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Preparation of viable myocardial slices from biopsies of neonates and infants with congenital heart disease for physiological measurements --Manuscript Draft--

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| Abstract: | <p>In cardiovascular research, diverse ex vivo models are utilized to investigate cardiac function. These models can be categorized according to their complexity ranging from isolated cardiomyocytes to multicellular 3-dimensional tissue preparations such as the Langendorffperfused heart or coronary-perfused wedges. Cardiac tissue slices bridge the gap between these models as their relatively low thickness overcomes the need for arterial perfusion while native cellular alignment and extracellular matrix structure are preserved. This enables the use of tissue when coronary perfusion is not available, e.g. tissue excised during surgery for congenital heart disease. The present protocol describes the preparation of viable cardiac slices from myocardial explants from neonates and infantile patients undergoing surgery for congenital heart disease. Upon extraction, myocardial tissue is transferred into</p> |

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| | <p>oxygenated ice-cold low-calcium solution and is transported to the laboratory. Thereafter, the tissue is pre-cut, embedded into low-melting agarose and sectioned with a vibratome. Tissue recovery is promoted by stepwise increase of calcium concentration followed by gradual rewarming to 37 °C for 1 h in the measurement solution. Afterwards, the obtained acute myocardial slices can be used for physiological experiments. Representative results for isometric force measurements and action potential recordings are provided. The importance of the used solution and vibratome parameters for preparation of viable cardiac slices as well as limitations regarding control of fiber alignment and long-term culture are discussed.</p> |
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Köln, 07.04.2017

Dear Dr Mukherjee,

Please find enclosed a revised version of our manuscript entitled „Preparation of viable myocardial slices from biopsies of neonates and infants with congenital heart disease for physiological measurements“.

We revised the discussion and conclusions according to the reviewers' comments and we have the feeling that the conclusions are now much more adequate.

We hope that this revised version of our manuscript will be considered appropriate for publication in *JoVE*.

All authors have read and approved the submission of the manuscript. The manuscript has not been published and is not being considered for publication elsewhere in whole or part in any language.

Sincerely yours,



Tobias Hannes, MD

TITLE:

Preparation of Acute Myocardial Tissue Slices from Biopsies of Human Neonates and Infants with Congenital Heart Disease for Physiological Measurements

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tissue slices, myocardium, human, Tetralogy of Fallot, hypoplastic left heart syndrome, isometric force measurements, cardiac electrophysiology

SHORT ABSTRACT:

Cardiac slices are a unique model for cardiovascular research and bridge the gap between single-cell and whole-heart models. This protocol describes the preparation of viable cardiac slices from myocardial tissue samples excised during surgery for congenital heart disease.

LONG ABSTRACT:

In cardiovascular research, diverse *ex vivo* models are used to investigate cardiac function. These models can be categorized according to their complexity, ranging from isolated cardiomyocytes to multicellular 3-dimensional tissue preparations, such as the Langendorff-perfused heart or coronary-perfused wedges. Cardiac tissue slices bridge the gap between these models, as their relatively low thickness overcomes the need for arterial perfusion, while the native cellular alignment and extracellular matrix structure are preserved. This enables the use of tissue when coronary perfusion is not available (*e.g.*, tissue excised during surgery for congenital heart disease). The present protocol describes the preparation of viable cardiac slices from myocardial explants from neonate and infant patients undergoing surgery for congenital heart disease. Upon extraction, the myocardial tissue is transferred to oxygenated, ice-cold, low-calcium solution and transported to the laboratory. Thereafter, the tissue is pre-cut, embedded into low-melting agarose, and sectioned with a vibratome. Tissue recovery is promoted by the stepwise increase of calcium concentration, followed by gradual rewarming to 37 °C for 1 h in the measurement solution. Afterwards, the obtained acute myocardial slices can be used for physiological experiments. Representative results for isometric force measurements and action potential recordings are provided. The importance of the solution and the vibratome parameters to the preparation of viable cardiac slices, as well as limitations regarding the control of the fiber alignment and long-term culture, are discussed.

INTRODUCTION:

Ex vivo cellular studies on myocardial function rely on a spectrum of models, ranging from isolated single cells to whole-heart preparations such as the Langendorff-perfused heart.

Although isolated cardiomyocytes are the key model for many research questions, they do not entirely reflect the *in vivo* situation because intercellular interactions and connections to an extracellular matrix (ECM) are missing^{1,2}. Moreover, enzymatic digestion during the dissociation of myocardial tissue can modify the electrophysiological properties of cardiomyocytes. For instance, Yue *et al.*³ demonstrated that delayed rectifier potassium channel configuration was dependent upon the isolation protocol.

Multicellular preparations, such as the Langendorff-perfused heart or coronary-perfused wedge preparation, on the other hand, provide cardiomyocytes in their native cellular and extracellular environment. This allows for the investigation of phenomena that require interactions such as the development of arrhythmia. To provide proper oxygenation and to cover the metabolic demands, they require arterial perfusion due to their relatively great thickness^{1,4}. This restricts the use of these techniques, especially in human myocardial preparations, as explanted whole hearts or at least large tissue samples with an intact coronary artery are required.

Organotypic tissue slices have been a popular *in vitro* model for physiological and pathophysiological investigations for many decades. Although well-established for organs such as the brain, liver, and kidney, the use of slice preparations for functional cardiovascular research has gained more interest only recently^{1,5}. With a few exceptions¹, myocardial slices have extended the methodological repertoire of cardiovascular research only in the last decade. Many studies have demonstrated that viable cardiac tissue slices of high integrity can be obtained from diverse species, including mouse⁶⁻⁸, dog⁴, guinea pig^{2,9}, rabbit², zebrafish¹⁰, and human^{4,5,11}, and at different developmental stages.

Since the precision vibratome sectioning of slices thinner than 400 µm is feasible, adequate oxygenation and nutrient supply by diffusion can be ensured for cardiac slice preparations¹. Cardiac tissue slices show a more *in vivo*-like profile in terms of cell composition and extracellular matrix than single cardiomyocytes or cell culture models². As arterial perfusion is not required, this preparation technique can be used for small patient biopsies.

The present protocol describes a method to prepare viable myocardial slices obtained from right ventricular tissue samples. These biopsies are an essential component during surgery on neonate and infant patients with hypoplastic left heart syndrome (HLHS) and Tetralogy of Fallot (TOF), respectively, and are discarded if not used for experimental purposes.

PROTOCOL:

This study was approved by the local ethics committee of the Medical Faculty of the University of Cologne (reference no. 07-045) and complied with the World Medical Association Declaration of Helsinki (7th revision, Fortaleza, Brazil, 2013). Written informed consent was given by the parents of each patient.

1. Laboratory Preparation

1.1. Switch on the water bath that controls the temperature of a custom-made jacketed vessel (**Figure 1**). Set the temperature to 42 °C to maintain the temperature of melted agarose in a beaker, which will be inserted later, at 37 °C .

1.2. Add 500 µL of the calcium solution to 1 L of solution A (**Table 1**) to obtain a low-calcium solution with a Ca^{2+} concentration of 0.05 mmol/L.

1.3. Fill the vibratome chamber with low-calcium solution A. Oxygenate the solution in the vibratome chamber by bubbling with pure O_2 . Cool the solution around the vibratome chamber with ice.

1.4. Insert a new steel blade according to the manufacturer's manual.

Note: The steel blade must be handled carefully to prevent injury. One blade is sufficient for the whole slicing procedure on one experimental day.

1.5. Prepare the “slice collector” tool by carefully breaking the tip of a glass Pasteur pipette and mounting a small Peleus ball on the broken side. Use a glass cutter to make a score on one side, close to where the taper starts. Wrap the pipette in paper tissue and gently press at the point of the score to break the glass.

Note: Breaking glass and handling broken glass increases the risk for injuries. Work carefully and wear protective clothes (*i.e.*, cut-resistant gloves and goggles). Alternatively, the opening of the plastic Pasteur pipettes can be widened by cutting them with scissors. However, the tissue slices tend to stick to the inside of plastic Pasteur pipettes more frequently than to glass Pasteur pipettes.

1.6. Weigh 0.4 g of low-melting agarose into a beaker and add 10 mL of low-calcium solution A and a magnetic stir bar.

1.7. Transfer the beaker to a microwave, set the power to 750 W, and heat for 10 s. Swirl the beaker a few times and heat for an additional 5 s to completely dissolve the agarose. Cover the beaker opening with aluminum foil.

1.8. Transfer the beaker into the custom-made jacketed vessel, which stands on a magnetic stirring device. Stir the agarose briefly at a high speed and then at a moderate speed to avoid bubble formation in the agarose. Keep the temperature at 37 °C.

1.9. Fill a “collection beaker” with 40 mL of low-calcium solution A and store it on ice. Oxygenate by bubbling with pure O_2 .

1.10. Store a 10-cm Petri dish filled with 10-15 mL of oxygenated low-calcium solution A on ice.

1.11. Fill a small “transport Erlenmeyer flask” with low-calcium solution A and place it in a portable container with ice. Cover the Erlenmeyer flask opening with aluminum foil or thermoplastic paraffin sealing film.

2. Transferring the Tissue from the Operating Room to the Laboratory

2.1. Arrive at the operating room early. Define a time trigger indicating when to leave the lab depending upon local circumstances.

Note: Here, researchers leave the lab approximately at the time of the sternotomy.

2.2. Upon arrival to the operating room, briefly discuss the tissue transfer with the surgeon’s assistant at a convenient moment, specifically concerning the proper waiting area, the transfer procedure, and the signal for tissue receipt.

Note: It is crucial that the tissue pieces are transferred into low-calcium solution A immediately after excision. Arranging with the surgeon’s assistant helps to minimize the time delay between the myectomy and the transfer of the tissue into the solution without interfering with the sterility of the procedure.

2.3. Start oxygenating the ice-cold low-calcium solution A upon arrival to the operating room using a portable oxygen cylinder.

2.4. Before the surgeon starts the myectomy, take the transport Erlenmeyer flask out of the ice and wipe it dry. Wait at the arranged position until the surgeon’s assistant is ready to drop the tissue directly into the low-calcium solution A.

2.5. Return the transport Erlenmeyer flask to the portable container for cooling, restart oxygenation, and return to the lab.

Note: Here, the time interval between excision and arrival in the laboratory was 17 ± 4.5 min (mean \pm standard deviation, $n = 21$).

3. Slicing

3.1. Transfer the tissue (collected in step 2) to a Petri dish filled with oxygenated low-calcium solution A on ice.

3.2. Pre-cut the tissue into smaller blocks (approximately 3 mm x 3 mm x 3 mm) using a scalpel.

3.3. Transfer a single tissue block into a cylindrical steel chamber (inner: \varnothing 1.5 cm, 0.9 cm depth; outer: \varnothing 2.0 cm, 1.1 cm depth). Remove the fluid using a pipette.

3.4. Pour the liquid agarose solution into the steel chamber and move the tissue block into the middle using a pipette tip.

3.5. Immediately cool down the steel chamber on ice to solidify the agarose.

3.6. Carefully retrieve the agarose block with the aid of a scalpel and glue it on the vibratome specimen holder using instant adhesive, applying gentle pressure. Remove excess instant adhesive with a scalpel and insert the specimen holder into the vibratome chamber.

3.7. Set the cutting thickness to 300 μm . Advance the blade to the tissue. Before entering the tissue, slow down the speed at which the blade is advancing to a minimum and set the oscillation frequency to between 70 and 80 Hz.

3.8. Transfer the tissue slices in the collection beaker filled with oxygenated, ice-cold, low-calcium solution A using the slice collector tool.

4. Preparation of Tissue Slices for Physiological Measurements

4.1. After the slicing is finished, fill up the collection beaker to 90 mL with oxygenated, ice-cold, low-calcium solution A.

4.2. Add 405 μL of calcium solution (**Table 1**) to increase the Ca^{2+} concentration to 0.50 mmol/L. Incubate on ice for 15 min.

4.3. After 15 min, add other 405 μL of calcium solution to further increase the Ca^{2+} concentration to 0.95 mmol/L.

4.4. After another 15 min on ice, collect the tissue slices and transfer them to a Petri dish at 4 °C and filled with the solution suitable for the intended type of subsequent measurement.

Note: Physiological buffer solutions such as Tyrode's solution (solution A) or Krebs-Henseleit buffer, as well as cell culture media, such as Iscove's modified Dulbecco's medium, can be used for subsequent measurements. Particular attention should be paid to ensuring an appropriate calcium concentration between 1.2 and 2.0 mmol/L.

4.5. Place the Petri dish into a humidified incubator at 37 °C to slowly rewarm the tissue slices.

Note: 37 °C is typically reached after 1 h.

REPRESENTATIVE RESULTS:

Pictures of typical myocardial tissue slices obtained using the present protocol are shown in **Figure 2**. The prepared slices can be used for physiological measurements, such as force measurements or electrophysiological recordings.

For force measurements, the tissue slices were mounted onto J-shaped steel needles connected to an isometric force transducer. The slices were immersed in the measurement chamber, which was filled with IMDM. The temperature was maintained at 37 °C and was continuously bubbling with carbogen (95% O₂ and 5% CO₂), pH 7.4. Contractions were triggered by field stimulation at 2 Hz. One major intrinsic regulatory mechanism for cardiac contractility is the Frank-Starling mechanism. When the length of the tissue slices was increased stepwise after mounting on an isometric force measurement setup, a positive force-length relationship was observed. A representative force-length experiment is depicted in **Figure 3A**, showing that contractility is elevated at a greater length. The same experiment demonstrated that passive tension of the tissue slices and force of contraction (FOC) increase with length (**Figure 3B**). The FOC is less elevated after the last length increment than was observed in previous steps, indicating that the length of maximum FOC (L_{max}) was reached. However, passive tension still increased substantially after the last length increment. After L_{max} was reached, the FOC attained routinely steady-state conditions within 15 min (**Figure 3C**). The FOC can be maintained for longer measurement periods (**Figure 3D**).

Representative action potential recordings are depicted in **Figure 4A**. Recordings were performed with microelectrodes pulled from borosilicate capillaries (30-60 M Ω) in DMEM and maintained at 37 °C under constant bubbling with carbogen (95% O₂ and 5% CO₂). Tissue slices were electrically stimulated at 1 Hz with a glass micropipette filled with DMEM and connected to a stimulation device. Application of the I_{Kr} blocker E4031 (0.1 μ mol/L) led to the expected prolongation of the action potential duration, and this effect was antagonized by the subsequent addition of the β -adrenergic agonist isoproterenol (0.1 μ mol/L) (**Figure 4A-B**). Action potential properties did not change after prolonged measurements (**Figure 4C**). More detailed methodological descriptions are available elsewhere^{6-8,10}.

FIGURE LEGENDS:

Figure 1. Jacketed vessel for the maintenance of the agarose temperature. The custom-made jacketed vessel is warmed by water flow from a water bath (42 °C). The agarose is stirred constantly by a magnetic stir bar.

Figure 2. Representative pictures of myocardial tissue slices. (A-C) The pictures show individual slice preparation before functional measurements were performed.

Figure 3. Isometric force measurements. (A) Representative force-length relationship experiment of a slice obtained from a patient with HLHS at day 7 of life who underwent the implantation of a shunt between the right ventricle and pulmonary arteries. The time course of the experiment is depicted in the lower trace, while the upper traces (a, b) show enlarged excerpts. The length was increased stepwise every 2 min. (B) Changes in the FOC and passive tension with increasing length during the same experiment. (C) After the length of maximum FOC is obtained, the FOC is maintained for at least 15 min. The mean FOC of 15 min for each experiment was used for normalization. The data is presented as the mean \pm SEM, n = 4. Slices were obtained from 3 HLHS patients undergoing the implantation of a shunt between the right

ventricle and pulmonary arteries at 2, 7, and 8 days of life. (D) Examples of long-term measurements over 90 min from two slices. The right traces (c-f) show averaged contractions (interval: 2 min) from the begin and end of the recordings. These slices were obtained from the right ventricular outflow tract of a patient undergoing TOF repair at 6 months of age.

Figure 4. Microelectrode recordings. (A) Representative action potential recordings of a myocardial tissue slice before (baseline, black) and after (dark gray) the administration of 0.1 $\mu\text{mol/L}$ E4031 and the subsequent addition of 0.1 $\mu\text{mol/L}$ isoproterenol (ISO, light gray), stimulated at 1 Hz. (B) Changes in the action potential duration (APD) of the same experiment, before and after drug administration. This slice was obtained from an HLHS patient who received a shunt between the right ventricle and pulmonary arteries at 69 days of life. (C) Representative action potential recordings of a myocardial tissue slice 1 min (black) and 22 min (red) after impalement with a microelectrode (not corrected for baseline drift). This slice was obtained from the right ventricular outflow tract of a patient undergoing TOF repair at 6 months of age.

Table 1. Materials: The essential supplies, reagents, and equipment.

DISCUSSION:

Cardiac slices bridge the gap between single-cell and complex multicellular models for physiological research^{1,2}. Here a protocol has been introduced describing the preparation of viable cardiac tissue slices from human myocardial explants obtained from neonatal and infantile patients undergoing surgery for congenital heart disease. These slices can be used for studying contractile behavior and electrophysiological properties, among other physiological parameters.

A particular challenge for the preparation of viable cardiac tissue slices is to minimize tissue damage. The cessation of electrical activity and contractions does not only reduce the metabolic demand during storage and vibratome sectioning but also facilitates the sectioning process. This is most commonly achieved by transferring the tissue into oxygenated, ice-cold (4 °C) solutions^{2,4-11}. Additional strategies involve the use of low-calcium¹¹ or calcium-free^{6-8,10} solutions, the use of high potassium concentrations⁹, and pharmacological uncoupling of excitation and contraction with 2,3-butanedione monoxime (BDM)^{2,4,5,7-9,11} or blebbistatin². In the present protocol, oxygenated, ice-cold, low-calcium solution supplemented with BDM was successfully used. To prevent calcium paradox-related tissue injury (*i.e.*, necrosis of cardiomyocytes upon calcium repletion after a period of calcium depletion)¹², the calcium concentration was increased stepwise back to a physiological level after slicing. Additionally, gradual warming to 37 °C and sufficient incubation time are required to promote tissue recovery to an adequate electrophysiological steady state².

The slicing process is the second source of tissue injury, and most difficulties arise from the high degree of elasticity of myocardial tissue⁹. Embedding in low-melting agarose stabilizes the tissue during slicing, and the low gelling temperature assists with the prevention of heat-induced damage. A very slow blade advancing speed, less than 50 $\mu\text{m/s}$, promotes homogeneous slice thickness and obviates undesired crushing of the myocardial tissue². Choosing an appropriate thickness is important to facilitate oxygen and nutrient supply as well as the removal of metabolic

waste by diffusion. Slice thicknesses below 400 μm are suggested to prevent hypoxia in the inner core of the slice². The lower boundary for intact slices is approximately 100-150 μm . It was shown that the preparation of slices from adult murine hearts with a thickness of 150 μm is feasible⁷.

For optimal results, it is crucial to establish a fast workflow for tissue transfer into the ice-cold oxygenated solution in the operating room and to limit the time for the whole slicing procedure. We observed that omitting to immediately drop the tissue explant into the solution dramatically reduced the chance of measuring contractions or action potentials after slicing. Restriction of the sectioning time to 1 h also led to a higher number of exploitable slices. One reason for this might be the use of BDM as cardioprotective reagent. Although BDM is widely used for the slicing of myocardial tissues, there are unfavorable side effects on calcium handling¹³ and the electrophysiological properties¹⁴ of cardiomyocytes. Blebbistatin is suggested to be a more specific myosin II ATPase inhibitor¹⁵ and thus might constitute a potentially superior yet more expensive alternative.

A limitation of human neonatal and infant myocardial explants is that it is challenging to control fiber orientation. This must be taken into account for several applications, such as the assessment of conduction velocities. When sectioned in the epicardium-tangential plane, cardiac slices display predominantly longitudinal fibers^{2,4}. However, the researcher must know the tissue orientation before embedding and sectioning, which might not always be possible, depending on the surgical procedure.

Following the present protocol, measurements can be performed for 4-8 h on the generated cardiac slices. An adapted culture protocol should be established when aiming for long-term experiments, such as screening for chronic drug effects. Brandenburger *et al.*¹¹ developed a culture system that allows for the maintenance of adult human cardiac slices for at least 28 days. Validation of the reliability of long-term cultures, however, is still lacking, as alterations of the physiological parameters should be expected over longer periods in culture.

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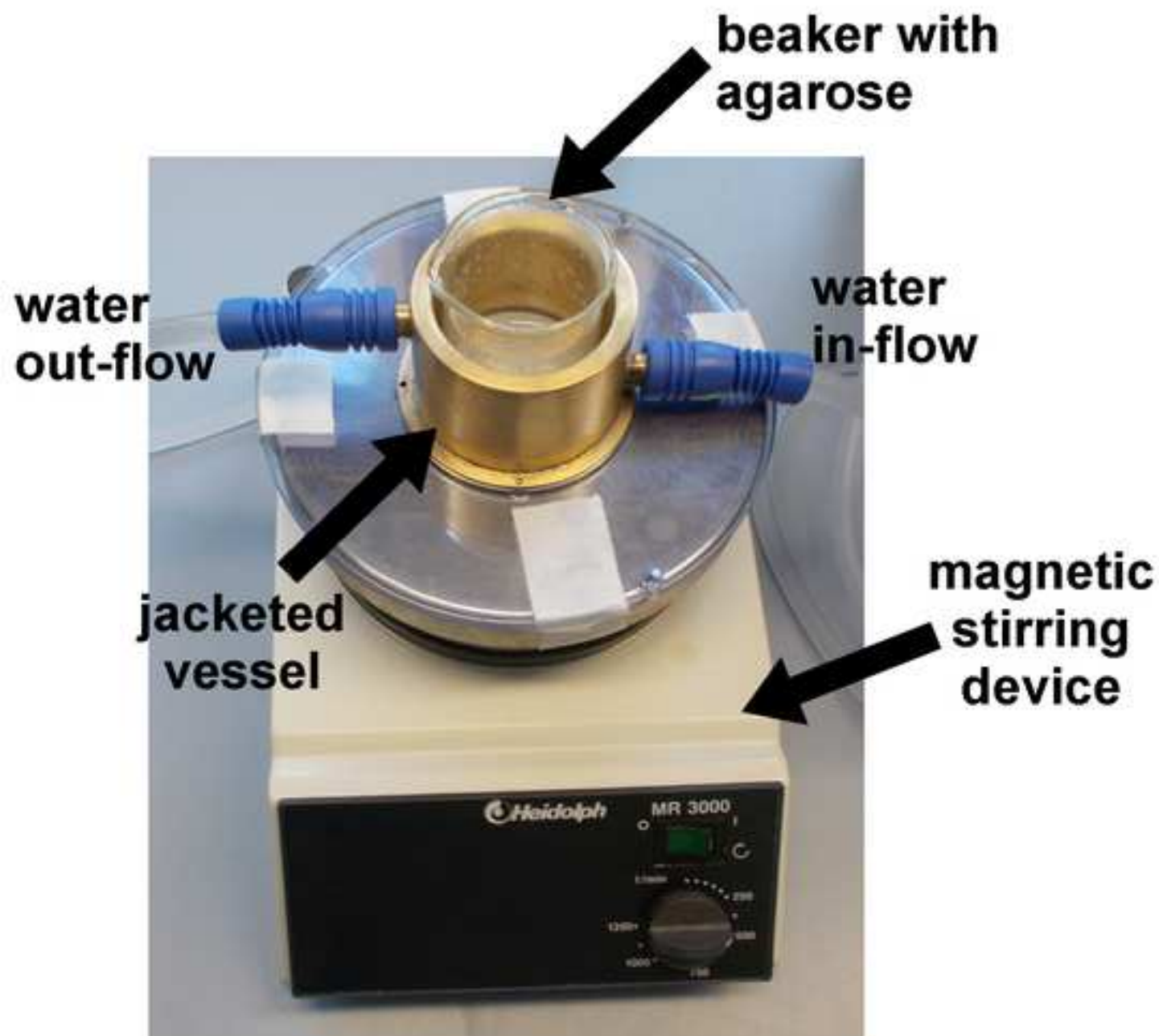
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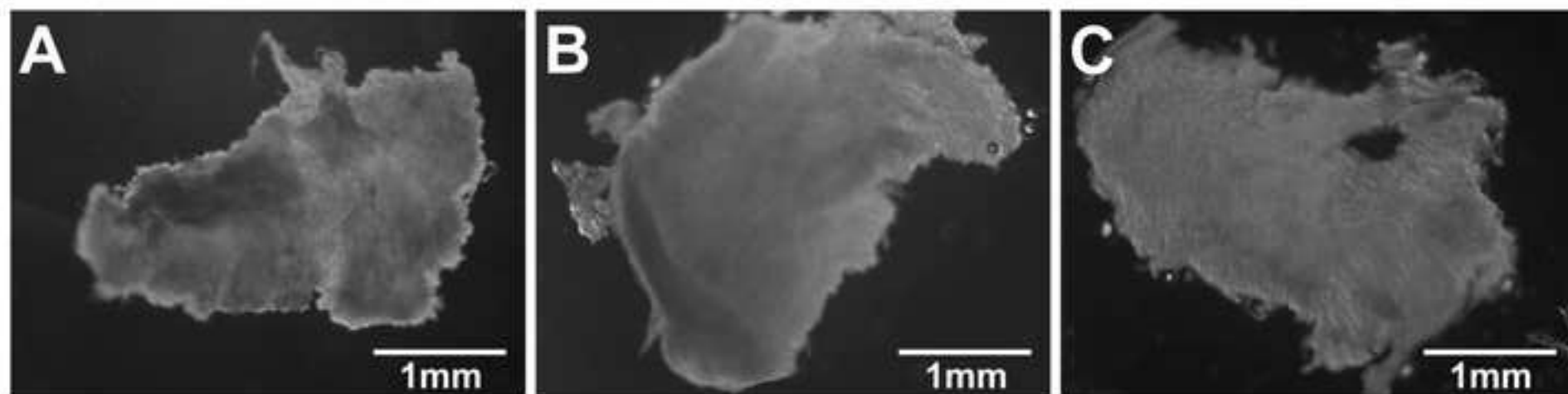
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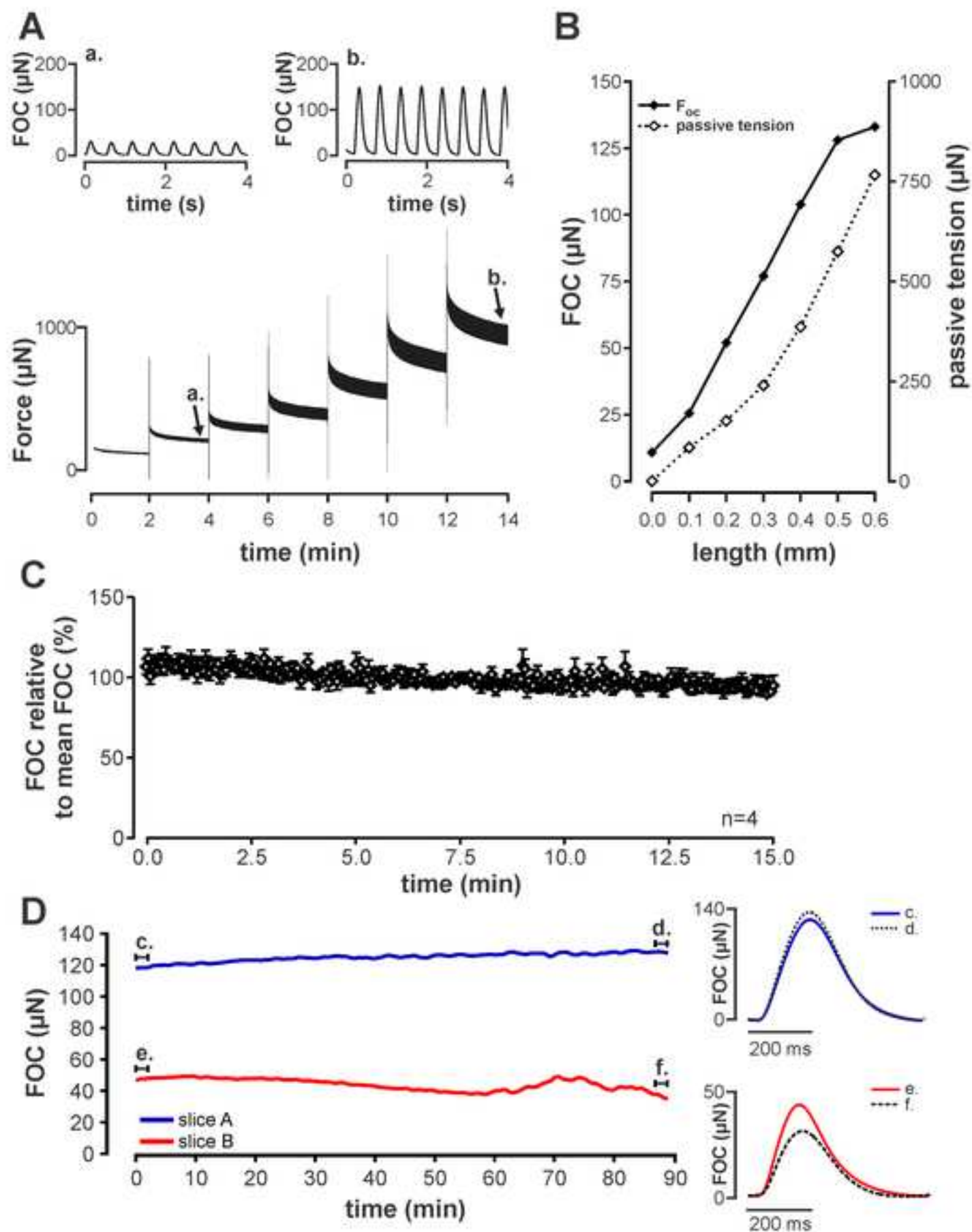
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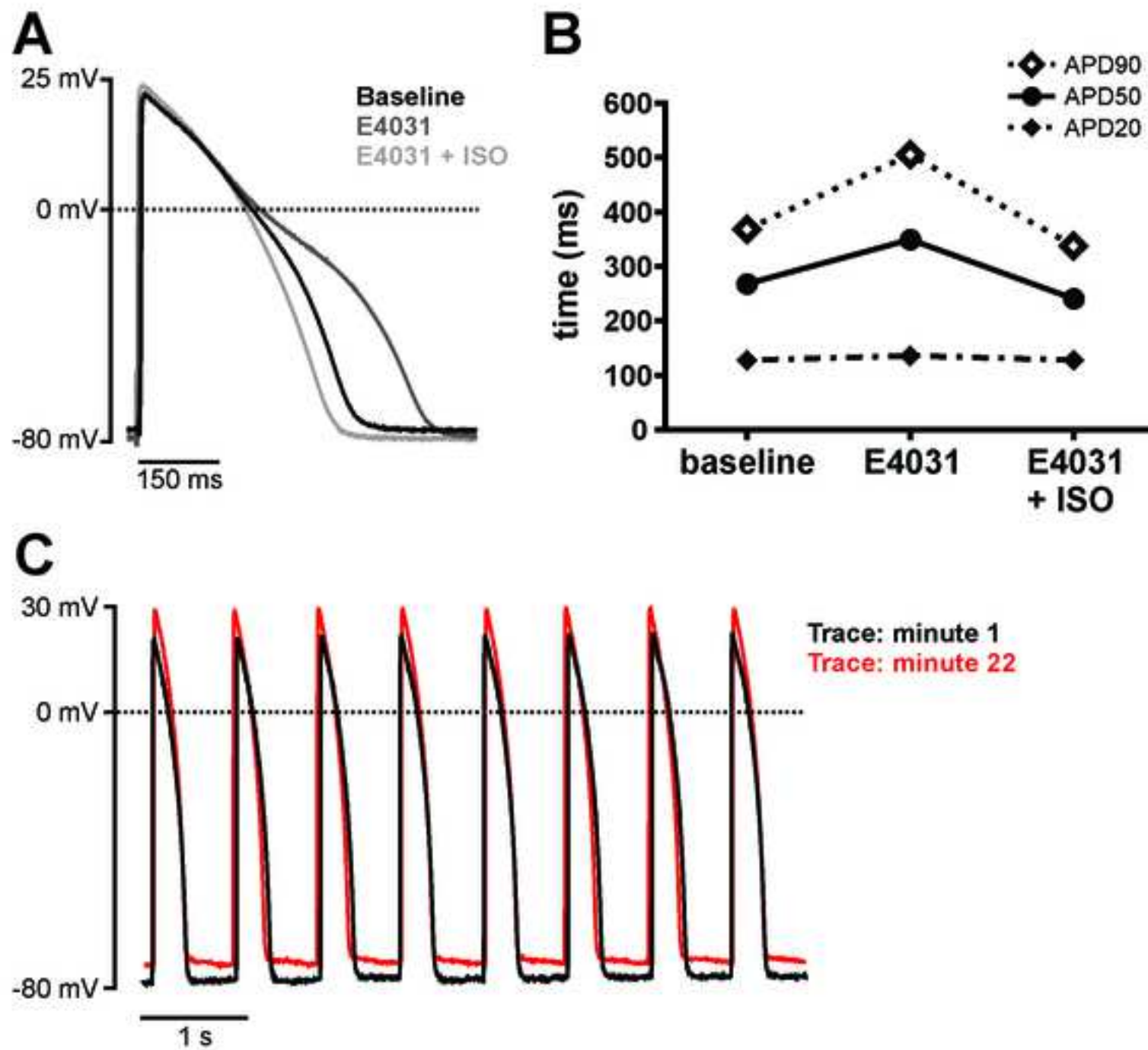
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| Water bath | Grant | Sub 14 |
| Water pump | Aquarium Systems | Mini Jet MN404 |
| IMDM | Life Technologies | 31980022 |
| DMEM, high glucose | Life Technologies | 61965026 |
| E4031 dihydrochloride | Abcam | ab120158 |

Comments

solution A: 136 mmol/L in aqua dest.

solution A: 5.4 mmol/L

solution A: 0.33 mmol/L

solution A: 1 mmol/L

solution A: 10 mmol/L

solution A: 5 mmol/L

solution A: 30 mmol/L

solution A: use to adjust pH to 7.4

calcium solution: 100 mmol/L in aqua dest.

for slicing

for slicing

for slicing

for 'slice collector' tool

for 'Slice collector' tool

for embedding tissue into agarose;

cylindric shape (inner: Ø: 1.5 cm, depth 0.9 cm;

outer: Ø: 2.0 cm, depth 1.1 cm)

Pattex Ultra Gel; for slicing

to maintain temperature of agarose in beaker at 37°C; made of steel with a plexiglass bottom

for temperature control of jacketed vessel

for temperature control of jacketed vessel

for force measurements

for microelectrode recordings

for microelectrode recordings

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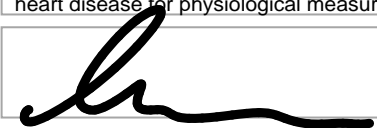
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| Department: | Paediatric Cardiology | |
| Institution: | University of Cologne | |
| Article Title: | Preparation of viable myocardial slices from biopsies of neonates and infants with congenital heart disease for physiological measurements | |
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Editorial comments:

-Please provide a little more information about the "custom-made jacketed vessel" in steps 1.1 and 1.8. If the construction has been mentioned elsewhere then a citation will be sufficient; otherwise a brief description would be helpful.

We have added a new figure (Figure 1) showing the construction in detail. The other figures have been renumbered accordingly.

Clarification is requested: Do the authors wish to include Section 2 in the video? This section includes obtaining tissue from the operating room during a neonatal or pediatric surgical procedure, which does not pose any technical problems but may require additional release forms or sensitivity in filming. Please use highlighting to indicate which sections of the protocol should be filmed.

We do not believe that filming in the operating theatre is essential for the description of the method. Weighing the additional information against the sensitivity in filming procedures in patients we prefer not to include this into the video.

Reviewer #1:

1. When working with human cardiac specimen, improper tissue handling during transportation between the surgery room and the laboratory drastically reduces survival. The author did not clearly describe the transportation time. As the tissue was not cardioplegically arrested with high KCl solution, a long transportation time will damage large percentage of cells.

We have added information on transportation time in our experiments (page 5): **In our experiments, the time interval between excision and arrival in the laboratory was 17 ± 4.5 minutes (mean \pm standard deviation, n=21).**

2. Slicing in a low calcium solution prior to performing acute experiment in normal calcium solution should avoid calcium related tissue injury. It would be interesting to compare mechanical and electrical parameters using this calcium reintroduction method versus using the working calcium concentration directly.

This is beyond the scope of our study. Patient material can only be obtained from procedures which are performed infrequently. We understand that this is an important issue indeed, however, we are not able to investigate this point due to low patient numbers.

3. The "solution A" described in the manuscript is pH calibrated to 7.4 with NaOH, however it also contains NaH_2PO_4 which when bubbled with carbogen changes pH. It is not clear if the author used pure O_2 or carbogen in the slice preparation step.

We have clarified this in the manuscript:

Page 3: Oxygenate the solution in the vibratome chamber **by bubbling with pure O₂**.

Page 4: Oxygenate **by bubbling with pure O₂**.

4. 30 mM BDM is used for EC uncoupling. As authors mentioned in the discussion, BDM can alter calcium handling, especially at this high concentration. Is there a reason why 30 mM is used over 10 presented in some of the cited studies?

We have used this concentration in the past and we are not aware of data suggesting that calcium handling remains altered after washing out BDM. To avoid a confounding effect of BDM we made sure that exposure was limited to approximately 1 hr.

5. Z-axis vibration is critical for the viability and longevity of slice preparation. Did the author track amount of z-axis vibration in their vibratome?

The vibratome we used for this study was not able to track z-axis vibration. We did not aim for longevity.

6. Mechanical study and electrical study used two different media (IMDM and DMEM). Both these culture media have different ionic concentration compared to Tyrodes which most studies are performed in. Author should indicate why these medias are chosen for acute studies over standard Tyrodes.

The media can be adapted as required. This was already discussed in the submitted manuscript on page 5-6: **Notes:** Physiological buffer solutions such as Tyrode's solution (solution A) or Krebs-Henseleit buffer as well as cell culture medium such as Iscove's modified Dulbecco's medium can be used for subsequent measurements. Particular attention should be paid on an appropriated calcium concentration between 1.2 – 2.0 mmol/L.

7. Author should clarify the pacing frequency used to calculate APD in figure 3.

We have added this information:

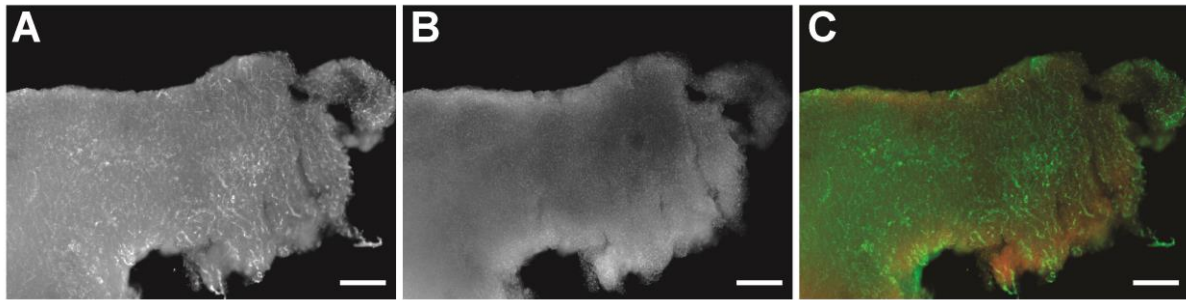
Page 6: Tissue slices were electrically stimulated **at 1 Hz** with a glass micropipette filled with DMEM and connected to a stimulation device.

Page 7: (A) Representative action potential recordings of a myocardial tissue slice before (baseline, black) and after administration of 0.1 µmol/L E4031 (dark gray) and subsequent addition of 0.1 µmol/L Isoproterenol (ISO, light gray) **stimulated at 1 Hz**.

8. Author should indicate typically what percentage of cells in slices are viable. Mechanical properties will likely change due to number of contracting cells.

We are unable to determine the absolute number of viable and non-viable cardiomyocytes and non-myocytes, respectively. To give an overall impression of the viability of the preparation, we

performed live/dead cell viability stainings using Calcein-AM and propidium iodide (Rebuttal-Figure 1).



Rebuttal-Figure 1: Live/dead cell viability staining. (A) Staining of viable cells using Calcein-AM. (B) Staining of non-vital cells using propidium iodide. (C) Merged (green: Calcein-AM, red: propidium iodide).

9. This protocol lacks organotypic culture step, which limits its applicability for chronic studies. For example, a recent study (ref. 5) has demonstrated significant difference between acute and chronic effects of phenylephrine. This protocol also lacks electrical or optical mapping techniques. Please discuss these limitations.

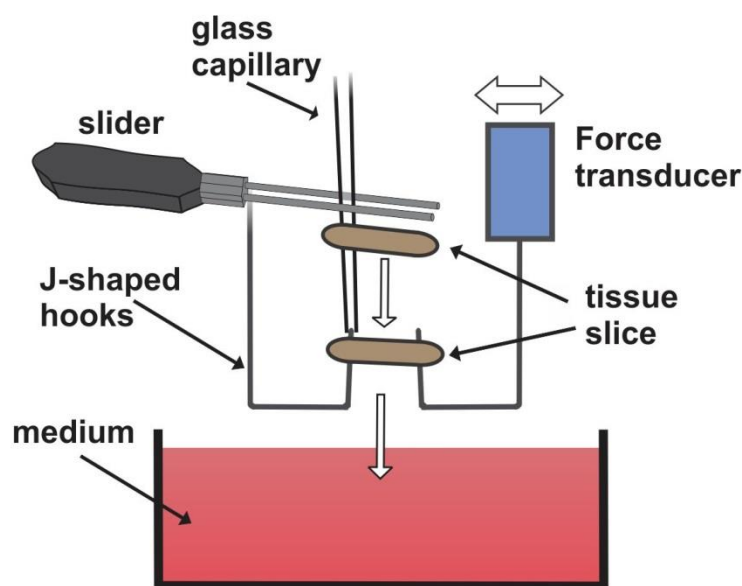
This is beyond the scope of our study and was already discussed on page 8: Following the present protocol, measurements can be performed for 4-8 h with the generated cardiac slices. An adapted culture protocol should be established when aiming for long-term experiments such as screening for chronic drug effects. Brandenburger *et al.*¹¹ developed a culture system allowing the maintenance of human adult cardiac slices for at least 28 days. Validation of the reliability of long-term cultures is however still lacking as alterations of physiological parameters should be expected over longer periods in culture.

Reviewer #2:

1. It is not clear how the authors "mount" the tissue slice on a J-shaped needle - do they use it as a hook? If yes, how are the forces within the whole slice are transferred to the isometric force transducer Instead of showing 3 sample slices in Fig. 1 where 1 slice would suffice, I suggest that the authors use the space for a picture of what the mounted tissue slice would look like.

We thank the Reviewer for the suggestion, but we are afraid that including a figure about mounting of the tissue slices would be beyond the scope of our protocol and would therefore rather confuse the reader. Our intention is to present a protocol enabling the reader to prepare slices for functional measurements, but not to describe the functional measurements themselves. We included representative force and electrophysiological data to give the reader ideas about possible measurement methods and to show that slices prepared by our protocol demonstrate functional properties of heart tissue.

To give the Reviewer an overview, we describe our mounting technique. The setup consists basically of two J-shaped needle hooks, one fixed to the setup assembly and the other connected to the force transducer (Rebuttal-Figure 2). One side of the slice is impaled on a glass capillary with a conic tip. The lumen of the capillary is placed on the tip of the fixed J-shaped hooks and the tissue slice is pushed down in a way that the other side of the slice is pinned to the J-shaped hook of the force transducer. Afterwards, the slice is lowered into the medium-containing measuring chamber. As the horizontal position of the force transducer can be altered, the distance between both hooks is stepwise increased by a motor system until L_{\max} is reached.



Rebuttal-Figure 2: Technique used for mounting of tissue slices.

2. The authors should provide real time-matched controls in order to demonstrate the stability of the preparations for the entire experimental period and not only for the "pre-intervention" (physiological, pharmacological) time slot. It is essential that the preparations are stable for the equilibration plus the experimental period. *[Editorial comment: We understand your concern over having to include data from several additional experiments in order to address the reviewers' comments. While we do not require in depth results for publication in JoVE, the results must accurately demonstrate the efficacy of the proposed method. Additionally, the results must substantiate all claims presented within the manuscript. Please ensure that all claims you make are*

We have included representative control measurements (Figure 3D).

3. Time-matched controls are also required for action potential measurements. We have included a representative control measurement (Figure 4C).

Reviewer #3:

1. The authors describe that myocardial slices were prepared from right ventricular tissue samples of neonates and infantile patients undergoing surgery for congenital heart disease. Please further specify in the legends with each figure the precise origin of the tissue used for the experiments (e.g. age of patient, reason for surgery).

We have added this information as required.

Page 6: Representative force-length relationship experiment of a slice obtained from a patient with HLHS at day 7 of life who underwent implantation of a shunt between right ventricle and pulmonary arteries.

Page 7: Slices were obtained from 3 HLHS patients undergoing implantation of a shunt between right ventricle and pulmonary arteries at 2, 7, and 8 days of life.

Page 7: (D) Examples of long-term measurements over 90 minutes from two slices. Right traces (c.-f.) show averaged contractions (interval: 2 minutes) from begin and end of the recordings. These slices were obtained from the right ventricular outflow tract of a patient undergoing TOF repair at 6 months of age.

Page 7: This slice was obtained from a HLHS patient who received a shunt between right ventricle and pulmonary arteries at 69 days of life.

2. For sake of reproducibility could you please further specify how the very small (3x3x3mm) blocks of tissue are prepared.

" 3.2 Pre-cut the tissue into blocks of approximately 3 mm x 3 mm x 3 mm using a scalpel."

Pre-cutting the tissue makes slicing easier; however, the exact size is not important. We therefore use eyeballing to estimate the size of the blocks. To make this clearer, we have changed the sentence:

" 3.2 Pre-cut the tissue into smaller blocks (approximately 3 mm x 3 mm x 3 mm) using a scalpel."

3. Table 1 is somewhat unclear. A table describing the content of solution A and a table describing the necessary equipment would be more appealing.

We agree with the reviewer. It is however an editorial requirement to not divide this information into multiple tables.

4. You used low calcium solution combined with BDM to ensure the absence of contractions during the slicing process, thereby lowering tissue injury. Could you please clarify the choice for

both low calcium and BDM instead of either of these options on its own, which would have been easier?

This is a very interesting point we are unable to answer. We have used this method in various settings in the past (Pillekamp F 2005, Halbach M 2006, Halbach M 2007, Pillekamp F 2007, Halbach M 2012). Given the small number of preparations (approx. 10-15 per year), we are unable to test whether using either of these options alone would be non-inferior or better.

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Pillekamp, F., *et al.* Establishment and characterization of a mouse embryonic heart slice preparation. *Cell. Physiol. Biochem.* **16**(1-3), 127-132 (2005)

Halbach, M., Pillekamp, F., Brockmeier, K., Hescheler, J., Müller-Ehmsen, J., & Reppel, M. Ventricular slices of adult mouse hearts--a new multicellular in vitro model for electrophysiological studies. *Cell. Physiol. Biochem.* **18**(1-3), 1-8 (2006)

Halbach M, Pfannkuche K, Pillekamp F, Ziomka A, Hannes T, Reppel M, Hescheler J, Müller-Ehmsen J. Electrophysiological maturation and integration of murine fetal cardiomyocytes after transplantation. *Circ. Res.* **101**(5):484-92 (2007)

Pillekamp, F., *et al.* Neonatal murine heart slices. A robust model to study ventricular isometric contractions. *Cell. Physiol. Biochem.* **20**(6), 837-846 (2007)

Halbach M, Krausgrill B, Hannes T, Wiedey M, Peinkofer G, Baumgartner S, Sahito RG, Pfannkuche K, Pillekamp F, Reppel M, Müller-Ehmsen J, Hescheler J. Time-course of the electrophysiological maturation and integration of transplanted cardiomyocytes. *J Mol. Cell. Cardiol.* **53**(3):401-8 (2012)

5. It would be nice to see how the electrophysiology results of the slices differ compared to other techniques, single cell patch clamping and wedge or whole heart preparations. Could you please add a historical comparison with other studies? [Editorial comment: The above comment may be addressed in the Discussion using relevant references.]

This is a very interesting point. However, there are no electrophysiological data from HLHS patient biopsies or from other neonatal myocardial biopsies available at all in the literature. This unfortunately makes a comparison impossible.

6. An Y axis with a line indicating 0 mV should be added to figure 3A.

We have changed former Figure 3, now Figure 4 accordingly.