obtain an atrial and ventricular electrophysiological phenotype of a single mouse. This removes statistical obstacles posed by previous isolation protocols only isolating either atrial or ventricular cells at high quality and helps to reduce the number of animals needed for a specific study. This can be especially important if interventions as for example the implantation of osmotic minipumps filled with expensive agents are part of the experiments and it can be a vital factor in animal welfare considerations.

DISCLOSURES

None

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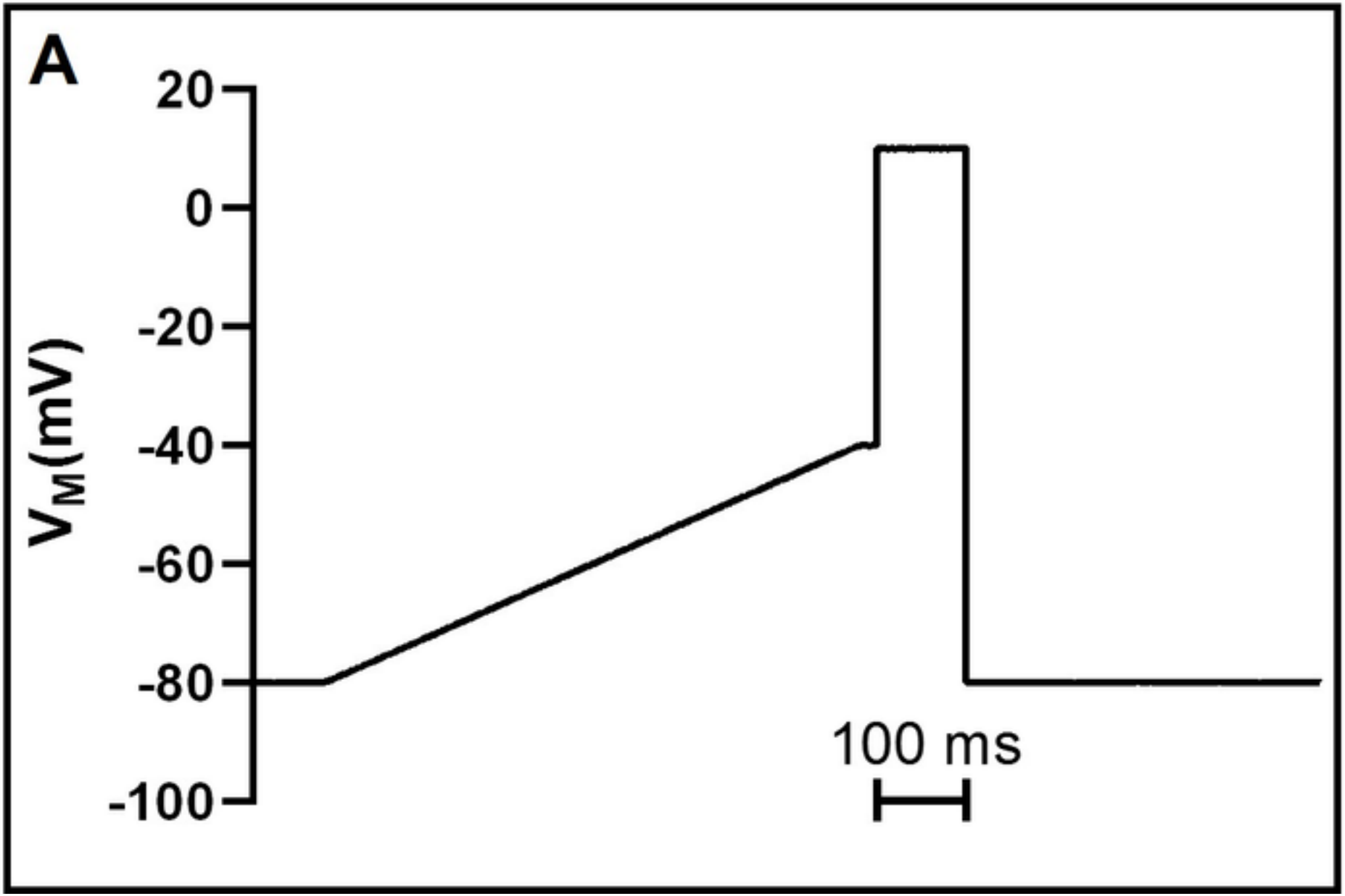

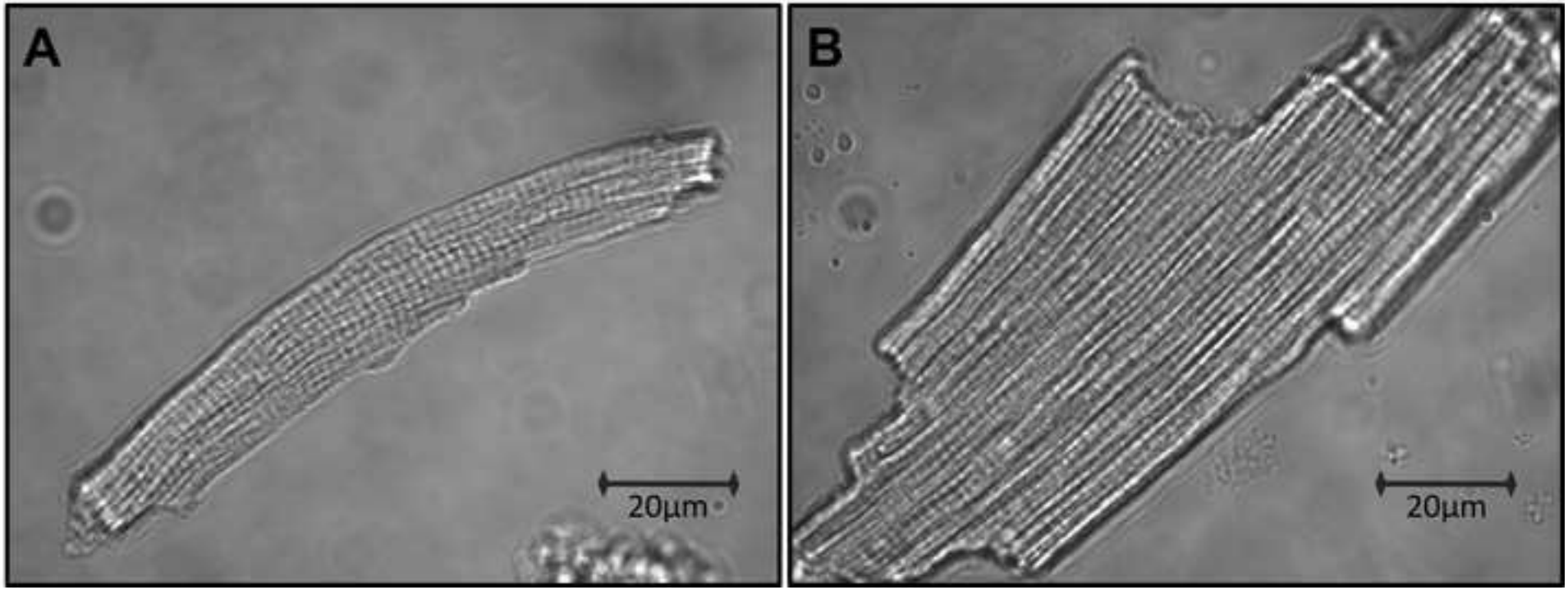

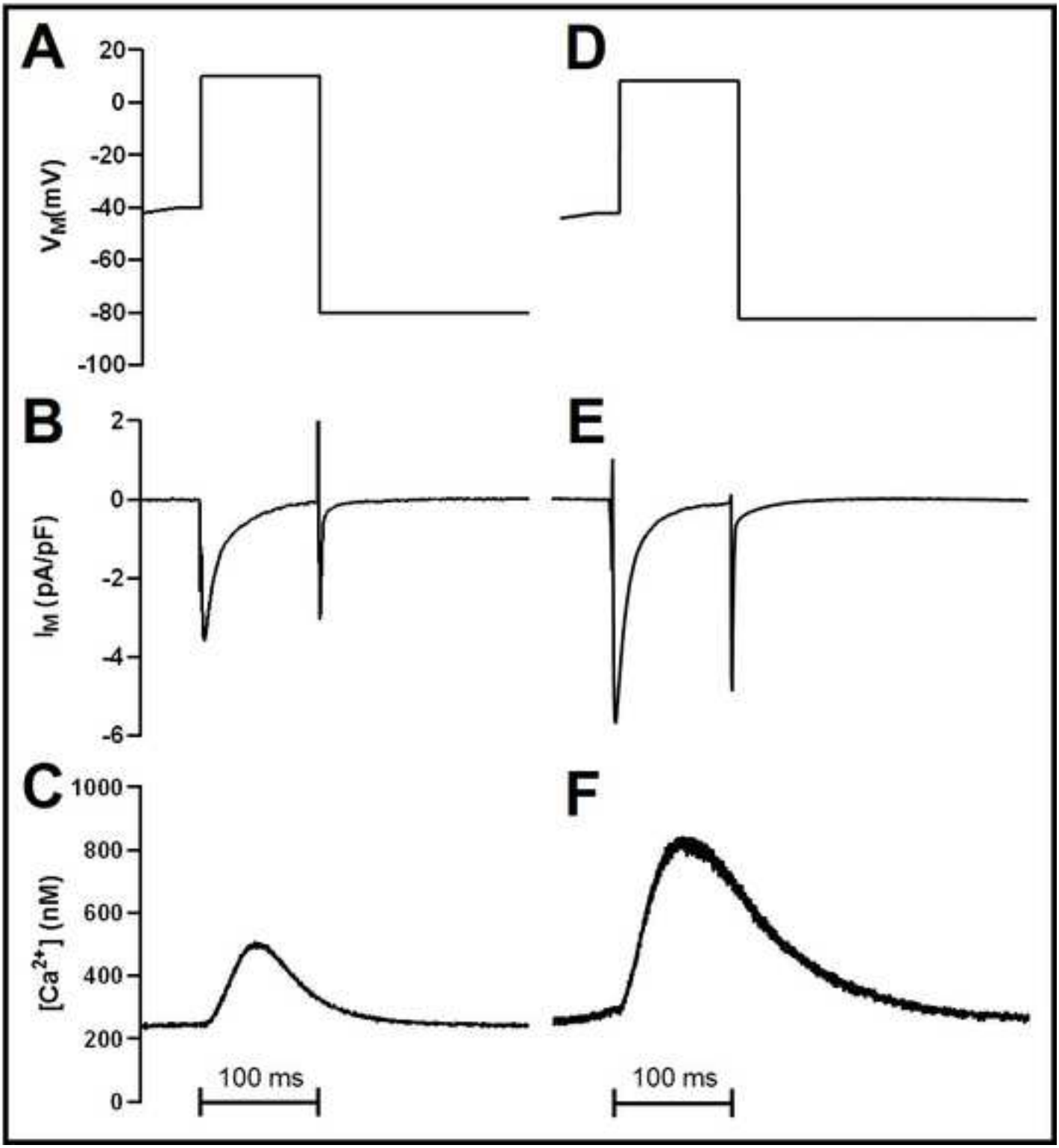

Table 1

	Final concentration (mM)
NaCl	120.4
KCl	14.7
KH_2PO_4	0.6
Na_2HPO_4 x $2H_2O$	0.6
$MgSO_4$ x $7H_2O$	1.2
HEPES	10

Table 2

	Final concentration (mM)
$NaHCO_3$	4.6
Taurin	30
2,3-Butanedione monoxime	10
Glucose	5.5
10x perfusion buffer	50ml
double destilled H_2O (18,2 MΩ-cm)	add 500ml

Table 3

	Final concentration
Collagenase Type II	~600 U/ml
$CaCl_2$	40 µM
1x perfusion buffer	50ml

Table 4

	Final concentration
Bovine Calf Serum	10%
$CaCl_2$	12.5 µM
1x perfusion buffer	10ml

Table 5

	Final concentration (mM)	
	Tyrode	4-AP
NaCl	140	140
HEPES	10	10
Glucose	10	10
KCl	4	4
$MgCl \times 6H_2O$	1	1
Probenecid	2	2
4-Aminopyridine		5
$BaCl_2$		0.1
$CaCl_2$	1	1
Caffeine		
pH	7.35 adjusted with 1 M NaOH at room temperature	7.35 adjusted with 1 M NaOH at room temperature

Table 6

	Final concentration (mM)
DL-aspartat K^+-salt	92
KCl	48
Na_2ATP	5
EGTA	0.02
Guanosine 5'-triphosphate tris salt	0.1
HEPES	10
Fluo-3	0.1
pH	7.20 adjusted with 1 M KOH at room temperature

Table 7

	Company	Catalogue number
Fluo-3 AM	Invitrogen	F1242
Pluronic Acid F-127	Sigma-Aldrich	P2443
Anhydrous DMSO	Sigma-Aldrich	D12345

Table 8

Problem observed	Possible reason	Solution
Low cell yield, tissue chunks left	Underdigestion	Increase digestion time in 15 seconds increments
Low cell yield, tissue chunks left	Underdigestion	Increase collagenase amount in 50 U/ml steps
mix of over- and underdigested cells, tissue chunks left	No coronary perfusion	Make sure not to insert aortic cannula into the ventricle
mix of over- and underdigested cells, tissue chunks left	Air bubbles	Make sure not to insert any air into the perfusion system
Low overall cell quality	Ischemic time too long	Decrease ischemic time, consider alternative methods to maintain circulation as long as possible
Many dead cells after calcium reintroduction	Overdigestion	Reduce digestion time in 15 seconds increments
Many dead cells after calcium reintroduction	Overdigestion	Reduce collagenase amount in 50 U/ml steps

Many dead cells after calcium reintroduction	Shear stress	Reduce shear stress by widening tips even more and pipetting more carefully
Many cells contracting after calcium reintroduction	Overdigestion	Reduce digestion time and/or collagenase amount

Table of Materials

Click here to access/download;Table of Materials;table of materials.xlsx

	Company	Catalogue number
2,3-Butanedione monoxime	Sigma-Aldrich	31550
27G cannula	Servoprax	L10220
4-Aminopyridine	Sigma-Aldrich	A78403
Anhydrous DMSO	Sigma-Aldrich	D12345
Aortic cannula	Radnoti	130163-20
$BaCl_2$	Sigma-Aldrich	342920
blunt surgical forceps	Kent Scientific	INS650915-4
Bovine Calf Serum	Sigma-Aldrich	12133C
$CaCl_2$	Sigma-Aldrich	C5080
Caffeine	Sigma-Aldrich	C0750
Circulating heated water bath	Julabo	ME
Collagenase Type II	Worthington	LS994177
disscetion scissors	Kent Scientific	INS600124
DL-aspartat K^+-salt	Sigma-Aldrich	A2025
EGTA	Sigma-Aldrich	E4378
Fluo-3	Invitrogen	F3715
Fluo-3 AM	Invitrogen	F1242
Glucose	Sigma-Aldrich	G8270
Guanosine 5'-triphosphate tris salt	Sigma-Aldrich	G9002
Heating coil	Radnoti	158821
Heparin	Ratiopharm	25.000 IE/5ml

HEPES	Sigma-Aldrich	H9136
induction chamber	CWE incorporated	13-40020
Isoflurane	Cp-pharma	1214
Jacketed heart chamber	Radnoti	130160
KCl	Merck	1049360250
KH_2PO_4	Sigma-Aldrich	P5655
MgCl x $6H_2O$	Sigma-Aldrich	M0250
$MgSO_4$ x $7H_2O$	Sigma-Aldrich	M9397
Na_2ATP	Sigma-Aldrich	A2383
Na_2HPO_4 x $2H_2O$	Sigma-Aldrich	S5136
NaCl	Sigma-Aldrich	S3014
$NaHCO_3$	Sigma-Aldrich	S5761
Nylon mesh (200 µm)	VWR-Germany	510-9527
pasteur pipette	Sigma Aldrich	Z331759
petri-dishes	Thermo Fisher	150318
Pluronic Acid F-127	Sigma-Aldrich	P2443
Probenecid	Sigma-Aldrich	P8761
Roller Pump	Ismatec	ISM597D
surgical forceps	Kent Scientific	INS650908-4
surgical scissors	Kent Scientific	INS700540
suturing silk	Fine Science Tools	NC9416241
syringe	Merck	Z683531-100EA
Taurin	Sigma-Aldrich	86330

EDITORIAL COMMENTS:

Changes to be made by the Author(s):

Response: We would like to thank the editor for his time and his suggestions to improve the quality of our protocol.

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling, grammar, and language (e.g., line 66: severely improved) issues. Please use American English.

Response: Thorough proofreading has been done, British English was changed to American English to the best of our knowledge.

2. Please provide an email address for each author.

Response: Email addresses for each author have been added to the revised submission.

3. Please use periods and not commas to indicate decimal throughout the manuscript (e.g., 2.1, line 131; 3.8, line 183 etc).

Response: All decimals use periods now.

4. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Response: Personal pronouns have been removed from the isolation protocol

5. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

Response: thank you for this suggestion, clearly, an easy replication is the main goal of this protocol. We therefore added more detailed descriptions to numerous steps.

i) What is the age of the mice used in the study?

Response: The age of the mice used in this study was 6 ± 1 months. This information has been added to the protocol.

ii) 1.1: How much of these buffers are to be prepared?

Response: clarification of this issue has been added to the text.

iii) 1.4: Please clarify how the aortic cannula is to be prepared.

Response: The cannula has to be cleared from air by rinsing it with perfusion buffer. In order to be able to cannulate the heart, it has to be fixed under a microscope. Better explanation has been added to the text.

iv) Please indicate what instruments would be most suitable to perform steps in 2.4 and 2.5.

Response: thank you for this comment, clarification was added to the text and instruments which are needed were added to the materials table.

v) How much of perfusion buffer (2.6) and digestion buffer (3.4) should be used to immerse the heart?

Response: Specific amounts were not mentioned since they depend on the size of the used dishes. The goal is to keep the tissue covered and therefore keep it from drying out. Information about this issue has been added to the text.

vi) In 3.9 and 3.12, do you mean “carefully filter” or “carefully pass”?

Response: Carefully pass – the text has been changed accordingly.

vii) In 4.3 and 4.9, if only atrial cells are to be centrifuged at 5 x g for 1 minute, what should the speed and duration be for ventricular cells?

Response: Ventricular cells do not need to be centrifuged, they settle by sedimentation. An additional sentence has been added to clarify this issue.

viii) What do you mean by 330 u/mg dw?

Response: Units per milligram dry weight – since this was part of a sentence using commercial language, we entirely left out the sentence, enzyme concentration and activity is mentioned within the digestion buffer table.

6. Please highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

Response: highlights have been added to the revised version.

7. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.

Response: highlights have been added to the revised version.

8. Please cite figures in order. After Fig.1, you have cited Fig. 3 in Representative Results.

Response: Figure citation is now in numerical order.

9. Please have a separate Table of materials and relabel the other tables from 1 to 8.

Response: tables have been changed as required.

10. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please

remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: Worthington Type II collagenase (LOT # F7P17939A), and the sentence "Worthington Biochemical Corporation … convenient" (lines 367-369)

Response: Commercial language has been removed.

11. Please discuss some limitations of the protocol in the Discussion.

Response: thank you for this comment, limitations are now addressed in the discussion.

13. Please write journal names fully for all references in the list.

Response:.changes have been made to display full journal names.

14. Please check spelling (e.g., Typ II in Table 4) and symbols (e.g., NaCL in Table 2 and CaCL2 in Table 4).

Response: spelling was checked again and chemicals are now written correctly.

15. Please select a section in the submission site for your manuscript.

Response: A section was selected for the revised resubmission.

_____

REVIEWER COMMENTS TO THE AUTHORS:

Reviewer #1:
Manuscript Summary:
The authors have done a good job describing a detailed protocol for isolating myocytes from mouse atrium and ventricles. My concerns and suggestions are follows:

1) The authors should clearly summarize in Abstract and Discussion what they have done to modify/improve the currently used protocols in literature to increase the yield and quality of the cells

Response: First of all, we would like to thank the reviewer for taking his time to review our manuscript. We appreciate your comment, we agree that this was not made entirely clear in the initial manuscript and thus have rewritten the discussion to point out the modifications we have made to currently used protocols. We tried adding the information to the abstract, but due to word limits it turned out to be redundant and we decided to leave it as it was.

2) Blebbistatin, a myosin inhibitor has been used in many labs to increase yield greatly. The authors may need to discuss why they choose not to use it. It is conceivable that blebbistatin-treated cells are not suitable for contraction evaluation.

Response: we appreciate the reviewers comment. Blebbistatin can increase yield, but current publications[1,2] have shown that Blebbistatin has a significant effect on cardiac electrophysiology. Its use in electrophysiologic studies has to be treated with caution and

should be limited to circumstances where uncoupling is mandatory. We have discussed this important issue and added these two references to the article.

3) Fig. 2 shows good quality of a single cell isolated from atrium and ventricle, respectively. Photos with low magnification should also be provided to show the percentile yield in each case

Response: thank you for this comment. We considered this carefully but have purposely left out such a figure, because we think it does not add significantly to this article. The cell amount reflected in such a figure highly depends on the initial volume in which the cell pellet has been resolved and does not necessarily correlate with total yield or cell quality for patch-clamp experiments. Criterions for good cell yield and quality which are commonly reached with this protocol have been emphasized within this manuscript and are to our minds more important to researchers in the field.

Reviewer #2:

Manuscript Summary:

This study entitled "Isolation of high quality murine atrial and ventricular myocytes for simultaneous measurements of Ca2+ transients and L-type calcium current" describes the methodological procedure to isolate murine cardiomyocytes in order to perform calcium transient measurements and calcium current recordings.

Major Concerns:

None

Response: Thank you for your time to review our manuscript and for the important suggestions made below.

Minor Concerns:

Point 1.2. Due to the loss of temperature between the bath and the extremity of the cannula, perhaps it will be useful for the reader, as an example, to mention at which temperature in general you setup your bath to reach 37°C at the extremity of the cannula.

Response: thank you for this consideration, we do absolutely agree and have added clarification to the protocol. We wrote: 'In order to have a temperature of 37°C at the tip of the perfusion needle, water bath temperature has to be set slightly above at around approximately 40°C. This should be tested regularly by measuring temperature at the perfusion tip'

Point 2.6. Can you precise if you put the heart into cold or RT perfusion buffer? Are you also cleaning the heart to remove piece of lung or fatty tissue (extracted during the point 2.5) at this stage or it is not necessary?

Response: clarification of these questions have been added to the protocol. We wrote: 'put the heart into room temperature perfusion buffer and cannulate the aorta with a blunt end needle under the microscope as quickly as possible. **NOTE:** Remove any lung tissue and fatty tissue attached to the organ without losing too much time on it. While

cannulating, make sure that the end of the needle does not extend through the aortic valve, as this will impair your results by preventing buffers from entering the coronary arteries.'

After the point 2.7 in the ''Note'' section: Can you precise if the time mentioned (90-180 seconds) is the time between the cervical dislocation and the cannulation or if it the time between the heart extraction and the cannulation?

Response: clarification has been added to the protocol. We wrote: 'We recommend not taking longer than 90-180 seconds from removing the heart until the start of perfusion.'

Point 3.2. Is it exactly 37°C as mentioned or can we have a windows of freedom and if yes how much?

Response: temperature should be exactly 37°C, nevertheless we know that this is hard to achieve under real conditions. We have not tested different temperatures and their effects, we therefore recommend to aim for 37° and consider natural inaccuracy the 'window of operation'.

Point 3.7. For the pipette tip. Are you also melting a little bit the opening after the cutting to avoid a sharp opening? If yes, can you precise it.

Response: we do not heat polish or melt the pipet tips after cutting, this information has been added to the manuscript.

Point 6.3. Do you have a ref for the glass pipettes?

Response: since this section is a brief summary, added for completeness and refers to other publications containing this information we did not add the glass pipettes to the table of Materials. In our lab we use WPI (world precision instruments) 1.5/0.75mm PG101504 borosilicate glass.

In the discussion, line 326. Can you precise the windows of ages for which you can use this protocol?

Response: we added this information to the discussion.

In the discussion, line 355. For the pipette tip. Are you also melting a little bit the opening after the cutting to avoid a sharp opening? If yes, can you precise it.

Response: as stated above, we do not melt the openings.

Figure 1: Do you have a bubble trap in you system? Perhaps it will be useful to precise it in the legend.

Response: thank you for this comment, we do not use a bubble trap in our system since we do not experience problems with air bubbles after carefully filling the Langendorff-apparatus prior to experiments. Nevertheless we added this possibility to the protocol for researchers experiencing problems with air trapping. We wrote: '**NOTE:** It is crucial to avoid any air inside the perfusion system, as this will directly affect coronary perfusion

and thus digestion effectiveness. If necessary, a bubble trap might be added to the setup to safely avoid any air trapping.'

Figure 2: The cardiomyocytes are beautiful, however I was expected an atrial cardiomyocytes with a less pronounced striated pattern and much more filiform. Do you have other example of atrial cardiomyocytes that you can add to this one or all the atrial cardiomyocytes, using this protocol, look like the one on figure 1A?

Response: thank your consideration. Atrial cells summarize a variety of different cell types containing cells of the cardiac conduction system, especially the sinus node, as well as different myocytes from the right and left atrium as well as the atrial appendages. In this isolation protocol we do not separate these regions prior to dissociation, and this results in various cell morphologies within the isolated cells. All atrial cells are small with cell capacitances ranging from around 35 to 100 pF, some are more filiform than others. The cell we show is a typical cell from the atrial working myocardium obtained with this protocol. We have added an elaboration of this matter to the discussion.

Reviewer #3:

Manuscript Summary:

The manuscript describes the method used by the authors for isolation of functional cardiac myocytes of mice. Although it is of potential interest to many labs, the aims of this contribution should be better targeted. Specifically, to clearly point to advancement in the proposed method over the previously published methods, including their own publication (Ref. 9), since the authors claim in the Abstract and Introduction, that current methods are not so good. From reading the manuscript, I could not identify the improvements or differences. Moreover, I was neither convinced about the claimed quality of the presented procedure just on the basis of presented exemplar experiments. Therefore, I propose additions/modifications of the study.

Response: We thank the reviewer for his important suggestions and we highly appreciate his effort for reviewing our manuscript.

Major Concerns:

1/ The title is very specific, consider a simpler title like - Standardization of the isolation procedure for highly functional murine cardiac myocytes

Response: Thank you for your comment. Indeed, the title is quite specific. We thoroughly discussed if we should change our manuscript's title. Nevertheless, the aim of this article is to share a method of isolating murine atrial and ventricular cardiomyocytes for simultaneous patch-clamp and calcium transient measurements. We did not use the isolated cells for other measurements and consequently cannot judge their suitability for other measurements. A less specific title would thus imply more generality, which we think is improper. That's why we prefer to keep the title as it is.

2/ The aim should be better elaborated. Is it preparation procedure itself or how to record calcium signals from isolated myocytes? Scientists working in the field would welcome

standardization of the isolation procedure. This needs to define explicitly a set of quality control check points in the procedure and the evaluation criteria for quality standards of isolated myocytes. The authors certainly have plenty of experience and data to do this.

Response: We thank the reviewer for his comments. He is correct, the aim was insufficiently elaborated. The aim of this article is to provide a functional and simple step by step protocol for simultaneous isolation of atrial and ventricular murine myocytes for patch-clamp studies and calcium measurements. We tried to clarify this issue throughout our manuscript and strengthened corresponding sections. We carefully revised the abstract, introduction and discussion to better elaborate the aims and scope of this manuscript. Further, we emphasize that patch-clamp measurement are not topic of this article within the protocol itself and thus only give a brief summary and refer to different publications describing the method in detail. The reviewer is correct, standardization of the isolation procedure and the development of evaluation criteria for quality standards would be of help for the field. The aims and needs of many researchers differ widely, which makes defining general criterions challenging and we think that the development of quality standards is beyond the scope of this protocol. Nevertheless, the ultimate evaluation for quality of the isolated cells is simple and done by answering one question: 'am I able to perform the desired measurements with high quality results in my isolated cells of each isolation'. To reach this goal, we provide an easy and functioning protocol with elaboration and discussion of major pitfalls and quality criterions that we feel meets the needs of many researchers within the field.

3/ The study limits on calcium currents and calcium transient, nevertheless, isolated myocytes are widely used for many other purposes like action potential, membrane currents, contractility, morphological studies, calcium sparks, protein interactions and co-localization, cellular energetics, mitochondrial function, etc., but also for protein and genomic profiling of contaminant free cardiac myocytes, to name just the most common ones. This fact should be mentioned in the introduction and elaborated in the discussion.

Response: We appreciate this comment. We are aware of the fact that many researchers within the field work on different aspects of cardiac electrophysiology and function using isolated cardiomyozytes. We added this information to our manuscript and discussed it.

4/ What is the trick that this protocol provides both the atrial and ventricular myocytes tolerant to calcium? I found only atrial dissection step, without any treatment difference to ventricles.

Response: thank you for this question. In order to obtain high quality atrial cells many aspects were adapted from previously published protocols. Perfusion time with digestion buffer is slightly shorter than it would be for ventricular isolation only. The amount of collagenase is a little less and the atrial dissection is very gentle by just pulling the tissue apart instead of chopping it into pieces. Reducing shear stress is another crucial step that is important to obtain good results. The shortening of the organ perfusion with digestion buffer and subsequent atrial dissection with meanwhile ongoing digestion of the ventricular tissue is what – to our mind – leads to calcium tolerant cells of both, atrium and ventricle. Additionally, it is clear that an experienced researcher is more likely to

obtain good results. We tried to clarify crucial steps and potential pitfalls and added information about these issues to our manuscript

5/ Quantitative criteria of isolation quality are not provided. Any users would like to know what would be the expected efficiency, yield, average percentage of viable isolated myocytes, of calcium tolerant myocytes, of excitable and contracting myocytes, oxygen consumption level, and so on. What was the typical heart rate before dissection and upon Tyrode perfusion?

Response: Quantitative criteria of isolation quality are mentioned within the representative results section of the manuscript. More than 100 viable, rod-shaped, non-contracting cells/10 µl for atrial cell isolation and more than 1000 viable, rod-shaped, non-contracting cells/10 µl for ventricular cell isolation are considered as sufficient yield and these results are commonly obtained using this protocol. Yield is determined after calcium reintroduction. The fraction of viable myocytes is around 80% and these myocytes are only of good quality if they are non-contracting. One major difference in patch-clamp studies compared to other experiments following cell isolation is that one viable calcium tolerating myocyte that leads to a good seal formation and subsequent experiment is worth more than thousands of non-measurable cells. Thus, quantitative evaluation alone does not give good information about cell usability and does not add significantly to this manuscript. This is the reason why we decided not to display large magnification images of cell isolations. Oxygen consumption levels have not been measured within this study, nor have heart rates been recorded before organ harvest or while perfusing the heart.

6/ An image of a typical isolated myocyte is not really the evidence of isolation quality. Basic morphometric descriptors like the average sarcomere length and cell shortening, would be much more useful for assessing the claimed quality of the procedure, and for comparisons in future studies using the same or modified procedures, as well.

Response: Thank you for this comment. The aim of this protocol is to obtain cells suitable for patch clamp experiments and calcium transient measurements and neither sarcomere length nor cell shortening are predictors for good seal formation, rupture and subsequent stable measurements. We therefore do not assess average sarcomere length and average cell shortening routinely to assess cell quality. Increased sarcomer length is associated with more frequent calcium sparks and calcium release possibly leading to spontaneous contractions. Cells showing this behavior – if present – are not of good quality for our intended purposes and will not be included into experiments.

7/ The composition of experimental solution is described just formally. To my experience, the quality of solutions should be verified before isolation by their pH and osmolarity values in all cases, but also according to the free calcium concentration in the case of Ca-free solutions. The quality of ddH2O (double distilled?) was not mentioned, at least the conductivity is usually reported. Was the age, weight, health state of mice taken into experiment a factor? Make order in names and preparation of solutions, i.e, perfusion buffer, Tyrode solution calcium free Tyrode, etc. What is BDM, BCS, ddH2O, GTP-Tris, …?

Response: Thank you for your important considerations and suggestions. First, we included information about pH adjustments in those steps, solutions and buffers where necessary (see table 5. for example). We do not measure osmolarity within this isolation protocol routinely. The conductivity of double distilled water (ddH_2O) has been added to the manuscript. The age and weight of the mice taken into experiment was documented but adaptions of the protocol are not needed as we did not experience any significant differences in outcomes. All animals taken into experiments were of good health. The manuscript has been proofread again and we tried our best to clarify misleading statements and insufficient descriptions of steps, solutions and buffer and improved overall readability. Abbreviations have been erased from the manuscript and tables or been introduced before use.

8/ The costs analysis would be also useful, especially for those who prepare their grant budget.

Response: We agree that a cost analysis would be of interest for someone preparing his or her grant budget. Nevertheless, costs highly depend on already present laboratory equipment, countries where studies are conducted and on the exact aim of each project and therefore costs unfortunately vary substantially. We consequently decided not to include a detailed cost analysis and think that our manuscript provides a highly useful and functioning isolation protocol even without this analysis.

1 Brack, K. E., Narang, R., Winter, J. & Ng, G. A. The mechanical uncoupler blebbistatin is associated with significant electrophysiological effects in the isolated rabbit heart. *Exp Physiol.* **98** (5), 1009-1027, (2013).

2 Kappadan, V. *et al.* High-Resolution Optical Measurement of Cardiac Restitution, Contraction, and Fibrillation Dynamics in Beating vs. Blebbistatin-Uncoupled Isolated Rabbit Hearts. *Front Physiol.* **11** 464, (2020).