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Preparation of viable adult ventricular myocardial slices

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KEYWORDS:

myocardial slice, organotypic cardiac tissue slice, cardiac tissue slice, heart slice, heart, cardiac model

EDITORIAL SUMMARY:

This protocol describes how to obtain 100-400µm-thick slices of living myocardium that retain the native multicellularity, architecture and physiology of the heart.

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ABSTRACT:

This protocol describes a robust and reproducible method for the preparation of highly viable adult ventricular myocardial slices from small and large mammalian hearts. Adult ventricular myocardial slices are 100-400µm-thick slices of living myocardium that retain the native multicellularity, architecture and physiology of the heart. This protocol provides a list of the equipment and reagents required alongside a detailed description of methodology for heart explantation, tissue preparation, slicing with a vibratome and handling of myocardial slices. Supplementary videos are included to visually demonstrate these steps. A number of critical steps are addressed that must be followed in order to prepare highly viable myocardial slices. These include: identification of myocardial fiber direction and fiber alignment within tissue block, careful temperature control, use of an excitation-contraction uncoupler and optimal vibratome settings and correctly handling myocardial slices. Many aspects of cardiac structure and function can be studied using myocardial slices *in vitro*. Typical results obtained from rat (small mammal) & human heart failure (large mammal) samples are shown, demonstrating myocardial slice viability, maximum contractility, Ca^{2+} handling & structure. This protocol can be completed in ~4 hours.

INTRODUCTION:

Myocardial slices are 100-400µm-thick slices of living adult ventricular myocardium, prepared using a high precision vibratome(1–25) (Fig. 1). They retain the multicellularity, complex architecture and physiology of adult cardiac tissue and their thinness allows the diffusion of oxygen and other metabolic substrates to their innermost cells, maintaining viability in the absence of coronary perfusion *in vitro*(2). Slices have been produced from a number of small and large mammals, including mice(3,15,16,21), rats(4,8–14,17–20,22–24), guinea pigs(5,13,14), rabbits(5), dogs(3,6,25), pigs and human tissue biopsies(2,3,6,7,25). Multiple slices can be

produced from each heart or biopsy, reducing the number of animals required for studies.

[Insert Fig. 1 here]

Development & applications

In recent years, myocardial slices have been increasingly utilised, however, they were first used as far back as the 1970s. Early studies investigated a number of aspects of cardiac function including biochemistry (8), metabolism(9,18–21), electrophysiology(10,11) and pharmacological cardiotoxicity(4). These studies demonstrated that myocardial slices had wide-ranging applications, but it was not until the early 2000s that they became more widely recognized. During this period, myocardial slices were found to have more representative electrophysiological properties than other cardiac models(12) and other functional parameters, including myocardial slice contractility(15), were measured for the first time. Importantly, techniques to culture slices for up to one week were developed(15) and more recent use of an air-liquid interface has demonstrated that slices can be maintained for up to one month *in vitro*(2).

As myocardial slices provide a clinically relevant and representative human model system *in vitro* they are particularly useful for translational studies(7). A substantial amount of work has demonstrated that myocardial slices can be used for pharmacological testing and *in vitro* drug safety screening(6,7,13,14,22). They have also been used to study the integrative capacity of a number of cell types as part of the development of cell-replacement strategies(16,26,27). More recently, slices have been used to test the clinical applicability of conductive polymer patches (17).

Despite the number of studies published, the protocols used have varied greatly. There are a number of critical steps that need to be carefully controlled to produce myocardial slices with high viability and this optimized protocol has significantly improved their contractility (Supplementary Fig. 1) alongside other structural and functional parameters. A critical step, that is often overlooked, is the identification of myocardial fiber direction and fiber alignment within the tissue block. In a large number of papers published, hearts are sliced in the short-axis. As myocardial fibers run parallel to the epicardium, this results in a large numbers being transected and substantial tissue damage. Other critical steps include: carefully maintaining the correct temperature, the use of an excitation-contraction uncoupler and optimal vibratome settings and correctly handling the myocardial slices. These factors and the techniques used to control them are discussed in depth in this protocol.

Comparison with other methods

Cardiovascular research has been conducted using a variety of model systems.

Isolated adult cardiomyocytes. Isolated adult cardiomyocytes have been used widely. During isolation, cardiomyocytes lose connections with other cardiac cells and the ECM, both of which are known to modulate myocardial function(28,29), and the outcomes of different isolation protocols are known to vary noticeably(30).

Isolated cardiomyocytes also undergo significant remodeling within hours(31,32), with significant implications for the relevance of chronic studies(32).

Whole hearts. Whole heart studies avoid the issues associated with cell isolation. However, it is extremely difficult to use isolated whole hearts for more than a few hours and investigations therefore require large numbers of animals. Their complexity also makes it difficult to study events at the cellular level and these studies require specific and expensive set ups. Other intermediate preparations exist, including cardiac wedges and papillary muscles, but these often become ischaemic *ex vivo*(33), particularly when prepared from large mammals.

Stem cell derived cardiomyocytes. In recent years, human pluripotent stem cell-derived cardiomyocytes (PSC-CMs) have been utilised. While these cells have the advantage of recreating pathological phenotypes *in vitro*(34), they also have a relatively naïve phenotype(35), with many aspects including morphology, metabolism(36), electrophysiology and Ca^{2+} handling(37) regarded as particularly immature.

Myocardial slices. Myocardial slices bridge the gap between isolated cardiomyocytes and whole heart studies. The multicellular composition and non-cellular architecture of the myocardium are almost fully maintained during slicing, resulting in a model with the correct cell ratios and cell-cell / cell-ECM interactions. This allows all cardiac cell populations, including fibroblasts and endothelial cells, to be studied alongside one another, which is required to improve our understanding of cardiac physiology and pathology(38). Additionally, chronic culture is facilitated by the ability of oxygen and metabolic substrates to diffuse to all of the cells within the preparation, which is not possible or very difficult to achieve with other cardiac multicellular preparations(2).

Experimental design

When designing experiments using myocardial slices, the number of slices that can be produced must be considered. Smaller mammals, including mice and rats, can yield 3-6 slices per heart, while large mammals, including canine or human biopsies, can yield 20-30 slices per tissue block and several tissue blocks can be prepared from the left ventricle. As multiple slices can be produced from each animal, many experiments can be conducted, ultimately reducing the number of animals used in studies. The age and phenotype of the animal can also contribute to the thickness of the ventricular wall and should be considered. Another important consideration is the region of the ventricular wall from which the slice has been derived. The endocardium, midmyocardium and epicardium have differing electrophysiological and contractile properties(39) and also display varied responses to pharmacological stimuli(40). This can be overcome by using slices from specific regions or by randomizing slices and increasing the number of repeats. The timings of experiments should also be considered carefully. Slices should be used as swiftly as possible, but can be kept in ice-cold, oxygenated Tyrode's solution for up to 4 hours prior to being used. Exceeding this time period may result in degradation of the myocardial slices.

Limitations & expertise required

While myocardial slices have a number of benefits over other cardiac preparations, they also have their limitations. The biggest determinant of myocardial slice quality is the method by which the cardiac tissue block is dissected & prepared. Due to the manual nature of this process there is a relatively steep learning curve and a number of weeks and animals (10-15 hearts) should be dedicated to practicing before high quality tissue blocks can be consistently prepared. The quality of the tissue block preparation directly correlates to the quality of data that can be collected (See “Anticipated Results – Viability”). Access to fresh cardiac tissue / animal facilities and the expense of equipment (See “Equipment”) may also be prohibitive for some laboratories.

Summary

In summary, myocardial slices provide significant advantages over other commonly utilised cardiac preparations and can be produced from a large variety of species. The protocols used to date vary greatly and there are a number of critical steps that need to be addressed in order to produce myocardial slices with high viability and preserved structure and function. This protocol describes a robust and reproducible technique for producing myocardial slices and aims to standardize the methodology by which they are produced, reducing the variability in the quality of myocardial slices produced in different laboratories.

MATERIALS:

Reagents

- Fresh mammalian cardiac tissue. Rodent, human heart failure, porcine and canine cardiac tissues were used to produce myocardial slices. All animal experiments must comply with Institutional and National regulations. Our use of living cardiac tissue was approved by Imperial College London. The procedures we describe here were performed under license by the UK Home Office, in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986. Animals were killed following guidelines established by the European Directive on the protection of animals used for scientific purposes (2010/63/EU). Human samples were provided by the NIHR Cardiovascular Biomedical Research Unit at the Royal Brompton and Harefield NHS Foundation Trust and Imperial College London. The inferior 1/3rd of explanted hearts were obtained from end-stage heart failure patients undergoing cardiac transplantation. The study performed conformed to the principles outlined in the Declaration of Helsinki and the investigation was approved by a UK institutional ethics committee (Ref: 09/H0504/104+5 CBRU Biobank). **!CAUTION** Informed consent was obtained from each patient involved in this study. Porcine hearts were obtained from the Translational Biomedical Research Centre, University of Bristol. Procedures were approved by the University of Bristol. 18 month old healthy Beagle dog hearts were donated by GlaxoSmithKline, after the animals were necessarily sacrificed at the end of pharmacology/toxicology studies. Only control animals were used.
- 2,3-Butandione Monoxime (Acros Organics, cat. no. AC150375000)
- Agarose (Sigma, cat. no. A9539)
- Bovine Serum Albumin (Sigma, A7030)

- Calcium Chloride (1M Solution) (VWR, cat. no. E506-500ML)
- D-Glucose Anhydrous (Fisher Chemical, cat. no. D16-1)
- Ethanol (VWR, cat. no. 64-17-5)
- Fetal Bovine Serum (Gibco, cat. no. 10270)
- Formaldehyde Solution (Sigma, cat. no. F8775)
- Heparin Sodium 1000IU/ml (Fannin, PL 20417/0109)
- HEPES (Sigma, cat. no. H3375)
- Horse Serum (Heat Inactivated) (Gibco, cat. no. 26050088)
- ITS Liquid Media Supplement (100x) (Sigma, cat. no. I3146)
- Magnesium Chloride Hexahydrate (VWR, cat. no. 97061-356)
- Magnesium Sulphate (Sigma, cat. no. M2643)
- Medium-199 (Sigma, cat. no. M5430)
- Penicillin-Streptomycin (Sigma, cat. no. P4333)
- Potassium Chloride (Sigma, cat. no. P9541)
- Phosphate Buffered Saline (Oxoid, cat. no. BR0014G)
- Sodium Chloride (VWR, cat. no. 470302-522)
- Sodium Bicarbonate (Sigma, cat. no. S5761)
- Sodium Hydroxide (Fisher Chemical, cat. no. S/4880/60)
- Sodium Phosphate Monobasic Monohydrate (Sigma, cat. no. S3522)
- Triton X-100 (Sigma, cat. no. X100)

Equipment

- 100% Medical Grade Oxygen Gas
- 150 x 15mm Petri Dish (Falcon, cat. no. 351058)
- 28mm Syringe Filter (0.2 Micron) (SFCA+PF Membrane) (Corning, cat. no. 431219); other 0.2 micron filters are appropriate
- 40µm Cell Strainer (Corning, cat. no. 352340)
- 6 Well Plate (Customized with holes in base of each well – See “Fig. 2A”) (Corning, cat. no. CLS3506)
- Ceramic Vibratome Blades (Campden Instruments, cat. no. 7550-1-C); stainless steel blades can be used for single use as they wear quickly
- Disposable Sterile Scalpel - Surgical Steel Blades (Swann-Morton, cat. no. 05XX)
- Electronic Balance
- Flat Spatula
- Gauze
- Glass Beaker (1L / 0.25L) (Duran, cat. no. Z231894 / Z231843); other glass beakers are appropriate
- Glass Petri Dish 100x20mm Soda-Lime (VWR, cat. no. CLS70165101); other glass petri dishes are appropriate
- Graefe Forceps (0.8x0.7mm) (Fine Science Tools, cat. no. 11050-10); other small forceps are appropriate
- Histoacryl Surgical Glue (Braun, cat. no. 1050052)
- Incubator; 37 degrees & 5% CO₂ required
- Laboratory Bottles (1L / 0.5L) - High-Density Polyethylene, Wide Mouth (Nalgene, cat. no. 10547341 / 10633141); other laboratory bottles are appropriate
- Laminar flow cabinet with UV light

- Large glass dish ± lid (See “FIGURE 3C”)
- Magnetic Stirring Bars
- MS-H-S Circular-top Analog Hotplate Stirrer (Scilogex, cat. no. 811121029999); other hotplate stirrers are appropriate
- Noyes Spring Scissors (14mm Blade - 15012-12) (Fine Science Tools, cat. no. 15012-12); other small dissecting scissors are appropriate
- Parafilm "M" Laboratory Film (Bemis, cat. no. PM992)
- Pasteur Pipette (Sterile) (VWR, cat. no. 612-1685)
- pH Meter
- Polythene Bag
- Rapid-Flow Sterile Disposable Filter Unit (PES Membrane - 0.2µm) (Nalgene, cat. no. 167-0045); other 0.2µm filters are appropriate
- Razor Blade
- Scissors Iris Straight 11.5cm (4.5") (Rociale, cat. no. RSPU100-093); other surgical scissors are appropriate
- Self-Seal Sterilization Pouch (Westfield Medical, cat. no. WPSS135255)
- Stainless Steel Plain Washer (RS, cat. no. 189-636)
- Sterilin Polystyrene Containers (Thermo Scientific, cat. no. 11319143)
- Transwell® Polyester Membrane Cell Culture Inserts (Corning, cat. no. CLS3450)
- Vibrating Microtome 7000smz-2 (Campden Instruments, cat. no. 7000smz-2)
Note: Alternative vibratomes can be used. **CAUTION** Alternative vibratomes must be able to check and calibrate Z-axis error, be capable of cooling slicing solution <8°C (ideally solution should be kept at 4°C) and be capable of advancing at slow speeds (0.03mm/s).
- Vibrating Microtome Bath (Campden Instruments, cat. no. 7000-3-1A)

Reagent Setup

Preparation of 1L slicing solution (Tyrode's Solution + 30mM BDM)

Cardiac tissue is sliced in Tyrode's solution with the addition of an excitation-contraction uncoupler (2,3-Butanedione Monoxime). Add the following to 1L distilled water while mixing using a magnetic stirrer: 3.00g 2,3-Butanedione Monoxime (30mM), 8.18g Sodium Chloride (140mM), 0.45g Potassium Chloride (6mM), 1.86g Glucose (10mM), 2.38g HEPES (10mM), 1ml 1M Magnesium Chloride solution (1mM) & 1.8ml 1M Calcium Chloride solution (1.8mM; see also Table 1 for details of the components required). Wait for the reagents to dissolve and then measure the pH. Add drops of 2M Sodium Hydroxide solution until the solution is pH 7.40. Filter the solution with a 0.2 micron pore filter and transfer to the previously sterilized 1L container. Cool the solution to 4°C. **CAUTION** For large mammalian hearts >1L may be required. **CRITICAL** Must be made up fresh on the day of the experiment. **CRITICAL** Prepare slicing solution first. This needs to be cooled to ~4°C prior to starting procedure. **CRITICAL** If myocardial slices are to be cultured, all solutions must be filter-sterilized prior to starting procedure.

Table 1

Table 1: Preparation of 1L slicing solution (Tyrode's solution + 30mM BDM)		
Add the following to 1L distilled water:		
Solid reagents:		
	Mass (g)	Final concentration (mM)
2,3-Butanedione Monoxime	3.00	30.00
Glucose	1.86	1.00
HEPES	2.38	10.00
Potassium Chloride	0.45	6.00
Sodium Chloride	8.18	140.00
Solutions:		
	Volume (ml)	Final concentration (mM)
1M Magnesium Chloride	1.00	1.00
1M Calcium Chloride	1.80	1.80

Preparation of hot & cold heparinized slicing solution

Take 2x 60ml containers and fill with slicing solution. Add 100IU Heparin Sodium (1000IU/ml) to each container (100µl in 50ml slicing solution). Cool one container to 4°C and warm the other to 37°C. **CRITICAL** Must be made up fresh on the day of the experiment.

Preparation of 1L cardioplegia solution

This is only required for the cardioplegic arrest of large mammalian hearts *in situ* or the transportation of cardiac tissue specimens. Alternatively, pre-made clinical grade cardioplegia can be purchased and used.

To make up, add the following to 1L distilled water while mixing using a magnetic stirrer: 0.99g Glucose (5.50mM), 0.13g Magnesium Sulphate (0.50mM), 1.79g Potassium Chloride (24.00mM), 1.68g Sodium Bicarbonate (20.00mM), 6.37g Sodium Chloride (109.00mM), 0.12g Sodium Phosphate Monobasic Monohydrate (0.90mM) & 1.8ml 1M Calcium Chloride solution (1.8mM; see also Table 2 for details of the components required). Wait for the reagents to dissolve and then measure the pH. Add drops of 2M Sodium Hydroxide solution until the solution is pH 7.40. Filter the solution with a 0.2 micron pore filter and transfer to the previously sterilized 1L container. Cool the solution to 4°C. **CRITICAL** Must be made up fresh on the day of the experiment.

Table 2: Preparation of 1L cardioplegia solution		
Add the following to 1L distilled water		
Solid reagents:		
	Mass (g)	Final concentration (mM)
Glucose	0.99	5.50

Magnesium Sulphate	0.13	0.50
Potassium Chloride	1.79	24.00
Sodium Bicarbonate	1.68	20.00
Sodium Chloride	6.37	109.00
Sodium Phosphate Monobasic Monohydrate	0.12	0.90
Solutions:		
	Volume (ml)	Final concentration (mM)
1M Calcium Chloride	1.80	1.80

Preparation of 4% Agarose

Dissolve 4% agarose (4g in 100ml) in distilled water. Heat and stir until the agarose has melted (~80°C). Pour the hot agarose solution into large glass petri dishes to a height of 2-3mm. Leave to cool on a flat surface. Once the agarose has cooled and solidified, cover with Parafilm and keep at 4°C for up to 1 month.

CRITICAL It is crucial that the agarose is as flat as possible.

Equipment Setup

Sterilization of tools & equipment

This is only required for chronic studies that require myocardial slice culture.

To preserve sterility and prevent infections during culture, it is important to keep the vibratome and tools as clean as possible. Place all the tools, including small tweezers, micro dissecting spring scissors, surgical scissors and the vibratome specimen holder in self-seal sterilization pouch and autoclave. Transfer the non-autoclavable pieces of equipment (1L container + lid, vibratome bath, glass dish ± lid & customized 6 well plate) to a sterile laminar flow hood, spray with 70% ethanol and expose to UV light for one hour. **CRITICAL** Sterilization takes 1 hour. Sterilize equipment before setting up other pieces of equipment.

Vibratome calibration

Ensure the vibratome is clean by spraying with 70% ethanol and wiping dry. To work in complete sterility, place the vibratome in a sterile laminar flow hood. Mount a new ceramic blade. It is crucial to adjust the blade alignment until the Z-axis error is <1.0µm. If it is not possible to achieve a Z-axis error <1.0µm, remove the blade and wipe clean with ethanol before remounting. If achieving a Z-axis error <1.0µm is still not possible, the blade may be damaged and will need to be replaced. Once satisfied with blade alignment, mount the blade protector to avoid injuries.

TROUBLESHOOTING

Mounting of agarose on specimen holder

Remove the 4% agarose from the fridge. Cut a 2.5cm² piece of agarose. Place a large drop of histoacryl glue in the center of the base of the specimen holder. Using tweezers carefully pick up the square of agarose and place on the base of the specimen holder. Wait 30 seconds for the agarose to attach. Cut another 2.5cm² of agarose. Apply histoacryl glue to the back of the specimen holder and place the second square here. Wait 30 seconds for it to attach. The agarose-covered specimen holder should be as pictured in Fig. 2B. Place the specimen holder in a polythene bag and keep at 4°C. Specimen holder should be prepared on day of experiment. **CRITICAL** Make sure the agarose is completely flat as the tissue will be mounted on top. Uneven agarose can result in poor alignment of myocardial fibers and subsequent tissue damage.

Preparation of tissue dissection area

The tissue dissection area needs to be prepared in advance to minimize delays. Choose an area of the bench or sterile laminar flow hood close to the vibratome. The following will be required for dissection: 15cm Petri Dish, sterile surgical scalpel/razor blade, small tweezers, micro-scissors, Histoacryl surgical glue, surgical scissors (large mammal only) and specimen holder with agarose. Organise the dissection area as pictured in Fig. 2D.

Preparation of vibratome & holding bath

Mount the vibratome bath on the vibratome. Connect the waste tubing to a 500ml container. Fill the outer part of the vibratome bath with ice or set the bath cooling system to 4°C. Remove the slicing solution from the fridge and pour into the inner part of the vibratome bath. Bubble the solution with filtered 100% oxygen, making sure the tip of the tubing has been sterilized. The myocardial slice holding bath, which can hold slices prior to experiments, should also be prepared. Place the glass dish on ice. The 6 well dish with holes (Fig. 2A) should be placed inside and a cell strainers inserted into each well. Fill the dish with enough cold slicing solution to cover all the wells. Oxygenate the solution and cover the container with a lid. The set up should be as pictured in Fig. 2C/E.

[Insert Fig. 2 here]

PROCEDURE

Excision of mammalian heart

!CAUTION Experiments involving live animals must conform to relevant Institutional and National guidelines.

1 Follow option A if using a small mammalian heart (for example, rodents) or options B, C or D if using a large mammalian heart (for example human, pig and dog, respectively).

A) Excision of small mammalian heart (Rodents)

Time required: 5 minutes

CRITICAL The following procedure minimizes the pain, suffering and distress of rodents, while allowing the rapid excision of the rodent heart following sacrifice. This is crucial to obtain highly viable slices.

- i. Sedate the rodent with isoflurane (4% isoflurane and 4L/min oxygen).
- ii. Sacrifice the rodent following Institutional & National guidelines. Our laboratory performs cervical dislocation and death is confirmed via dissection of the carotid arteries.
- iii. Place the rodent in the supine position.
- iv. Locate the sternum. Hold the overlying skin with tweezers and make a small incision distally.
- v. Once the sternum is exposed, hold with tweezers. Make an incision along the entirety of the costal margin using scissors.
- vi. Once the diaphragm has been exposed, make another incision along costal junction to remove the diaphragm and allow access to the mediastinum.
- vii. Hold the lung parenchyma with your tweezers (pulling the heart and lungs away from the posterior wall of the thorax) and gently move your scissors behind the heart and lungs. Cut through the aorta and venae cavae.
- viii. Remove the heart and lungs from the animal by pulling the lungs. Never directly hold the heart with the tweezers.

CRITICAL The excision of a small mammalian heart should take no longer than 30 seconds following the sacrifice of the animal. The heart should still be vigorously beating following excision.

TROUBLESHOOTING.

- ix. Rapidly transfer the heart and lungs to your warm (37°C) heparinized slicing solution.
- x. With two fingers, gently and repeatedly compress the heart for 5-10 seconds to eject remaining blood from the ventricles.
- xi. Transfer the heart and lungs to the cold (4°C) heparinized solution and gently compress the heart to wash the remaining blood for 5 seconds. Keep the cold solution on ice and quickly transfer to the dissection area.

B) Excision of large mammalian heart (Human)

Time required: Excision = 5 mins, Transport to laboratory = 1-4 Hours

- i. Perfuse the heart with cardioplegia prior to explantation.

- ii. Following explantation and dissection of the failing heart, place the specimen in a 1L container with freshly made, cold (4°C) cardioplegia solution.
- iii. Place the container on ice and transfer to the laboratory.

PAUSEPOINT Heart remains viable for further studies if kept on ice for up to 4 hours.

C) Excision of large mammalian heart (Porcine)

- i. Carry out procedure in a controlled manner in theatre, under general anesthesia, with ongoing full monitoring and with antibiotic cover.
- ii. Perform sternotomy and isolate the aorta and venae cavae.
- iii. Administer heparin (300IU/Kg) and allow to circulate for 2 minutes.
- iv. Clamp the aorta and venae cavae.
- v. Deliver 2L cold cardioplegia into the aortic route at a delivery pressure of 300mmHg. Open the inferior vena cava at the junction of the right atrium to vent excess cardioplegia from the coronary sinus.
- vi. Cool the ventricular surface with cold saline / ice.
- vii. Carefully remove the heart by transecting the venae cavae and pulmonary veins.
- viii. Place the whole heart or biopsies in sterile containers with cold cardioplegia solution and put on ice.

PAUSEPOINT Heart remains viable for further studies if kept on ice for up to 4 hours.

D) Excision of large mammalian heart (Canine)

- i. Kill dog with an overdose of pentobarbital sodium (Dolethal 200mg/ml solution for injection).
- ii. Confirm death.
- iii. Remove the rib cage to expose the thoracic cavity. Remove the heart immediately and place into a 1L container with freshly made, cold (4°C) cardioplegia solution.
- iv. Place the container on ice and transport to laboratory.

PAUSEPOINT Heart remains viable for further studies if kept on ice for up to 4 hours.

Preparation of left ventricular tissue block

2 Follow option A and Supplementary Videos 1-3 if using a small mammalian heart or option B if using a large mammalian heart.

Time required: 5-10 minutes

A) Preparation of small mammalian (Rat) left ventricular tissue block

CRITICAL See **Supplementary Video 1** to visualize steps 2A i-vii

- i. Carefully transfer the heart and lungs to the large petri dish at the tissue dissection area.
- ii. Fill the petri dish with enough 4°C slicing solution to cover at least half the heart and keep it cold.
- iii. Using a scalpel/razor blade, dissect off the lungs and other tissues to leave an intact heart (Fig. 3A).
- iv. Visualize the atria and make an incision slightly inferiorly (through the base of the heart) (Fig. 3B).
- v. From the superior position, visualize the left and right ventricular walls and the septum. Locate the right ventricle, which is crescentic in shape and has a thinner wall. With small tweezers, hold the right ventricle by its free wall (Fig. 3C).
- vi. With micro-scissors in your other hand, make an incision along the right ventricular-septal junction towards the apex (Fig. 3D).
- vii. Continue to hold the right ventricle by its free wall (Fig. 3E) and cut along the other ventricular-septal junction to remove it (Fig. 3F). Some papillary muscles may also have to be cut to remove the right ventricle.

CRITICAL See **Supplementary Video 2** to visualize steps 2A viii – x

- viii. You should now have the left ventricle and septum. Locate the septum and make an incision down towards the apex using micro-scissors (Fig. 3G).
- ix. Open the left ventricle and allow it to flatten (Fig. 3H).
- x. If further flattening is required, make small incisions along the superior and inferior borders of the tissue block. If necessary, also cut the papillary muscles (Fig. 3I).

CRITICAL See **Supplementary Video 3** to visualize step 2A xi

- xi. Turn the block over and use a scalpel/razor blade to remove remaining septal tissue from the edges of the tissue block (Fig. 3J). You should now have a relatively flat block of left ventricular tissue (Fig. 3K/L).

CRITICAL It is critical to make the tissue as flat as possible. Use micro-scissors to cut large papillary muscles from the endocardial surface and remove any fibrous tissue or sub-valvular tissue from the basal part of the ventricle. It may also be necessary to make very small incisions along the borders of the tissue block to make it truly flat. During tissue handling it is important to keep the tissue cold to prevent the myocardium from contracting.

TROUBLESHOOTING

[Insert Fig. 3 here]

B) Preparation of large mammalian (Human) left ventricular tissue block

- i. Transfer the heart from the container of cold cardioplegia solution to the tissue dissection area.
- ii. Remove the heart from the container and place in the large petri dish.
- iii. Using the scalpel/razor blade make an incision inferior to the atria and atrioventricular valves to leave the left and right ventricles and the septum.
- iv. Identify the left ventricle and locate the area of the left ventricular free wall with the least epicardial curvature.
- v. Dissect out a 1.5cm² tissue block from this area by making incisions through the full thickness of the ventricular wall with a scalpel/razor blade.

TROUBLESHOOTING

CRITICAL Human specimens may contain regions of pathology (E.g. infarct scarring). Unless specifically required for experiments, these areas should be avoided to produce highly viable myocardial slices.

Mounting tissue block

CRITICAL See **Supplementary Video 4** to visualize steps 3-8

CRITICAL Tissue blocks are attached to the agarose-coated specimen holder epicardial surface down. The epicardium is significantly flatter than the endocardium and this helps align the myocardial fibers in the same plane within the tissue. This is crucial to obtain myocardial slice with high viability.

3 Lightly blot the epicardial surface of the tissue block with tissue paper to remove excess solution from its surface.

4 Apply a drop of Histoacryl glue to the agarose attached to the base of the specimen holder. Spread to ensure a thin and even coating of glue over the entire surface.

5 Using small tweezers, hold and pick up the tissue block by one of its lateral edges.

6 Move the left ventricle so that it is positioned directly over the specimen holder. Slowly lower the ventricle until the free edge of the ventricle makes contact with the agarose.

!CAUTION Tissue will attach to the surface immediately upon contact. It is not possible to remove tissue once stuck.

7 Smoothly roll the rest of the ventricle onto the agarose until the entire epicardial surface is in contact.

8 Using a flat spatula, apply gentle pressure to the endocardial surface to ensure the tissue attaches.

!CAUTION The sample should not be placed too close to the back of the specimen holder as this can cause slices to become trapped during slicing.

9 Rapidly transfer the specimen holder to inner part of the vibratome bath.

TROUBLESHOOTING

Slicing

Time required: 0.5-1.5 Hours

CRITICAL See **Supplementary Video 5** to visualize steps 10-14

10 Once the tissue block is in position, move the blade to the correct starting position. Move the blade forward until it reaches the anterior border of the tissue block. Adjust the height of the blade to the superior border of the tissue block. The following settings have been optimized to maintain tissue viability while slicing. The blade should be set to vibrate at a frequency of 80Hz and an amplitude 2.00mm. The section thickness should be 300µm (although slices of thickness 100-400µm can be produced). While slicing, advance the blade at 0.03mm/s. Ensure Z-axis error <1.0µm.

11 Start slicing. The blade should advance slowly and make contact with the tissue.

12 Once the blade reaches the posterior border of the tissue block, stop the blade advancing.

13 Return the blade to the starting position. Once the blade has returned to this position, it will automatically lower by 300µm (or the selected slice thickness).

14 Start slicing. Repeat steps 11-13 until you have sliced through the full thickness of the ventricle.

TROUBLESHOOTING

Handling & storage of myocardial slices

Time required: 1 minute

CRITICAL See **Supplementary Video 6** to visualize steps 15-16

15 Once the blade has completely cut through the tissue block, a slice will detach from the superior surface of the tissue block. It takes 5-10 minutes to cut a slice (depending on tissue block dimensions) due to the slow speed at which the blade advances. Use a Pasteur pipette to transfer a slice to the holding bath. Move the pipette tip under the slice and gently lift. The slice should drape over the pipette tip.

TROUBLESHOOTING

!CAUTION It is important not to suck the slice inside the Pasteur pipette as this creates tissue damage.

16 Submerge the pipette into one of the cell strainers in the holding bath to release the slice. Make sure the slice is flat on the cell strainer gauze. Place a circular gauze over the top of the slice and hold down using a washer.

PAUSEPOINT Slices can be kept in the holding bath for up to 4 hours. Slices must be used for acute experiments or put in culture within this time period.

17 Repeat further slicing.

18 Ceramic blades can be used to produce myocardial slices from several animals. To avoid damage to the blade, we do not recommend removing it between experiments. Thoroughly clean the blade with 70% ethanol after each experiment. If multiple tissue blocks are sliced in a single day, clean the blade between each and recheck the z-axis error.

Additional Steps:

Culturing, fixing & staining of myocardial slices

19 Follow step A for culturing or step B for fixing and staining.

A) Culturing – Air-liquid interface method

Time required: 10-15 minutes for 6 slices

CRITICAL See **Supplementary Video 7** to visualize steps 19 A i-viii

!CAUTION The following steps must be carried out in a sterile manner in a laminar flow hood. Spray all items with 70% ethanol before placing them in flow hood and observe standard cell culture practices.

- i. Transfer a myocardial slice from the holding bath to a sterile petri dish using a Pasteur pipette. Gently pipette some cold Tyrode's solution onto the slice surface to keep it cool and prevent it from drying out. Quickly transfer to flow hood. **CRITICAL** Handle myocardial slices as described in "Handling & storage of myocardial slices".
- ii. Wash the slice with PBS + 3% Penicillin-Streptomycin at room temperature.
- iii. Myocardial slices can be cultured on an air-liquid interface using Transwell® membrane cell culture inserts. Use a Pasteur pipette to transfer the slice onto the surface of the Transwell® membrane and pipette some cold Tyrode's solution into the insert.
- iv. Gently remove the solution from the insert using a Pasteur pipette. Make sure the slice is lying flat on the Transwell® membrane.
- v. Remove as much liquid as possible from the edges of the slice using a Pasteur pipette.
- vi. Place 1ml of medium (Medium-199 + 3% Penicillin-Streptomycin + 0.001% ITS liquid media supplement) into the well in which the insert is placed.
- vii. Cover the 6 well plate with lid and transfer to an incubator. Slices should be incubated in humidified air at 37°C with 5%CO₂.
- viii. Replace medium every 24 hours. Penicillin-Streptomycin concentration can be reduced to 1% after 24 hours. An additional wash in PBS + 3% Penicillin-Streptomycin can be carried out after 24 hours to prevent infections.

B) Fixing & Staining

- i. Myocardial slices are fixed in 4% formaldehyde solution for 15 minutes at room temperature.

PAUSEPOINT Fixed myocardial slices can be kept in PBS at 4°C for several months.

- ii. If permeabilization is required, permeabilize the fixed slice in 1% Triton X-100 for 1 hour at room temperature.
- iii. Block the slice using 10% FBS, 5% BSA and 10% horse serum in PBS solution (for several antibodies a weaker blocking (1%BSA) is sufficient).
- iv. Incubate slice in primary antibody in PBS at 4°C overnight.
- v. Wash the slice in PBS for 30 minutes. Repeat three times.

- vi. Incubate slice in secondary antibody in PBS for 3 hours at room temperature.
- vii. Wash the slice in PBS for 30 minutes. Repeat three times.

PAUSEPOINT Stained myocardial slices can be kept at 4°C covered in aluminum foil for up to one week. Please be aware that secondary antibody fluorescence decreases with time and imaging samples directly after staining is recommended.

TIMING

Total time required: ~ 4 hours (excluding additional steps)

- Reagent set up – 1 hour
- Equipment set up – 1 hour
- Excision of heart (step 1)– 5 minutes (if excision cannot take place in laboratory, additional tissue transport time will be needed)
- Preparation of left ventricular tissue block (steps 2-9) – 5-10 minutes
- Slicing (steps 10-18) – 0.5-1.5 hours (dependent on thickness of ventricle)
- *Additional steps: Culturing – 10-15 minutes to put 6 slices in culture, Fixing & Staining – 24 hours*

TROUBLESHOOTING

For troubleshooting guidance see Table 3.

Table 3: Troubleshooting table			
Step	Problem	Possible Reason	Solution
Equipment Setup - Vibratome Calibration	Z-axis error >1.0µm	Blade is dirty	Clean blade and blade holder with 70% ethanol.
		Blade is damaged	Replace blade
		Blade not mounted correctly	Remove blade and remount. Make sure the blade is positioned in the middle of the blade holder.
Step 1A - Excision of small mammalian heart	Heart is not beating following excision	Excision took too long	Discard heart. Make sure you have been adequately trained and have understood the methodology for excision.
		Animal too deeply anaesthetized	Closely observe the animal during anesthesia. Monitor

			level of consciousness (E.g. pedal reflex).
Step 2A - Preparation of small mammalian left ventricular tissue block	Heart is spontaneously contracting during dissection	Heart is not sufficiently cooled	Transfer the heart to the holding bath. Submerge in the cold (4°C) Tyrode's solution for 30 seconds to 1 minute. Transfer heart to dissecting area and continue. Dissection needs to be as quick as possible.
		Excitation-Contraction uncoupler not added to slicing solution	Ensure 2,3-Butandione Monoxime has been added to slicing solution.
Step 2B - Preparation of large mammalian left ventricular tissue block	Whole heart not received	Biopsy / part of the ventricle received	Locate the epicardial surface of the tissue. Locate the flattest portion of the epicardium and dissect the tissue block from here.
Step 9 - Mounting tissue block	Tissue does not look flat on specimen holder	Agarose not flat	Ensure the agarose used to coat the specimen holder is flat.
		Tissue mounted poorly	Make sure the tissue is rolled onto the specimen holder to ensure even attachment over the entirety of the tissue
		Tissue mounted endocardium-down	The endocardium is significantly less flat than the epicardium. Discard tissue block and start again.
Step 14 - Slicing	Foam is appearing in slicing solution	Slicing solution incorrectly made	Tissue damage is occurring - check viability of slices prior to use
		Tissue block poorly mounted	Tissue damage is occurring - check viability of slices prior to use

Step 15 - Handling myocardial slices	Slice has not detached from the superior surface of the tissue block	Fibrous tissue is present	Use micro-scissors to free the slice by carefully cutting through any fibrous tissue. Make sure the blade has reached its final position and stopped vibrating before attempting this.
		Vibratome hasn't cut through entirety of tissue	Continue to slice until the slice detaches from the surface. Take care that the blade does not hit the back of the specimen holder.

ANTICIPATED RESULTS:

In this section we present typical data that we collected from healthy rat (small mammal) and failing human hearts (Human HF) (large mammal) myocardial slices. When we present N values this indicates: number of slices/number of hearts (E.g. 10/5 = 10 slices derived from 5 hearts). All data is presented as mean $\pm$ standard error.

For structural and functional studies only aligned areas (myocardial fibers running parallel to each other) should be used. To visualize the aligned areas, place the myocardial slice on a petri dish and remove excess solution. Visualize the structure of a slice using a macroscope and determine the aligned area of the slice (Fig. 4). Using a scalpel/razor blade, trim the slice to isolate the aligned area and use for structural and functional studies.

[Insert Fig. 4 here]

Viability

The viability of the surface of myocardial slices can be assessed using Live/Dead staining (Thermo Fisher Scientific). Live cells are stained green by calcein-AM and the nuclei of dead cells are stained red by ethidium homodimer-1. The surface of myocardial slices can be imaged with widefield microscopy (Fig. 5A) or confocal microscopy (Fig. 5B). The % area of living cells on the surface can be quantified using Image J (National Institute of Health, USA). We found $59.4 \pm 6.4\%$ of cells were alive on the surface of rat myocardial slices (N=11/6 – Fig. 5C) and $47.4 \pm 4.1\%$ were alive on the surface of human HF slices (N=10/4 – Fig. 5D).

[Insert Fig. 5 here]

Imaging of the layer of cardiomyocytes directly below the slice surface revealed almost all of the cardiomyocytes were alive (Fig. 6A), indicating that tissue damage

was limited to the slice surface. It was not possible to image the full thickness of the myocardial slice using confocal microscopy as the tissue is too optically dense. Calculations were used to estimate the viability of the myocardial slices. These revealed that the damaged cardiomyocytes on the myocardial slice surface account for ~3% of the total cardiomyocyte population within the slice, demonstrating preserved viability (See box below for full calculations).

Estimation of the viability of adult rat ventricular myocardial slice		
The following calculations assume cardiomyocytes below the myocardial slice surface are 100% viable and a 7x7mm myocardial slice is used		
Myocardial slice volume: 14700000000μm³		
Estimation of average adult rat cardiomyocyte volume(41)		
	Percentage (%)	Volume (μm3)
Mononucleated cardiomyocytes	3.50	18006
Binucleatued cardiomyocytes	96.50	25453
∴ Average cardiomyocyte volume: 25192μm³		
Total cardiomyocytes in myocardial slice: 583519 (Myocardial slice volume / Average cardiomyocyte volume)		
Estimation of adult rat cardiomyocyte surface area exposed at the myocardial slice surfaces(42)		
	Length (μm)	
Average cardiomyocyte length	112	
Average cardiomyocyte width	25	
∴ Average exposed area of surface cardiomyocytes: 2800μm² (Average cardiomyocyte length x Average cardiomyocyte width)		
Exposed myocardial slice surface area: 106400000μm² (Both cut surfaces & edges)		
Total cardiomyocytes at the myocardial slice surface: 38000 (Exposed myocardial slice surface area / exposed cardiomyocyte area)		
Number of dead cardiomyocytes at exposed myocardial slice surface: 15200 (If we consider 40% of surface cardiomyocytes are killed during slicing)		

% dead cardiomyocytes within myocardial slice: 2.60% (Number of dead cardiomyocytes at surface / total number of cardiomyocytes)

There are a number of critical steps that must be followed to produce slices of high viability. The method by which the tissue block is prepared is particularly important, especially for small mammals. The method described in this protocol ensures the left ventricle of small mammals is completely opened and consequently flattened. This is critical as the ventricles of small mammals have a pronounced curvature. Flattening of the ventricles ensures the long axes of the myocardial fibers lie in the same plane as the blade. When this is the case, the layers separate during slicing, resulting in minimal damage to cardiomyocytes within the slice. Due to the reduced curvature of the large mammalian ventricle, less dissection and flattening is required. However, with both small and large mammals, it is important to mount the tissue epicardium-side down (epicardial surface in contact with agarose). The epicardium is significantly flatter than the endocardium, which is covered with papillary muscles, and this helps to correctly orientate the fibers. When the tissue is mounted endocardium-side down, it will have an increased curvature and more damage will occur at the edges of slices (Fig. 6B). When the tissue block is not flat myocardial fibers will be transected, reducing slice viability (Fig. 6C/D). It is not possible to prepare highly viable myocardial slices by simply sticking biopsies onto agarose when fiber direction is ambiguous. This also applies to embedding biopsies in agarose, which disregards fiber direction.

[Insert Fig. 6 here]

There are a number of additional factors that must also be considered to preserve viability. An important determinant is the way in which the cardiac tissue is explanted. Small mammalian hearts can be directly removed from the animal following sacrifice, while large mammalian hearts require cardioplegic arrest prior to explantation. The faster the heart is explanted and cooled, the higher the viability of myocardial slices. Therapeutic hypothermia is cardioprotective(43,44), reducing the tissue's metabolic demand and the initiating protective signaling pathways(45). The time taken to slice is also crucial. Oxygen is unable to diffuse through the entirety of the ventricular wall and the tissue becomes increasingly ischaemic with time. This is minimized by tissue cooling, but any delays will impact on slice viability. Studies have shown that diffusion distances >200µm are generally poorly tolerated by tissues(33). 300µm slices have a maximum diffusion distance of 150µm from either surface and allow the rapid diffusion of substrates to their innermost cells(34). Thinner slices (100-250µm) can be prepared but they are more difficult to handle and, because of the lower number of cell layers, the proportion of damaged cardiomyocytes is increased. The use of an excitation-contraction uncoupler (2,3-butanedione monoxime) prevents the cardiac tissue from contracting during slicing and its omission results in poor slice viability. The vibratome settings are also important and were optimized in our laboratory, as described in Camelliti et al(5).

Other studies investigating optimal slicing solutions have been carried out and the solutions described in this protocol yielded the highest viability.

Contractility

A force transducer (Harvard Apparatus, USA) was used to assess the maximum contractility of myocardial slices. Rat and human HF slices were field stimulated at 1Hz (Fig. 7A) and 0.5Hz (Fig. 7C) (10-30V), respectively. The maximum contractility was recorded by stretching slices in a step-wise manner. Custom PTFE-coated silver rings were attached to opposite ends of myocardial slices orthogonal to longitudinal fiber direction using Histoacryl surgical glue. Slices were stretched by ~5% and then left to contract for 2 minutes. This was repeated until isometric contraction was achieved and maximum contractility was recorded. Rat slices had a maximum contractility of $11.6 \pm 1.3 \text{ mN/mm}^2$ (N=10/10 – Fig. 7B). Human HF slices had a maximum contractility of $13.8 \pm 3.5 \text{ mN/mm}^2$ (N=9/9 – Fig. 7D).

See **Supplementary Video 8** to visualize a human heart failure and rat myocardial slices contracting on a force transducer.

[Insert Fig. 7 here]

Rat slices were paced at 1Hz, while human HF slices were paced at 0.5Hz. Rat slices are derived from healthy animals and can follow a field stimulus of >5Hz (resting heart rate around ~300bpm(46)). Human HF slices can be paced at >0.5Hz but at these rates the tissue cannot fully relax, reflecting the disease state of the tissue (end-stage HF, NYHA class IV). Myocardial slices can also be point stimulated (1V) (See “Supplementary Video 7”).

Ca²⁺ Handling

To assess Ca²⁺ handling, myocardial slices were loaded in Medium-199 (Sigma) with Fluo-4 AM (5.56µg/ml) (Thermo Fisher Scientific) and Pluronic F-127 (0.001%) (Thermo Fisher Scientific). A circular gauze and washer were placed over the slice (same as “Procedure – Step 17” / Supplementary Video 6) to prevent contraction during loading. Rat myocardial slices were loaded for 10 minutes at 37°C and then directly transferred to the optical mapping set up. Human HF slices were loaded for 20 minutes and then left to de-esterify for a further 10 minutes in Tyrode's solution with 30mM 2,3-Butandione Monoxime at 37°C. 2,3-Butandione Monoxime prevents movement artefact during calcium transient acquisition. Rat and human HF slices were field stimulated at 1Hz (Fig. 8A) and 0.5Hz (Fig. 8B) (30V), respectively. The average time to peak, time to 50% decay and 90% decay were analysed (Fig. 8C). Rat myocardial slices have a time to peak of $30.3 \pm 1.3 \text{ ms}$, time to 50% decay of $61.9 \pm 2.5 \text{ ms}$ and time to 90% decay of $153.3 \pm 8.5 \text{ ms}$ (N=24/6 – Fig. 8D). Human HF myocardial slices had a time to peak of $134.1 \pm 16.5 \text{ ms}$, time to 50% decay of $408.5 \pm 38.5 \text{ ms}$ and time to 90% decay $981.7 \pm 98.4 \text{ ms}$ (N=10/3 – Fig. 8E).

[Insert Fig. 8 here]

See **Supplementary Video 9** to visualize Fluo-4 flashing on the surface of a rat myocardial slice.

Conduction Velocity

The conduction velocity of myocardial slices can be assessed using multielectrode arrays (Multichannel Systems), which allow non-invasive, synchronous, multifocal recording of extracellular field potential. Data were acquired with a 60 microelectrode plate, arranged in 8 x 8 matrix with a 700µm inter-electrode distance, providing a recording area of 4.9 x 4.9mm². Representative conduction maps show the propagation of electrical activity in rat and human HF slices longitudinal (Fig. 9A & 9D) and transverse (Fig. 9B & 9E) to myocardial fiber direction. Rat myocardial slices have a longitudinal conduction velocity of 60.9±7.7cm/s and a transverse conduction velocity of 21.9±2.1cm/s (both N=14/14 – Fig. 9C). Human HF myocardial slices have a longitudinal conduction velocity of 55.5±9.8cm/s and a transverse conduction velocity of 17.6±2.0cm/s (both N=11/4 – Fig. 9F).

[Insert Fig. 9 here]

Structural Studies

The structure of myocardial slices can be investigated using various techniques. We have used immunohistochemistry and confocal microscopy. As myocardial slices can be stained and imaged either fresh or fixed and do not require sectioning, they are an ideal model for assessing cardiac structure. Sectioning requires tissue freezing, handling and processing and can result in sample damage. Due to tissue density and light scattering, imaging is limited to the surface of the preparation (2-3 cell layers). A number of antibodies were used to visualize fundamental cardiac structures (see “Table 4” for further details). Caveolin 3 antibody was used to stain cardiomyocyte cell membranes and intercalated discs were stained with connexin-43 antibody (Fig. 10A). The structure of the sarcomeric apparatus was assessed with α-actinin staining (Fig. 10B). The microvasculature was stained with isolectin and the cardiac stromal cell population was stained with vimentin (Fig. 10C). More detailed assessment of myocardial slice ultrastructure can be carried out using scanning electron microscopy (Fig. 10D). To image the full thickness of the myocardial slice, optical clearing methods were used as described by Perbellini et. al(25). Second harmonic generation imaging was used to visualize collagen distribution within a human HF slice (Fig. 10E). The addition of antibody labelling for large blood vessels (vimentin) and high definition 3D reconstruction provided a precise map of the macrovascular network (Fig. 10F).

Table 4: Antibodies			
Primary Antibodies			
Caveolin 3	Raised in mouse	1 in 500	BD Biosciences
Connexin-43	Raised in rabbit	1 in 2000	Sigma

α -Actinin	Raised in mouse	1 in 2000	Sigma
Vimentin	Raised in chicken	1 in 3000	Thermo Scientific
Isolectin B4	Biotin conjugated	1 in 1000	Life Technologies
Secondary Antibodies			
Alexa 488	Raised in donkey, Anti-mouse	1 in 2000	Life Technologies
Alexa 546	Raised in donkey, Anti-rabbit	1 in 2000	Life Technologies
Alexa 647	Raised in goat, Anti-chicken	1 in 2000	Life Technologies
Nuclear Staining			
Hoechst 33342		1 in 1000	Life Technologies

[Insert Fig. 10 here]

Conclusions

Myocardial slices provide a unique platform that facilitate the study of myocardial structure and function at the cellular level *in vitro*. Myocardial slices have been produced for a number of years, but the protocols used have varied greatly (2,5,6,15). This protocol describes a robust and reproducible method for producing slices with high viability and addresses a number of critical steps that must be followed. The data presented represents a fraction of the studies that can be performed using myocardial slices. Data collected using myocardial slices has the advantage of being derived from native adult myocardium with preserved multicellularity and architecture. These aspects play important roles in myocardial function(28,29) and cannot be ignored. Due to their unique advantages and their ability to be chronically cultured, myocardial slices will be an important platform for future cardiovascular research.

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AUTHOR CONTRIBUTION STATEMENT:

SW wrote the manuscript, collected data and contributed to the optimization of the protocol. MS collected data and contributed to the optimization of the protocol. IB contributed to the optimization of the protocol. RA provided porcine specimens. CT contributed to the optimization of the protocol. FP collected data and contributed to the optimization of the protocol. All authors proof read the manuscript.

DISCLOSURES:

The authors have nothing to disclose.

FIGURE LEGENDS:

Fig. 1

Representative images of a rat myocardial slice. A) Original myocardial slice. Photograph taken directly after slicing. B) Aligned area. Photograph taken after the aligned area of the slice had been visualized under a macroscope and cut out using a scalpel (For further information, see “Fig. 4”).

Fig. 2

Apparatus required for the production of myocardial slices. A) Customized 6 well dish with holes drilled into the base of each well. B) Specimen holder covered with 4% agarose. Both the base and the back of the holder should be completely covered. C) Myocardial slice holding bath (without lid). Filled with cold slicing solution and bubbled with filtered 100% O₂. Bath surrounded with ice to maintain a temperature of 4°C. D) Suggested dissection area set up. E) Right hand side – vibratome with vibratome bath in place. Outer section of bath is filled with ice and inner section is filled with cold slicing solution, bubbled with filtered 100% O₂. Left hand side – myocardial slice holding bath covered with lid. Holding bath placed on ice.

Fig. 3

Preparation of small mammalian (rat) left ventricular tissue block. These images show dissection with a dry heart to aid visualization of the method. However, always dissect tissue in a petri dish with the tissue submerged in cold slicing solution. For further details, please consult step 2A. A) Using a surgical scalpel/razor blade, dissect off the lungs and other tissues. B) Visualize the atria and make an incision slightly inferiorly, through the base of the ventricle. C) Visualize the right ventricle, which has a thinner wall and is more crescentic in shape. Hold the free wall of the right ventricle with tweezers. D) Using micro-scissors, cut along the right ventricular-septal junction on one side. Cut towards apex. E) Lift the cut border of the right ventricle away from the heart. F) Cut along the other ventricular-septal junction to remove the right ventricle. G) Place one edge of the micro-scissors inside the left ventricle and cut along the septum towards the apex. H) The ventricle should now open. I) Make small incisions along the borders of the tissue block to allow the ventricle to fully flatten. Fibrotic tissue and large papillary muscles will also need to be cut. J) Turn the tissue block over so the epicardium is facing upwards. Visualize

the edges of the septum at the lateral edges of the tissue block. Remove with surgical scalpel/razor blade. K/L) Flat left ventricular tissue block. Tissue block is 20x12mm in size.

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Fig. 4

Rat myocardial slice on a 1mm grid visualized using a macroscope. Aligned area, where myocardial fibers are parallel, is highlighted in green. This area is appropriate for structural and functional studies. Non-aligned areas are highlighted in red.

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Fig. 5

Myocardial slice viability. Viability was assessed using a Live/Dead staining. Live cells are stained green (Calcein-AM), the nuclei of dead cells are stained red (Ethidium homodimer-1). A) Representative image of Live/Dead staining of a rat myocardial slice imaged using widefield microscopy. Scale bar = 1000µm B) Representative image of Live/Dead staining of a human HF myocardial slice imaged using confocal microscopy. Scale bar = 300µm C) 59.4±6.4% of the surface of rat myocardial slices was alive (N=11/6). D) 47.4±4.1% of the surface of human HF myocardial slices was alive (N=10/4).

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Data is presented as mean ± standard error
N = number of slices/number of hearts

Fig. 6

Understanding the viability of myocardial slices. A) ~50-60% of cardiomyocytes on the surface of myocardial slices were alive. Living cardiomyocytes were stained green (Calcein-AM), while the nuclei of dead cardiomyocytes were stained red (Ethidium Homodimer-1). Images were acquired using confocal microscopy. Deep imaging of the layers of cardiomyocytes below the surface revealed that almost 100% of cardiomyocytes below the surface were alive. The representative animation shows live cells in green and dead cells in red. Animation of slice surface shows ~50% of cells alive. Animation of layer of cardiomyocytes just below the slice surface shows almost all cardiomyocytes are alive. B) The tissue block should never be

mounted endocardium-down. When this is the case, the tissue block has an increased curvature which results in large portions of tissue being damaged. Damage usually occurs on the lateral portions of slices. C/D) When the tissue block is not flat, myocardial fibers can be transected. Slices were stained with α -actinin (see 'Table 4'; further information on fixing and staining myocardial slices can be found in "Procedure – Additional Steps – Fixing & Staining") and imaged using confocal microscopy. The images show areas of undamaged cardiomyocytes alongside areas where cardiomyocytes have been transected. Cardiomyocytes lying in the same plane as the blade are undamaged, while those lying perpendicular to the blade are transected.

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Fig. 7

Contractility of rat and human HF myocardial slices. A) Representative contractility traces from rat myocardial slice field stimulated at 1Hz. B) Maximum contractility of rat myocardial slices was $11.6 \pm 1.3 \text{ mN/mm}^2$ (N=10/10). C) Representative contractility traces from human HF myocardial slices field stimulated at 0.5Hz. D) Maximum contractility of human HF myocardial slices was $13.8 \pm 3.5 \text{ mN/mm}^2$ (N=9/9).

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Data is presented as mean $\pm$ standard error

N = number of slices/number of hearts

Fig. 8

Ca^{2+} handling of rat and human HF myocardial slices was assessed by loading slices with Fluo-4 AM and optical mapping. A) Representative Ca^{2+} transient from a rat myocardial slice field stimulated at 1Hz. B) Representative Ca^{2+} transient from a human HF myocardial slice field stimulated at 0.5Hz. C) Visual description of the aspects of Ca^{2+} handling kinetics assessed. D) Ca^{2+} handling kinetics of rat myocardial slices field stimulated at 1Hz. E) Ca^{2+} handling kinetics of human HF myocardial slices field stimulated at 0.5Hz.

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Data is presented as mean $\pm$ standard error

N = number of slices/number of hearts

Fig. 9

Conduction velocity of rat and human HF myocardial slices. A&B) Representative conduction maps from rat myocardial slices, with electrical propagation longitudinal (A) and transverse (B) to myocardial fiber direction. C) Rat myocardial slices have a longitudinal conduction velocity of 60.9 ± 7.7 cm/s and a transverse conduction velocity of 21.9 ± 2.1 cm/s (N=14/14). D&E) Representative conduction maps from human HF myocardial slices, with electrical propagation longitudinal (D) and transverse (E) to myocardial fiber orientation. F) Human HF myocardial slices have a longitudinal conduction velocity of 55.5 ± 9.8 cm/s and a transverse conduction velocity of 17.6 ± 2.0 cm/s (N=11/4).

Imperial College London provided permission for the use of the animals in this study. All procedures were performed under license by the UK Home Office, in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986. Animals were killed following guidelines established by the European Directive on the protection of animals used for scientific purposes (2010/63/EU).

Data is presented as mean $\pm$ standard error
N = number of slices/number of hearts

Fig. 10

Cardiac structure studied using myocardial slices. A-C) Immunohistochemical staining and confocal microscopy of rat myocardial slices. A) The cell membranes of cardiomyocytes have been stained with caveolin 3 antibody, the intercalated disc is stained with connexin-43 antibody & nuclei are labelled with Hoechst 33342. B) The sarcomeric apparatus is stained with α -actinin antibody and nuclei are labelled with Hoechst 33342. C) Microvasculature is stained with isolectin antibody, the cell membranes of cardiomyocytes are stained with caveolin 3 antibody, fibroblasts are stained with vimentin antibody and nuclei are labelled with Hoechst 33342. D) Scanning electron microscopy of human HF myocardial slice. Transverse section. Grey structures are cardiomyocytes and the smaller, darker substructures are mitochondria. E/F) Optical clearing was used to image the full thickness of myocardial slices (300 μ m)(25). Second harmonic generation imaging was used to visualize collagen distribution within a human HF myocardial slice (E). The macrovascular network was imaged using this method and the addition of a vimentin antibody (F). Further information on the antibodies used can be found in "Table 4". The fixing and staining protocol used is described in "Procedure – Additional Steps – Fixing & Staining".

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SUPPLEMENTARY VIDEO LEGENDS**Supplementary Video 1**

Preparation of left ventricular tissue block from small mammalian heart (Rat) – Part 1. Video depicts steps 2A i-vii.

The preparation of the tissue block in this video has been carried out slowly to aid the visualization of the technique. However, tissue block preparation should be carried out as quickly as possible. Tissue should be kept cool throughout. If tissue contracts during block preparation, transfer to holding bath and allow to cool for 30 seconds.

Supplementary Video 2

Preparation of left ventricular tissue block from small mammalian heart (Rat) – Part 2. Video depicts steps 2A viii-x.

The preparation of the tissue block in this video has been carried out slowly to aid the visualization of the technique. However, tissue block preparation should be carried out as quickly as possible. Tissue should be kept cool throughout. If tissue contracts during block preparation, transfer to holding bath and allow to cool for 30 seconds.

Supplementary Video 3

Preparation of left ventricular tissue block from small mammalian heart (Rat) – Part 3. Video depicts step 2A xi.

The preparation of the tissue block in this video has been carried out slowly to aid the visualization of the technique. However, tissue block preparation should be carried out as quickly as possible. Tissue should be kept cool throughout. If tissue contracts during block preparation, transfer to holding bath and allow to cool for 30 seconds.

Supplementary Video 4

Mounting of left ventricular tissue block on agarose-coated specimen holder. Video depicts how to handle, dry and mount tissue block correctly.

Supplementary Video 5

Slicing of left ventricular tissue block. Video depicts bringing blade to “starting position” and various stages of slicing.

Supplementary Video 6

Handling & storage of myocardial slices. Video depicts how to move a myocardial slice from vibratome bath to holding bath. Video also shows how myocardial slices should be kept in holding bath.

Supplementary Video 7

Culturing myocardial slices using air-liquid interface method. Video depicts how to culture myocardial slices on an air-liquid interface using Transwell® membranes as described by Brandenburger et al(2).

Supplementary Video 8

Myocardial slice contraction. Video depicts a human HF myocardial slice contracting (field stimulation, 0.5Hz, 30V) and a rat myocardial slice contracting (point stimulation, 1Hz, 10V).

Supplementary Video 9

Ca²⁺ handling of myocardial slices. Rat myocardial slice was loaded with Fluo-4 AM (as described in 'Anticipated Results – Ca²⁺ handling'). Myocardial slice was field stimulated at 1Hz, 10V. Cardiomyocytes at the slice surface flash with each calcium transient.

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Figure 1

Myocardial Slice - Rat

A Original Slice

10mm

B Aligned Area

10mm

Figure 2

Figure 3

Figure 4

A Rat: LiveDead Staining
Widefield Microscopy

Live (Calcein) Dead (Ethidium)

C Rat: Viability

B Human HF: LiveDead Staining
Confocal Microscopy

Live (Calcein) Dead (Ethidium)

D Human HF: Viability

Figure 6

Surface → One Cell Layer Deep

Tissue block mounted endocardium-down

A Rat: Representative Contractility Traces
1Hz Field Stimulation

B Rat: Max. Contractility

C Human HF: Representative Contractility Traces
0.5Hz Field Stimulation

D Human HF: Max. Contractility

A Rat: Representative Ca^{2+} Transient
1Hz Field Stimulation

B Human HF: Representative Ca^{2+} Transient
0.5Hz Field Stimulation

D Rat: Ca^{2+} Kinetics

E Human HF: Ca^{2+} Kinetics

A Rat: Longitudinal

B Rat: Transverse

C Rat: Conduction Velocities

D Human HF: Longitudinal

E Human HF: Transverse

F Human HF: Conduction Velocities

Figure 10