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Isolation of Cardiac and Vascular Smooth Muscle Cells From Adult, Juvenile, Larval and Embryonic Zebrafish for Electrophysiological Studies

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TITLE:

Isolation of Cardiac and Vascular Smooth Muscle Cells From Adult, Juvenile, Larval and Embryonic Zebrafish for Electrophysiological Studies

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SUMMARY:

The present protocol describes the acute isolation of viable cardiac and vascular smooth muscle cells from adult, juvenile, larval, and embryonic zebrafish (*Danio rerio*), suitable for electrophysiological studies.

ABSTRACT:

Zebrafish have long been used as a model vertebrate organism in cardiovascular research. The technical difficulties of isolating individual cells from the zebrafish cardiovascular tissues have been limiting in studying their electrophysiological properties. Previous methods have been described for dissection of zebrafish hearts and isolation of ventricular cardiac myocytes. However, the isolation of zebrafish atrial and vascular myocytes for electrophysiological characterization was not detailed. This work describes new and modified enzymatic protocols that routinely provide isolated juvenile and adult zebrafish ventricular and atrial cardiomyocytes, as well as vascular smooth muscle (VSM) cells from the bulbous arteriosus, suitable for patch-clamp experiments. There had been no literary evidence of any electrophysiological studies on zebrafish cardiovascular tissues isolated at embryonic and larval stages of development, causing a dearth in the understanding of zebrafish cardiovascular development. Partial dissociation techniques that allow patch-clamp experiments on individual cells from larval and embryonic hearts are demonstrated in this methodology.

INTRODUCTION:

Zebrafish are small teleost fish that has long been used as a model vertebrate organism[1] and have

recently come to prominence as a viable vertebrate system for high throughput screening for genes and drugs[2,3]. However, physiological analysis of zebrafish tissues is not well developed. In the cardiovascular system, methods have been described for dissection of zebrafish hearts[4] and isolation of ventricular cardiac myocytes[5-7]. There are few detailed descriptions of the effective isolation of atrial myocytes and no reports of vascular smooth muscle (VSM) preparation for patch-clamp studies.

The current work describes the methodology to isolate zebrafish cardiac and vascular myocytes, viable for electrophysiological and functional studies. This approach includes modifications of previously reported protocols for zebrafish ventricular myocyte isolation[5,6] and adapts methods from mammalian VSM cell isolations[8], allowing for the isolation of zebrafish vascular smooth muscle cells from the bulbous arteriosus (BA). The protocols result in efficient yields of isolated atrial, ventricular, and VSM cells from zebrafish that can be reliably used in patch-clamp studies for up to 8 h[9].

Despite their nearly transparent larvae that develop entirely outside the parental organism, exploring their promised ontogenetic potential in studying cardiovascular development has been limited by challenges in extracting and analyzing the tissues at a younger age. The current article addresses this limitation by demonstrating patch-clamp experiments on zebrafish hearts isolated as early as 3 days post-fertilization (dpf), using an adapted, published extraction method[10].

PROTOCOL:

All zebrafish (wild type strain AB, both male and female) were raised, maintained, and handled for the experiments following the guidelines of the Washington University Institutional Animal Care and Use Committee (IACUC).

1. Isolation of atrium, ventricle, and bulbous arteriosus from adult, juvenile, and larval zebrafish

1.1. Euthanize fish using cold-shock, i.e., by immersing in 4 °C water, for ~10 s.

1.2. Using curved forceps, transfer fish into a large Petri dish partially filled with Perfusion Buffer (PB) (**Table 1**) and place under a dissecting microscope.

1.2.1. Decapitate the fish just posterior to the eyes, using a pair of scissors.

1.3. Using curved forceps (fine forceps for larval fish), hold the fish in the non-dominant hand with the ventral side facing the dissection scope. With the second pair of fine forceps (or superfine for larval fish) in the dominant hand, gently tear open the pectoral muscles and fins to reveal the cardiovascular (CV) tissues (atrium, ventricle, and bulbous arteriosus, BA) (**Figure 1**).

1.4. Placing the fine forceps in the dominant hand, gently pull the BA at the intersecting tip of the BA and the ventral aorta.

NOTE: The BA and heart will come out of the body cavity.

1.4.1. Carefully separate the BA from the aorta by pinching the forceps and locating the atrium's tip into which multiple venous branches converge (sinus venosus). Using the fine forceps, 'pluck' the tip of the atrium off the sinus venosus. This isolates the CV tissues from the rest of the body (**Figure 1**).

2. Dissociation of cardiomyocytes from adult, juvenile, and larval zebrafish for electrophysiological studies

2.1. Using the fine forceps, 'pluck' the atrium and ventricle out of the CV tissue isolated in the earlier step.

NOTE: The process allows for clean dissection of each tissue with minimal cross-contamination and feels akin to plucking fruit from a tree.

2.1.1. Make a gentle tear into the ventricle using fine forceps to drain excess blood, which can be ensured when the cardiac tissue turns pale salmon color from bright red.

2.2. Pool the isolated atrium and ventricle into separate 1.5 mL centrifuge tubes containing Perfusion Buffer (PB; 1.5 mL).

NOTE: To guarantee successful dissociation of adult zebrafish atrial cardiomyocytes (ACMs) and ventricular cardiomyocytes (VCMs), typically four atria and three ventricles are needed. In the case of juvenile zebrafish (28-90 days), this number is doubled.

2.2.1. In the case of larval zebrafish (14–28 days), collect as much tissue as possible from ~30 larvae.

2.3. Replace the PB in the tubes with 750 µL of Digestion Buffer (DB) (**Table 1**) each and place the tubes on a thermoshaker at 37 °C and 800 rpm. Allow digestion of the tissues until they become translucent (~30 min for the atria and ~40 min for the ventricles).

2.4. End the digestion by gently spinning down the tissues in a benchtop mini centrifuge (2,000 x *g* at ambient temperature) for 3-5 s and replace the supernatant DB with 750 µL of Stopping Buffer (SB) each (**Table 1**).

NOTE: This centrifugation step is optional for VCM isolation.

2.5. Incubate the pelleted tissues in the SB at ambient temperature for 15 min.

2.6. Gently replace the SB with 500-750 µL of PB each (depending on the desired density of the final cells) and triturate the tissues (~30 times) using a flame-polished Pasteur pipette to disperse the cells.

NOTE: The cardiomyocytes (CMs) are now ready for electrophysiological studies and stored at room temperature for up to 8 h.

2.7. For uniform sampling, gently pipette the cells up and down a couple of times to resuspend before adding a drop of them for corresponding studies.

3. Dissociation of vascular smooth muscle (VSM) cells from adult and juvenile zebrafish for electrophysiological studies

3.1. Pluck bulbous arteriosus (BA) from the CV tissues using the same approach as step 2.1 and pool five adult BAs into a 1.5 mL centrifuge tube containing S1 buffer (1.5 mL) (**Table 2**). In the case of juvenile zebrafish, pool ten BAs and for larvae older than 2 weeks, pool 30-40 BAs.

NOTE: It is not practically feasible to isolate VSM cells from zebrafish larvae younger than 2 weeks.

3.2. Replace S1 with 400 µL of S2 buffer (**Table 2**) and allow papain (see **Table of Materials**) digestion of the BAs on a thermoshaker at 37 °C and 800 rpm for ~20 min.

3.3. Allow the partially digested BAs to settle down for 1 min and replace the supernatant S2 with 500 µL of S3 buffer (**Table 2**) containing collagenase. Digest the tissues on a thermoshaker at 37 °C and 800 rpm for 3-5 min.

3.4. End the digestion by gently spinning down the tissues in a benchtop mini centrifuge (2,000 x *g* at ambient temperature) for 3-5 s and replacing supernatant S3 with 500 µL of S1.

3.5. Gently triturate the pelleted tissues (~15 times) to disperse VSM cells and plate onto glass coverslips of appropriate size.

NOTE: The coverslip size is determined by the size of the patch-clamp recording chamber, in this case, 5 x 5 mm was used).

3.5.1. Keep the coverslips at room temperature for 30 min for the cells to attach and use them within the next 6 h.

4. Isolation of hearts from embryonic zebrafish for electrophysiological studies

4.1. Add tricaine (150 mg/L; 1 ml per 100 mm x 20 mm Petri dish) to ~300 embryos (2-5 dpf), collected from their incubated condition (**Figure 2A**).

4.1.1. Concentrate the anesthetized embryos by transferring them to a 5 mL centrifuge tube using a transfer pipette, removing excess media (E3, **Table 2**) from the centrifuge tube (**Figure 2B**).

4.2. Wash the embryos with 3 mL of cold Perfusion Buffer (PB) twice and resuspend in 2 mL of PB (**Figure 2B**).

4.3. Using the embryonic heart isolation apparatus (**Figure 2C**), use the syringe to draw ~1 mL of the embryos into the needle and immediately expel them back into the tube. Repeat this process 30 times, at a rate of 1 s per syringe motion (**Figure 2C**).

4.4. Pass the fragmented embryos through a 100 µm cell-strainer sieve placed in a funnel and collect the filtered hearts in another 5 mL centrifuge tube. Rinse the syringe with 1 mL of PB and collect this rinse as well into the tube through the sieve (**Figure 2C**).

4.5. Mix well and separate into 1.5 mL centrifuge tubes, with each tube containing 1 mL of the contents. Centrifuge all the tubes on a benchtop mini centrifuge for 5 s (2,000 x *g* at ambient temperature) and discard the supernatants.

4.6. Carry out serial resuspension of the pellets in the tubes using 1 mL of PB, i.e., resuspend the pellet in one tube using 1 mL of PB and use that resuspended PB to resuspend the pellet in the next tube.

4.7. Gently pipette the hearts up and down two times before adding a drop of them for corresponding studies.

5. Patch-clamp studies on isolated cardiovascular cells

NOTE: Inside-out and whole-cell patch-clamp recordings from the isolated cells can be obtained as previously reported for KATP channel currents in zebrafish cardiovasculature[9].

5.1. For adult and juvenile cardiomyocytes, add the dissociated cells (from step 2) directly to the recording chamber from suspension. For VSM cells, place the coverslip containing the plated VSM cells (from step 3) into the recording chamber.

5.2. Add isolated intact embryonic hearts (from step 4) to the chamber from suspension, and make patch-clamp recordings from the epicardial myocytes (**Figure 2D**).

REPRESENTATIVE RESULTS:

The above protocols reliably and routinely provide sufficient cardiac and vascular myocytes of consistent quality amenable for patch-clamp studies as recently reported in extensive studies of ATP-sensitive potassium (K_{ATP}) channels in wild-type and mutant zebrafish cardiovasculature[9]. Representative traces of recordings of such K_{ATP} channel activity from isolated cardiomyocytes are shown in **Figure 3A-C**. In the case of cells isolated from bulbous arteriosus, the vascular smooth muscle cells were confirmed by *Tg(tagln:egfp)* expression[11,12] (**Figure 1**). Successful single-channel recordings were obtained (n = 8 recordings from N = 5 preparations) in patches excised from the embryonic hearts (**Figure 3D**). Action potentials were recorded from isolated

adult zebrafish ventricular and atrial myocytes in whole-cell, current-clamp mode, using a patch-clamp amplifier along with a compatible digitizer (see **Table of Materials**). The extracellular recording solution for the action potential (AP) measurements contained NaCl (140 mM), KCl (4 mM), $CaCl_2$ (1.8 mM), $MgCl_2$ (1 mM), Glucose (10 mM), HEPES (10 mM), and Blebbistatin (0.01 mM, pH 7.4); the intracellular recording solution contained KCl (120 mM), EGTA (5 mM), K_2ATP (5 mM), $MgCl_2$ (5 mM), and HEPES (10 mM, pH 7.2). Patch pipettes were pulled from soda-lime glass and had resistances of 3 – 5 MΩ when filled with intracellular solution. Ventricular myocytes exhibit stable hyperpolarized membrane potentials, and action potentials were stimulated *via* current injection through the patch pipette (**Figure 3E**), whereas atrial myocytes exhibited spontaneous action potential firing (**Figure 3F**). Action potential properties are summarized in **Table 3**.

FIGURE AND TABLE LEGENDS:

Figure 1: Isolation of atrial, ventricular, and bulbous arteriosus cells. Schematic to illustrate the isolation of atrial (**A**), ventricular (**B**), and bulbous arteriosus (**C**) cells. The images depict each isolated cell type (bottom) have scale bars = 50 µm in each case. BA cells isolated from smooth muscle cell transgenic reporter zebrafish lines (*Tg(tagln:egfp)*[11,12]) are positive for green fluorescence.

Figure 2: Schematic to illustrate the isolation of embryonic hearts. (**A**) Fertilized embryos are removed from the breeding tank and placed in a Petri dish containing E3 water and maintained at 28 °C for up to 5 days. (**B**) At desired age, embryos are anesthetized *in situ* using tricaine and transferred into PB in 5 mL centrifuge tubes for dissociation. (**C**) Embryonic heart isolation apparatus consists of a 10 mL syringe attached to a 19 G needle mounted above the dissociation tube on a bench stand, allowing the hands to perform the trituration. (**D**) Image of the isolated heart (day 4) attached to a patch-clamp pipette.

Figure 3: Representative ion channel recordings from isolated zebrafish cardiovascular tissues. (**A, B**) ATP-sensitive K_{ATP} channels from inside-out excised patch-clamp recordings of atrial (**A**) and ventricular (**B**) cardiomyocytes isolated from adult zebrafish. (**C**) K_{ATP} channel conductance was recorded from whole-cell patch-clamping of VSM cells isolated from adult zebrafish. (**D**) Single K^+ channel activity in membrane patches excised from zebrafish embryonic hearts (4 dpf). All excised patch recordings were made with 140 mM KCl on both sides of the membrane, at -50 mV membrane potential. Whole-cell currents were recorded with the membrane potential clamped at -70 mV. (**E**) Example current-clamp recording from the isolated ventricular myocyte. Action potentials were stimulated by current injection as shown, for example, at the bottom. (**F**) Example current-clamp recording from isolated atrial myocyte showing spontaneous action potential firing.

Table 1: Solutions for isolation of Zebrafish cardiomyocytes.

Table 2: Solutions for isolation of Zebrafish VSM cells.

Table 3: Action potential properties from isolated Zebrafish cardiomyocytes. Recorded in current clamp mode. Data shown as mean ± S.D. V_m = membrane potential; APA = action potential amplitude; APD50 = duration of action potential to 50% repolarization; MDP = maximal diastolic potential.

DISCUSSION:

Previous methods for isolating ventricular myocytes[5,6], aimed at generating myocytes for culture or electrophysiological studies, provided cells of lower yield and involved lengthy steps of multiple centrifugations that adversely affected the cell quality and viability. The protocols described here are reliable, cover each of the significant cardiovascular tissues (ventricle, atria, and VSM), and importantly are pretty practical for acute isolation of live cells. Isolation approaches involving cannulation of the ventricle *via* the BA[13] can be a sophisticated alternative for ventricular cardiomyocytes but require a higher degree of dexterity and may negatively affect atrial isolation. The approach detailed in the current protocol provides a much simpler and efficient alternative with minimal mechanical disruption and appropriate enzymatic dissociation, followed by trituration, which may be less stressful to the cells.

Additional considerations for larval cardiac tissue isolation are: (1) In step 1.4, depending on the age of the fish and magnification available, the tissues might be visible even before removing the pectoral muscles, in which case, it is better to directly 'pluck' the tissues out of the fish without prior surgery, and then clean off the non-CV tissues using superfine forceps; (2) In step 2.2, for larvae younger than 14 days, the hearts can be used directly for electrophysiological studies without any need for enzymatic dissociation.

As noted above, and typically true of enzymatic dissociation methods in general, it has proven essential to use fresh buffers and enzymes each time. However, Perfusion Buffer (PB) and S1 Buffer can be prepared in advance and stored at 4-8 °C for 1 month.

Successful tissue isolation time should not exceed ~90 s per fish, and tissues should not be exposed to air. When dissociation takes longer, the cell quality (i.e., survival) is reduced. When triturating tissues, care should be taken to be gentle enough to avoid generating air bubbles which also reduces cell quality. VSM cell isolation is sensitive to the digestion by collagenase in S3 buffer. After the first 3 min of digestion, the tube should be taken out of the thermoshaker every minute and examined for floating dilated and translucent tissues. Once tissue fragmentation is apparent, the digestion step should be ceased.

It is important to note that these protocols are not extensively optimized for isolating calcium-tolerant cardiomyocytes, as required for contractility studies. However, as shown, reliable recording of cardiomyocyte action potentials can be achieved in the presence of physiological extracellular calcium concentrations[7,14]. Blebbistatin, included to decouple excitation and contraction, and improve giga-Ohm seal formation and recording efficiency in these experiments, has previously been shown to not significantly affect the AP parameters in intact zebrafish hearts[14]. For electrophysiology, the absolute yield of cells is also not routinely rate-limiting. This protocol has not been optimized for yield, as might be necessary for biochemical studies of the

isolated cells. Still, the yield achieved with these methods is well suited for electrophysiological studies and likely multiple other approaches.

ACKNOWLEDGMENTS:

This work was supported by NIH grants HL140024 to CGN and HL150277 to CMC. Figure 1 and Figure 2 were created with BioRender.com.

DISCLOSURES:

The authors declare no competing financial interests.

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Biochemistry. **25** (4-5), 419-424 (2010).

Figure 1

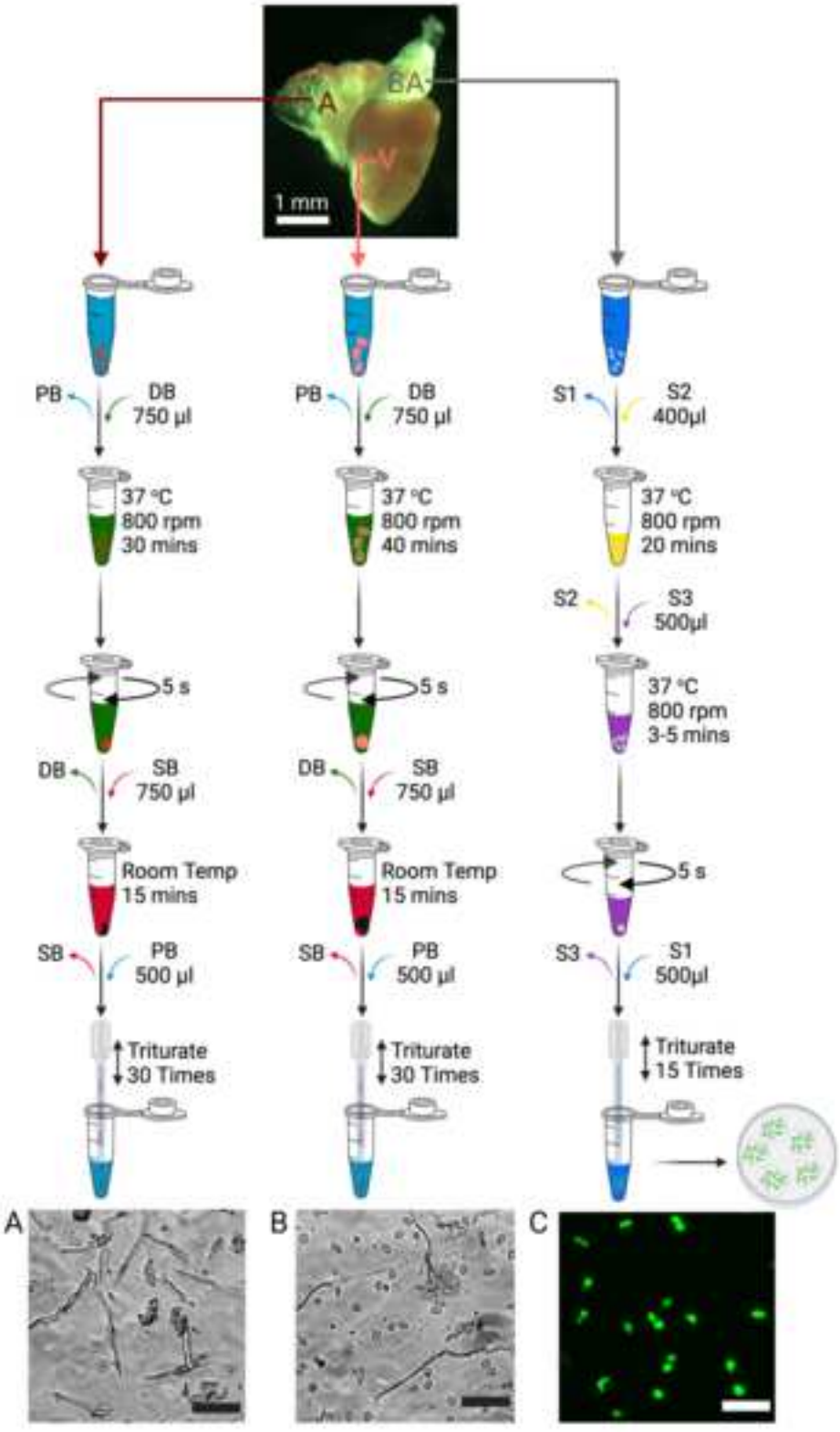


Figure 2

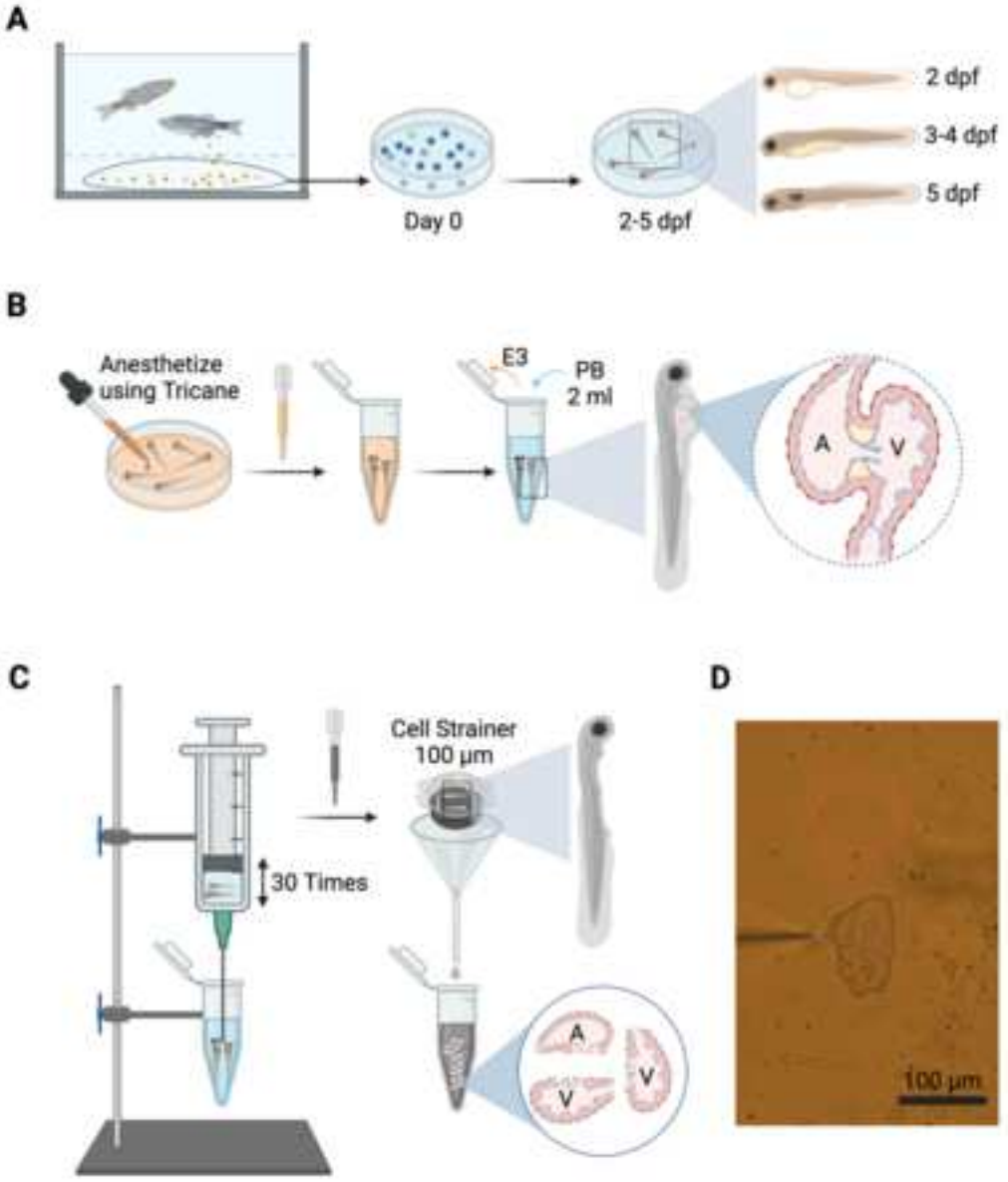
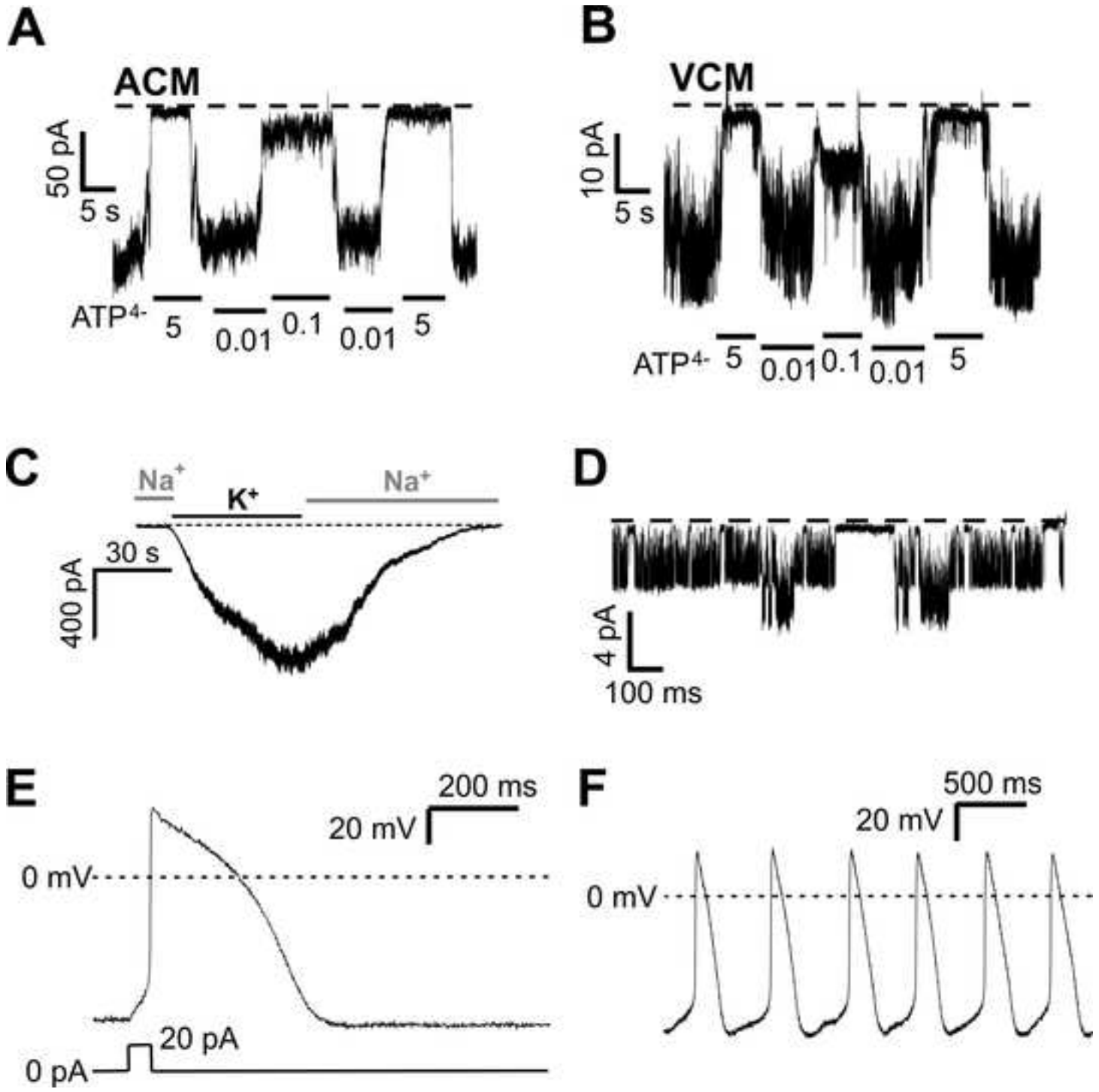


Figure 3



PERFUSION BUFFER (PB)	10 mM HEPES, 30 mM Taurine, 5.5 mM Glucose, 10 mM BDM. Make the solution in phosphate-buffered saline (PBS), adjust pH to 7.4
DIGESTION BUFFER (DB)	PB + 12.5 µM $CaCl_2$ + 5mg/mL Col II + 5 mg/mL Col IV + 5 ng/mL Insulin
STOPPING BUFFER (SB)	PB + 10% FBS + 12.5 µM $CaCl_2$ + 10 mg/ml BSA + 5 ng/mL Insulin

Table 2

S1 BUFFER	0.1% BSA (w/v), 145 mM NaCl, 4 mM KCl, 10 mM HEPES, 10 mM Glucose, 0.05 mM $CaCl_2$, 1 mM $MgCl_2$, adjusted to pH 7.4 using NaOH
S2 BUFFER	2 mL S1, 4 mg Papain, 2 mg DTT
S3 BUFFER	2 mL S1, 3 mg Collagenase Type H, 2 mg Trypsin Inhibitor, 1 mg Elastase

Table 3

Ventricular Myocytes (n = 3 Recordings)		
Vm (mV)	**APA (mV)**	**APD50 (ms)**
-75.7 ± 3.9	-119.6 ± 3.8	329 ± 163

Atrial myocytes (n = 3 Recordings)			
AP Rate (min^{-1})	**MDP (mV)**	**APA (mV)**	**APD50 (ms)**
107.3 ± 32.6	-71.2 ± 5.1	98.4 ± 4.7	141 ± 12

Response to Editorial and Reviewer comments

Original comments in black, responses in red

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Done.
2. Please concise the manuscript title to make within 150 characters in length, since shorter titles draw more readers and are easier to search. You may remove Danio rerio from the Title. Reduced to 139 characters.
3. Please provide a 150- 300-word Abstract. The current Abstract is 96 words. Increased to 155 words.
4. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.). Done.
5. Please ensure that the Introduction includes all of the following along with relevant citations:
a) A clear statement of the overall goal of this method
b) The rationale behind the development and/or use of this technique
c) The advantages over alternative techniques with applicable references to previous studies
d) A description of the context of the technique in the wider body of literature
e) Information to help readers to determine whether the method is appropriate for their application. The Introduction has been rewritten to include all these points, and with relevant citations added.

6. Please ensure that abbreviations are defined at first usage. Done.
7. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. Done.
8. The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion. The pertinent sections have been moved to the Discussion.
9. 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 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. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Relevant additional information is now added.
10. Please add more details to your protocol steps:
Step 2.1: How to ensure that the excess blood is completely drained out? Done.
Step 2.4/3.4: Please specify the centrifugation speed in x g and the temperature. Done.
Step 2.7: How long can the samples be kept at room temperature? We have added comment on how long they can be left at RT.
Step 3.5: Please mention the size of the coverslip used in this work. Done.
Step 4.1: What was the size of the Petridish used here? Added and updated the table of materials.
Step 4.4: Please provide the details of the sieve used in the Table of Materials. Done.
11. 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 and it should also be in line with the Title of the manuscript. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader. Done. These are highlighted in yellow.
12. Please modify the Result section to include all the observations and conclusions you can derive from the Figures. The Results section should focus on the effectiveness of your technique backed up with data. We have added additional data showing action potential recordings from atrial and ventricular myocytes as suggested by reviewers. These experiments are displayed in figure 3E-F and Table 3.
13. Figure 1: Please mark (A), (B), (C) panel in the Figure and explain in the legend the cell types isolated for each. Done.
14. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
(a) Critical steps within the protocol
(b) Any modifications and troubleshooting of the technique
(c) Any limitations of the technique
(d) The significance with respect to existing methods
(e) Any future applications of the technique The Discussion has been revised to more clearly address each of these points.
15. Line 232: The statement regarding BioRender can be included either in the respective Figure legends or in the Acknowledgement section. Please remove that from the Disclosure section. Done.
16. Please include a Disclosures section, providing information regarding the authors' competing financial interests or other conflicts of interest. If authors have no competing financial interests, then a statement indicating no competing financial interests must be included. Done.

Reviewer #1:

Manuscript Summary:

This study reports enzymatic protocols for isolation of ventricular and atrial cardiomyocytes, as well as vascular smooth muscle cells from juvenile and adult zebrafish. It is suggested that the isolated cells are suitable for patch-clamp experiments. Due to the small size of zebrafish, isolation of cardiac myocytes is more difficult than in larger animals. Therefore, improved methods for isolating cardiomyocytes and smooth muscle cells are welcome. However, it seems to me that the electrophysiological properties of the isolated cardiac myocytes and smooth muscle cells have not been sufficiently characterized to be considered as an alternative to the existing isolation methods. I encourage the authors to provide a more detailed analysis of ion currents in isolated myocytes and smooth muscle cells, particularly whole-cell ion currents and action potentials. Morphology of isolated cells should be shown in high quality images.
We appreciate the reviewer's comments. Since we have not exhaustively tested other published methods in which cells were isolated for non-electrophysiological, primarily biochemical, studies, we cannot readily compare 'quality' of isolated cells. Rather, we report our methods for their practical simplicity. In revising the manuscript, we have included more data and added a new results figure to illustrate the suitability of the cells for patch-clamp analysis.

Major Concerns:
Lines 37 and 38. "…for isolation of ventricular cardiac myocytes, but atrial myocytes have not been isolated, nor any vascular smooth muscle (VSM) preparation". There are several published articles on the action potential and ion currents of zebrafish cardiac myocytes, some of which include atrial myocytes (e.g., Nemtsas et al.). In particular, ion currents and action potentials of ventricular action potentials have been described in relatively great detail. We apologize for the poor wording here, which was meant to indicate the limited number of detailed published methods. We have revised the sentence to avoid misleading (lines 41 – 45).

Lines 163 and 164, and Figure 1. Due to poor resolution, it is almost impossible to evaluate the morphological quality of the preparations. However, it seems to me that cardiac tissue is not properly dissociated into single cells. Cardiomyocytes should be long, narrow and cross-striated. It seems as if the cells are rounded up and most of the preparation is groups of several cells? Better quality figures need to be provided. It difficult to say anything about the morphology of smoot muscle cells: they should be very long and very narrow. It would be important to mention something about the morphology of the cells in Results. We have added higher resolution images to indicate the cell quality. For cardiac atrial and ventricular myocytes, they are indeed long, narrow and cross-striated. In our hands, acutely isolated smooth muscle cells appear rounded during recording periods – this is true for mammalian as well as zebrafish cells. Only after longer culturing than we used (> 6 hours) do isolated smooth muscle cells take on a characteristic elongated shape.

Figure 2. In figure 2 the diagrams of atria and ventricle should be replaced by actual images of the corresponding organs. In order to image both the chambers *in vivo* at embryonic stage, the fish must be fixed in a particular orientation using agarose and unfortunately, we are unable to carry this out at this time.

Figure 3. Electrophysiological results show that the isolated cells are suitable for whole-cell and single channel recording of ATP-sensitive potassium currents. This may not, however, be a sufficient criterion for most other electrophysiological experiments. If current methods are intended to improve existing isolation methods for electrophysiological experiments, better criteria are needed. The fast component of the delayed rectifier potassium current (IKr) is shown to be sensitive to enzymatic isolation methods, and some previous studies have had difficulties in recording IKr in zebrafish atrial and ventricular myocytes under generally accepted experimental conditions (Nemtsas et al.). Therefore, it would be important to show that IKr can be recorded in atrial and ventricular myocytes under the whole-cell conditions. Another critical experiment is recording of sodium current (INa) under whole-cell conditions. Recording of INa requires negative hold potentials (-120 mV) and is therefore a good test for the robustness of the myocytes. Perhaps the most convincing indication for the robustness of the cells is generation of normal action potentials under whole-cell conditions. Rather than isolate individual currents under voltage clamp, we have, as suggested, illustrated action potentials measured under whole-cell current clamp conditions. We can routinely measure stable triggered action potentials from ventricular myocytes and spontaneous action potentials from atrial myocytes.

Lines 25-28 "We have developed modifications of existing protocols and developed new enzymatic protocols that routinely provide isolated juvenile and adult zebrafish ventricular and

atrial cardiomyocytes, as well as VSMs from the bulbous arteriosus, suitable for patch-clamp experiments." Lines 222 and 223. "It is perhaps important to note that our protocols are not designed, nor optimized, for isolation of calcium-tolerant myocytes, as would be required for contractility studies." If the cells are calcium-intolerant, they are probably unsuitable for most types of whole-cell patch-clamp experiments (not only for contractile studies). It should be therefore explicitly stated that the current preparations are suitable only for certain types of electrophysiological experiments and not useful in replacing the existing cell isolation methods used for ion current measurements in adult zebrafish atrial and ventricular myocytes. Or alternative, the authors should perform the experiments mentioned in the previous paragraph in order to show that that the current preparations really improve existing methods. We acknowledge that this protocol has not been *extensively* optimized for calcium-tolerant myocytes but we have modified the sentence to discuss how we are able to record action potentials in the presence of physiological extracellular calcium.

Reviewer #2:

Summary of the paper:

In this manuscript, Soma S. Singareddy and colleagues describe techniques for manual excision of zebrafish hearts and isolation of atrial, ventricular, and VSM cells from adult, juvenile, larval and embryonic zebrafish. The study describes adapted and extended procedures using enzymatic digestion and mechanical forces to collect viable cells for electrophysiological experiments. The authors show the use of acutely isolated cells by measuring K-sensitive currents from cardiac and vascular smooth muscle cells.

Comments:

The method developed by the authors for isolating cardiac and vascular smooth muscle cells is very simple and straightforward. Each step has been explained well. This provides a seemingly low-cost and rapid method of isolating cardiac myocytes and VSM cells. Isolation procedures for viable atrial and VSM cells have not previously been reported. The methodological descriptions for isolation of cells from larval fish is valuable given the advantages of cardiac research with embryonic fish.

1. The research group has extensive expertise in measuring KATP channel activity. However, is this the best assessment of whether isolated cells are ventricular vs atrial? Using the trasngelin line gives good indication that GFP+ cells are smooth muscle cells. It isn't clear however, how well the techniques are able to distinguish atrial vs ventricular cells. Can this be revealed from KATP current density? Might action potential recordings, immunological detection of tissue specific markers, or morphological features provide greater confidence in the nature of the cell type isolated? As noted, the atrium and ventricles were carefully separated at the A-V junction, making tissue cross-contamination highly unlikely. As evidenced in greater detail in our new *J Physiol* publication (Singareddy *et al*., 2021; Reference 9), there are differences between atrial and ventricular K_{ATP}, primarily in current density. As we now also show in Fig. 3E-F and Table 3, action potential characteristics are different, with stable resting potentials in ventricle, and pacemaker potentials evident in atrium.

2. In section 4.4, how well does this approach isolate just hearts? How is other non-cardiac tissue that is too large to pass through the filter removed? It has previously been shown that this syringe-based approach results in the successful and specific excision and purification of hearts

from embryonic zebrafish (Reference 10; Burns et al., 2006). The hearts are excised and remain intact as shown in Figure 2D with other tissues of the embryo intact. We have been able to confirm this using *Tg(αmhc:gfp)* zebrafish lines. The characteristic size and shape of the heart as shown in Figure 2D make selection of hearts simple for further steps.

3. There isn't description about how the isolated cardiomyocytes were replated for electrophysiological study. Was a matrix used to coat the glass? Cells were added in suspension and not plated; we have added these details.

4. Line 135: suggest to include percent tricaine used for anaesthetizing embryonic fish. Added.

5. Figure 3 legend: suggest to include electrode resistance range used for the patch clamp experiments. Added.

6. Before transferring the isolated cardiac tissue to perfusion buffer the authors seem to be keeping the tissue in water. Is there a specific reason for not keeping the fish in the buffer during isolation of the heart tissue? We have reworded to make clear that the tissue indeed maintained in perfusion buffer and not water.

7. Given the comment in the discussion about the importance of care in the centrifugation steps, it would be helpful to describe the centrifugation speed/force in sections 2.4, 3.4, 4.5. Added.

8. Line 168: Seems more appropriate for lab book notation and less so for publication. This has been removed.

Reviewer #3:

The authors describe the isolation of atrial, ventricular, and vascular smooth muscle (VSM) cells from zebrafish, along with the partial isolation from larval and embryonic hearts. They show successful isolation of all these cells and verify by conducting excised patch voltage-clamp technique. The isolation of atrial and VSM cells from zebrafish is new and novel.

However, there are major concerns of this protocol:

The authors only provide one trace example to show the reliability of the experiments. While I understand the difficulty of the excised patch technique, a minimum n value of recordings is required to quantify the viability of any protocol. How many times has the isolation been carried out? Our new publication (Reference 9) describes the results of using these techniques to thoroughly characterize zebrafish ATP-sensitive potassium channels in the cardiovascular system. These studies included > 30 isolations of each tissue resulting in viable cells on > 95% of occasions. We are now able to add reference to these studies (Reference 9) and have also added more data and a new figure, as well as more detail in the representative results, to address this.

The number of hearts/animals required for each isolation is impractical and does not align with the 3Rs of animal research. 4 adult fish for atria or ventricle; 8 fish if they are juvenile; 10 fish for bulbous arteriosus - to do one day of the experiment. In addition, the authors mention that the

protocol has not been optimised for yield while stating "seems to be quite high" is very vague. What is the total viable cell count? These are crucial as each failed isolation leads to 4-10 dead fish. We have added more detail to address this. Given that zebrafish breed in enormous numbers (~500 per clutch), then assuming easy genotyping strategies (i.e. avoiding the need to genotype experimental animals), then the numbers are actually quite practical, so long as (as we show) the isolation from each animal is quick.

They mention that their protocol is NOT optimized for calcium-tolerant myocytes. This in line with previous point, defeats the purpose of isolating atrial and ventricular cells if one cannot perform action potential or other ion channels patch recordings. We acknowledge that this protocol has not been *extensively* optimized for calcium-tolerant myocytes but we have modified the sentence to discuss how we are able to record action potentials in the presence of physiological extracellular calcium.

The authors claim the previous isolation methods used by Brette (2008) and Sander (2013) are impractical due to the use of centrifuge steps that are detrimental for cells. However, the authors use the similar technique of enzymatic digestion and table-top centrifuge albeit for shorter duration. Also, it is worth citing the recent publication by Kompella, S.N., et al., 2021 wherein enzymatic digestion by cannulation of bulbous arteriosus method was used, showing isolation of ventricular cells from just a single zebrafish ventricle. These are good points. We have added the suggested citations.

Overall, the authors present a detailed protocol for the isolation of atrial and VSM that has not previously been reported. However, the lack of detailed functional characterization and optimization of the protocol diminishes the reliability and efficacy of this protocol.