High spatial and temporal resolution Ca^{2+} imaging of myocardial strips from human, pig and rat

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Key references using this protocol

Burdyga, T. et al. Cell Calcium 34, 27-33 (2003): https://doi.org/10.1016/s0143-4160(03)00019-8
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EDITORIAL SUMMARY This describes how to undertake high spatial and temporal Ca^{2+} imaging of *ex vivo* multicellular myocardial strips that include the endocardial surface, allowing the study of the Ca^{2+} signaling underpinning cardiomyocyte contraction.

TWEET High spatial and temporal resolution calcium imaging of myocardial strips from mammalian hearts

COVER TEASER *Ca^{2+} imaging of the myocardium*

ABSTRACT

Ca^{2+} handling within cardiac myocytes underpins coordinated contractile function within the beating heart. This protocol enables high spatial and temporal Ca^{2+} imaging of *ex vivo* multicellular myocardial strips. The endocardial surface is retained, and strips of 150- to 300-μm thickness are dissected, loaded with Ca^{2+} indicators and mounted within 1.5 h. A list of the equipment and reagents used and the key methodological aspects allowing the use of this technique on strips from any chamber of the mammalian heart are described. We have successfully used this protocol on human, pig and rat biopsies. On use of this protocol with intact endocardial endothelium, we demonstrated that the myocytes develop asynchronous spontaneous Ca^{2+} events, which can be ablated by electrically-evoked Ca^{2+} transients, and subsequently redevelop spontaneously following cessation of stimulation. This protocol thus offers a rapid and reliable method for studying the Ca^{2+} signaling underpinning cardiomyocyte contraction, both in healthy and diseased tissue.

INTRODUCTION

Studies using isolated cardiac myocytes have revealed fundamental mechanisms underlying cardiac excitation-contraction coupling in health and disease.[1,2] However, whilst studies in single cells enable simultaneous measurements of membrane potential and intracellular Ca^{2+} concentration ($[Ca^{2+}]_{in}$), there are also disadvantages to isolating single cardiomyocytes from their surrounding extracellular matrix and cellular neighbourhood. For example, cell-to-cell interactions and synchronicity cannot be studied, and the precise anatomical source of the cells is not known. Additionally, cellular damage inevitably occurs during the digestion and preparation of isolated cells.[3,4] These drawbacks can be overcome by using multicellular preparations. Thus, techniques that enable investigation of spontaneous subcellular Ca^{2+} events in individual myocytes in a multicellular preparation, which are adaptable to humans, are most desirable.[5]

In this protocol, we describe a method to study Ca^{2+} signaling in a multicellular cardiac preparation that complements the myocardial tissue slice technique previously published in *Nature Protocols.*[6] By adapting the approach used in smooth muscle bundles to myocardial strips, *ex vivo* intracellular and intercellular communication can be investigated. The protocol achieves equivalent spatial resolution to that possible in single, dispersed cells, whilst retaining tissue architecture and hence including the effects of cell-cell contacts. This protocol can readily be applied to small biopsy sample sizes, an advantage for rodent and human ventricular biopsies,[7] and can be utilized in most cardiac physiology labs. It can be used to test drugs prior to embarking on clinical trials. When applied to biopsies, it can assess Ca^{2+} signaling events in individual patients, potentially enhancing the mechanistic understanding in patients with pre-operative atrial arrhythmias or predicting the occurrence of post-operative or future arrhythmia. We have previously used this protocol to study Ca^{2+} signaling in vascular and visceral smooth muscle, and here also describe how to adapt it to study Ca^{2+} signaling in cardiac muscle.

Development and applications

Abnormalities in Ca^{2+} signaling lead to a wide spectrum of pathophysiological conditions, and as such the significance of augmented spontaneous intracellular Ca^{2+} release[8-10] has been probed using isolated cells,[11,12] multicellular ventricular papillary trabeculae,[13-16] perfused tissue wedges,[17] and Langendorff-perfused hearts.[18-21] A growing number of recent publications highlight the promise of myocardial slices to address the deficits of current models.[22,23] Myocardial slices have been demonstrated to be a suitable approach for electrophysiological and pharmacological drug testing.[24-28] Organotypic slices preserve cellular electrical-mechanical coupling and connections with the extracellular matrix,[6,29-31] thereby sustaining differentiation of the adult cardiomyocyte

phenotype. However, studies using ventricular myocardial slices require relatively large biopsies for tissue slicing,[6] limiting the use of human tissue, and also preventing studies of the endocardial endothelial cells (EECs). We therefore decided to use intact ventricular papillary trabeculae from young rats instead.[14-16,32] This provides a viable myocardial strip preparation with an uncut endocardial surface for direct *ex vivo* imaging.

We previously used aluminium muscle clips to study intracellular Ca^{2+} signaling in microvascular networks,[33-35] visceral uterine smooth muscle,[36] and intact arteries.[34,37,38] In those studies, a custom built chamber was used to bathe the strips, which allowed the superfusion of temperature-controlled solution across the preparation (**Fig. 1**). Cardiac myocytes are highly sensitive to changes in extracellular Ca^{2+} concentration ($[Ca^{2+}]_{out}$), thus we assessed cellular damage during stepwise changes in temperature while reducing $[Ca^{2+}]_{out}$. The protocol we provide here provides reliable and consistent viability of the cardiac myocytes by maintaining the strips at either 25 ºC or 30 ºC, using 0.1 mM (low)-Ca^{2+} solution.

The myocardial strips can be used to study a wide range of physiological and pathological conditions. For example, the auto-oscillation[39] (calcium spontaneous oscillatory contractions [Ca-SPOC], or fasciculation) of individual myocytes and underlying Ca^{2+} events can be investigated in an intact tissue preparation. Furthermore, the intact endocardium allows simultaneous imaging of both EECs and cardiac myocytes, and signaling between the adjacent layers, opening a new avenue of investigation similar to those reported in the literature on vascular EC- to myocyte-interactions. Other applications include in depth analysis of cell-cell Ca^{2+} signaling, and mechanisms of cardiac dysrhythmia.

The developed protocol demonstrates that high spatial and temporal resolution imaging of pectinate muscle strips represents a suitable model to explore the Ca^{2+} events underpinning atrial cardiomyocyte excitation-contraction coupling, incorporating and expanding the advantages of previous techniques. Furthermore, the ability to align subcellular Ca^{2+} events of high spatial resolution with protein expression is demonstrated, adapted from our longstanding *ex vivo* microvascular studies.[40,41] Although our initial experiments focused on smooth muscle cells,[33-38] we show data here using atrial myocytes, and how the protocol can be further adapted to ventricular myocytes.

Experimental design

The procedure starts with the rapid and simple dissection of 150- to 300-µm thick myocardial strips with intact EECs from any chamber of the mammalian heart. The dissected strips are held between two clips mounted in a chamber (**Fig. 1**) and indicator dyes loaded into the cells under controlled conditions. Whilst the protocol retains the advantages of similar protocols using myocardial slices,[6] one surface of the strip remains uncut, retaining the cellular integrity and the arrangement of the myocytes adjacent to the EECs. In addition to the PROCEDURE reported below, **Supplementary Videos 1–6** demonstrate the key parts of the procedure, showing important steps in preparing and mounting the myocardial strips for study. We anticipate that all the techniques described can be learnt in a few days by a skilled researcher.

Cardiac tissue: We have successfully used myocardial strips from right atrial appendage biopsies from patients undergoing voluntary valve surgery, pigs undergoing experimental surgery and rats. As an example, we include results from the use of right atrial appendage strips from male and female patients undergoing aortic or mitral valve surgery. We also include results from atrial appendage strips collected from healthy female pigs undergoing equivalent surgery. The tissue itself should be used as soon as feasible upon arrival to the laboratory and, unless additional measures to maintain viability are made, on the same day. The duration of live cell Ca^{2+} imaging

experiments depends on retention of dyes within cells which varies between indicators. By using Cal-520® AM or Calbryte™-520 AM, consistent spontaneous Ca^{2+} events and reproducible responses to electrical field stimulation can be achieved for up to 2 h.

Alternative sources of tissue that we have used include:

- Human right atrial appendage (pectinate myocardial strips; atrial myocytes)
- Porcine right atrial appendage (pectinate myocardial strips; atrial myocytes)
- Rat right atrial appendage (pectinate myocardial strips; atrial myocytes)
- Rat left ventricle (papillary myocardial strips; ventricular myocytes)
- Rat ureter (arterioles, capillaries, venules; pericytes, endothelial cells, smooth muscle cells)
- Rat vas deferens (arterioles; endothelial cells, smooth muscle cells)
- Rat mesenteric artery (arteries, arterioles; endothelial cells, smooth muscle cells)

We anticipate the following tissues could be used:

- Human and porcine left ventricle (papillary myocardial strips; ventricular myocytes)
- Human and other animal model atrial and ventricular endocardial endothelial cells
- Human and other animal model heart valve preparations (structure, endocardial endothelial cells)
- Microvascular beds from the heart and other organs
- Skeletal muscle preparations
- Mouse and other animal models of cardiac and other tissues

When comparing results between experiments, it is important to consider confounding variables. For example, here we compare atrial biopsies collected under general anesthesia, packaged and couriered to the laboratory under identical conditions and over equivalent periods of up to 3 hours. The time frame of the protocol enables researchers interested in studying atrial myocytes from patients with identified and unidentified cardiac dysrhythmias to remove biopsies on the day of surgery and use them to potentially inform their clinical management. We anticipate that myocardial infarction and other heart failure and dysrhythmia models could be studied by using myocardial strips obtained from other parts of the heart. Thus, ventricular (and rat atrium and ventricle) biopsies collected at the termination of an experiment could enable responses in myocardial strips between the chambers of the same heart to be compared. The protocol can be readily adapted to the study of human and porcine ventricular myocardial strips, both fresh and following culture.[42,43] Rat hearts are a more readily available model to develop and establish the protocols, thus it is recommended these be used before moving to use material from larger mammals. Rodent models can provide valuable data including the effects of sex, chronic drug treatments, myocardial infarction and hypertension on the profile of cardiac myocyte Ca^{2+} events.

Details for isolating the porcine atrial myocardial strips are shown in **Supplementary Videos 1 and 2**. The use of mice myocardial strips and strips from other animal models could be accommodated with the use of appropriately sized clips to hold them into position.

Fluorescence imaging: The position of cells can be imaged using cell permeant cytosolic dyes such as high affinity Ca^{2+} indicator dyes (e.g. Cal-520® AM or Calbryte™-520 AM) (**Fig. 2**) and calcein AM. Experiments can be extended from cytosolic Ca^{2+} signaling to other indicator dyes, structural markers and protein expression studies. Membrane dyes such as wheat germ agglutinin and voltage-sensitive dyes (e.g. FluoVolt™) can be used to establish cell shape and points of contact between cells, and can be added in combination with nuclear (e.g. Hoechst 33342) (**Fig. 2**) and other fluorescent organelle markers. The Ca^{2+} indicator dyes also allow the rapid spatio-

temporal imaging of Ca^{2+} events across species and heart chambers (**Fig. 3 to Fig. 9**). The viability of cells is assessed by the ability of live cells to de-esterify dyes, and can be combined with cell impermeant nuclear dyes (**Fig. 7**). Tissue can be fixed following live cell imaging to establish protein expression, and align with structural markers (**Fig. 7 to Fig. 9**).

Imaging chamber: We provide 3 sources for the imaging chamber (**Fig. 1a**). Imaging chambers can be custom-made[33-38], or purchased from Warners Instruments or Danish Myo Technology (DMT). We anticipate that it would be easy to adapt the protocol to use different experimental imaging chambers to hold and stretch the strips. All three chambers shown here can be used to provide equivalent data, although the chamber from DMT has the advantage of a force transducer for tension measurement. However, although the DMT Confocal Cardiac Myograph includes a force transducer and heater, at present this technique has not been optimized for measurements of contractile force. Tissue oxygenation, a bicarbonate based buffer, and stepped increases to physiological $[Ca^{2+}]_{out}$ (~1.8 mM Ca^{2+}) in the absence of an inhibitor of excitation-contraction coupling are the key additions that still need to be added to the protocol to bring conditions in the imaging chamber closer to those seen in vivo.

Microscopy system: Implementation of this technique requires the use of high temporal and spatial resolution fluorescence microscopy. We have used an inverted microscope and a 40x, long working distance objective to image ~10 myocytes directly adjacent to the endocardial endothelium at a frame rate of ~30 Hz. The detection system settings must be optimized to avoid excitation light-induced damage and photobleaching. Live cells loaded with Ca^{2+}-indicator dye can be used to optimize settings. Time courses of repeated responses should be obtained to assess reproducibility within given cells.

We anticipate the procedure could be adapted to use multi-wavelength rapid image acquisition systems to simultaneously capture multiple live cell fluorescent indicator signals.

MATERIALS

BIOLOGICAL MATERIAL

- Sources of fresh mammalian cardiac tissue: The results shown here are from myocardial strips from right atrial appendage biopsies from patients undergoing voluntary valve surgery, pigs undergoing experimental surgery and sacrificed rats; the biopsies were collected as part of an ongoing study. Alternative tissue can be used as discussed in the Introduction.

CAUTION Procedures involving animals and human tissues must conform to relevant institutional and national regulations. Informed consent must be obtained from each patient for human tissue.

REAGENTS

- Sodium chloride (Sigma-Aldrich, cat. no. S7653)
- Potassium chloride (Sigma-Aldrich, cat. no. P9541)
- Magnesium chloride, 1 M solution (Fluka™, cat. no. 63020)
- Sodium pyruvate (Sigma-Aldrich, cat. No. P2256)
- d-Glucose, anhydrous (Millipore, cat. no. 1.08337)
- Calcium chloride, 1 M solution (Sigma-Aldrich, cat. no. 21115)
- HEPES (Sigma-Aldrich, cat. no. H3375)
- Sodium hydroxide (Sigma-Aldrich, cat. no. S5881) **! CAUTION** Corrosive, causes skin burns and eye damage; wear protective gloves and glasses

- 2,3-Butanedione monoxime (Alfa Aesar, cat. no. 14339)
- Ethanol (Fisher, cat. no. E/0665DF/17)
- Phosphate-buffered saline tablets (Sigma-Aldrich, cat. no. P4417)
- Paraformaldehyde solution, 4% (Electron Microscopy Services, 157-4) **! CAUTION** Harmful if swallowed or inhaled, skin, eye and respiratory irritant, may cause genetic defects and cancer; store and use in a fume hood, wear protective gloves and glasses
- 30 mL tubes with lids, Sterilin™ (Thermo Scientific™, cat. no. 128A/FS)
- Triton X-100 (Sigma-Aldrich, cat. no. T8532)
- Tween-20 (Sigma-Aldrich, cat. no. P2287)
- Bovine serum albumin (Sigma-Aldrich, cat. no. A3059)
- Dimethyl sulfoxide (Sigma-Aldrich, cat. no. 276855)
- Cal-520® AM, 10 x 50 µg pack (AAT Bioquest, cat. no. 21130)
- Calbryte™-520 AM, 10 x 50 µg pack (AAT Bioquest, cat. no. 20651)
- Calcein AM 20 x 50 µg pack (Invitrogen, cat. no. C3100MP)
- Pluronic® F-127, powder (Invitrogen, cat. No. P6867)
- FluoVolt™ membrane potential kit (Invitrogen, cat. no. F10488)
- Wheat germ agglutinin Alexa Fluor 555 conjugate (Invitrogen, cat. no. W32464)
- Hoechst 33342 (Invitrogen, cat. no. H3570)
- Propidium iodide (Sigma-Aldrich, cat. no. 81845) **! CAUTION** May cause genetic defects; wear protective gloves and glasses
- Anti-Sarcomeric Alpha Actinin antibody [EA-53] (Abcam, cat. no. ab9465, RRID: AB_307264; https://scicrunch.org/resolver/RRID:AB_307264)
- Anti-Von Willebrand Factor antibody (FITC) (Abcam, cat. no. ab8822, RRID: AB_306799; https://scicrunch.org/resolver/RRID:AB_306799)
- Anti-TRPC6 antibody (Alomone labs, cat. no. AGP-002, RRID: AB_11218954; https://scicrunch.org/resolver/RRID:AB_11218954)
- Anti-TRPV4 antibody (Sigma-Aldrich, cat. no. T9075, RRID: AB_532289; https://scicrunch.org/resolver/RRID:AB_532289)
- Goat Anti-Rabbit IgG H&L (Alexa Fluor® 488) (Invitrogen, cat. no. A-11034, RRID: AB_2576217; https://scicrunch.org/resolver/RRID:AB_2576217)
- Goat Anti-Guinea Pig IgG H&L (Alexa Fluor® 555) (Invitrogen, cat. no. A-21435, RRID: AB_2535856; https://scicrunch.org/resolver/RRID:AB_2535856)
- Goat Anti-Mouse IgG H&L (Alexa Fluor® 647) (Abcam, cat. no. ab150115, RRID: AB_2687948; https://scicrunch.org/resolver/RRID:AB_2687948)
- Vectashield® Hardset™ antifade mounting medium (Vector Laboratories, cat. no. H-1400)
- CoverGrip™ coverslip sealant (Biotium, cat. no. 23005)

EQUIPMENT

- Volumetric flask (1 L; Fisherbrand, cat. no. FB50159)
- Measuring cylinder (50 mL; Fisherbrand, cat. no. FB55207)
- pH meter (Mettler Toledo, FiveEasy F20) and electrode (Mettler Toledo, cat. no. LE438)
- Ice bucket, rectangular (2.5 L; Globe Scientific, cat. no. 445010)
- 50 mL falcon tubes (Corning, cat. no. 430921)
- Glass beaker (250 mL; Fisherbrand, cat. no. FB33112)
- Laboratory bottle (500 mL; Fisherbrand, cat. no. FB33146)
- Dissecting dish with silicone base (93- × 22-mm, Living Systems, cat. no. DD-90-S)
- Scissors, straight,ToughCut (Fine Science Tools, cat. no. 14058-11)
- Forceps, straight, student Dumont #5 (Fine Science Tools, cat. no. 91150-20)
- Forceps, straight, mirror finish Dumont #5 (Fine Science Tools, cat. no. 11252-23)

- Forceps, curved, Dumont #7 (Fine Science Tools, cat. no. 11271-30)
- Microscissors, straight, Vannas (Fine Science Tools, cat. no. 15000-00)
- Microscissors, curved, Vannas (World Precision Instruments, cat. no. 14122)
- Aluminium muscle clips (Large muscle clip from 1MIL aluminium foil, Laser Services, USA)
- Super Glue Power Flex Mini Trio (Loctite, cat. no. 5894784)
- Plastic platforms, weight boat cut to 20 mm x 15 mm shapes (Sigma Aldrich, cat. no. W2876)
- Coverslip, 64 mm x 22 mm (Scientific Laboratory Supplies, cat. no. MIC3208)
- Silicone grease, Dow Corning®, high-vacuum, (Sigma Aldrich, cat. no. Z273554)
- Imaging chamber: we have successfully used 3 options: #1, custom-built, 2 mL volume; #2, 2 mL volume (Warner Instruments, cat. no. RC-27); and #3, force transducer, electrical field stimulation, 5 mL volume (Danish Myo Technology, Confocal Cardiac Myograph)
- Micro clamps, S&T B1 (Fine Science Tools, cat. no. 00396-01)
- Temperature meter and probe (Physitemp, meter cat. no. BAT-7001H, microprobe cat. no. IT-1E)
- AccuBlock™, mini-compact dry bath (Fisher Scientific, cat.no. D0100)
- Rotator (20 rpm, Stuart, SB2)
- Vortexer (Grant, cat. no. PV-1)
- Eppendorf tubes® 3810X, 1.5 mL (Eppendorf, cat. no. 0030125177)
- 40x objective, for Nipkow disk confocal microscopy, 0.80 NA, WD 3.3 mm (Olympus, cat. no. LUMPLFLN 40XW)
- 40x objective, for linescan confocal microscopy, 1.15 NA, WD 0.25 mm (Olympus, cat. no. UAPON40XW340)
- 10x objective, for strip thickness, 0.40 NA, WD 3.1 mm (Olympus cat. no. UPLSAPO10X/0.4)
- Water purification system (Milli-Q® Integral 3 Water Purification System, cat. no. ZRXQ003WW; with Q-POD®, cat. no. ZMQSP0D01; and Biopak® Polisher, cat. no. CDUFBI001)

REAGENT SETUP

Human tissue

Before commencing studies with human tissue, protocol and ethical approvals must be obtained. The collection and use of tissue in this study was approved by the Oxford Research Ethics Committee and extended to the Bristol site (CS/2012/4200; REC Reference No: 10/H0606/36). Anonymized surplus right atrial appendage tissue (approx.1 cm x 1 cm) was obtained with informed consent from patients undergoing elective cardiac surgery for aortic and/or mitral valve replacement at the Bristol Heart Institute, University Hospital Bristol-Weston Foundation Trust, Bristol, UK. Atrial biopsy samples were packaged at clinical standards and promptly couriered to Oxford under a material transfer agreement. This research complies with the Helsinki Declaration and the Data Protection Act. This type of arrangement should be implemented for all future studies using this protocol. When biopsies arrive, store them at 8 ºC. At the completion of experiments, in accordance with our Human Tissue Authority licence, tissue was immediately disinfected and frozen, all within 24 h of arrival, and disposed of as clinical waste. This approach should be used for each human biopsy, and adapted to the requirements of each institution.

Pig: All procedures used to obtain the results shown here were approved by the University of Bristol Research Ethics committee and performed in accordance with the Guide for the Care and Use of Laboratory Animals[44] and the United Kingdom Animal (Scientific Procedures) Act, 1986, under Home Office project licences PPL 30/2854, PPL 30/3064, and PPL 7008975. Procedural aspects were as previously reported.[45] Briefly, juvenile, 5-6 month old female Landrace White pigs

(weight range 45–75 kg) were subjected to general anesthesia (pre-medication with ketamine, i.m. 15–20 mg/kg, induction with propofol i.v. 16–20 mg/kg and maintained with isoflurane). Animals were subject to median sternotomy and the right atrial appendage sample was removed before establishing cardiopulmonary bypass. Samples were packaged at clinical standards and promptly couriered to Oxford. Store tissue at 8 ºC until use and discard unused tissue using institutional guidelines.

Rat: The results shown here were obtained from rats housed locally in Oxford, in a temperature-controlled environment (20-22 °C) with a 24-hour light-dark cycle and water *ad libitum*. Animal use was approved by the University of Oxford ethical committee and complied with the Animals (Scientific Procedures) Act 1986 and European Directive 201/63/EU. These studies comply with ARRIVE guidelines.[46] Male Wistar rats (Charles River, weight: 220-270 g) were euthanised using CO_2, a Schedule 1 procedure, with death confirmed by cervical dislocation, and the heart rapidly excised. Store tissue at 8 ºC until use and discard unused tissue using institutional guidelines.

Preparation of 1 liter of working solution (low-Ca^{2+} HEPES solution)

Collect, transport, dissect, load and image tissue in a HEPES solution modified to contain 0.1 mM (low)-Ca^{2+} to avoid cellular damage. Add the components listed in the table below to 800 mL of ultra-pure H_2O:

Solid reagents		
	Mass (g)	Final concentration (mM)
Sodium chloride	7.60	130.00
Potassium chloride	0.37	5.00
Glucose	1.80	10.00
Sodium pyruvate	0.22	2.00
HEPES	2.38	10.00
Solutions		
	Volume (mL)	Final concentration (mM)
1 M Calcium chloride	0.10	0.10
1 M Magnesium chloride	1.20	1.20

Keep stirring until all chemicals dissolve and then measure the pH. Adjust the pH to 7.40 using 5 M NaOH, at RT, with continued mixing. Pour into a 1 liter volumetric flask and fill to 1 liter. 40 mL of the solution can be frozen in 50 mL falcon tubes for at least 3 months and thawed overnight in the fridge ready for the collection of human and porcine biopsies during surgical procedures. Alternatively, working solution should be freshly prepared on the day of the experiment and kept chilled to 4 ºC in the fridge until ready for use. Unused buffer should be discarded at the end of the day. ▲**CRITICAL** The biopsy transport solution is the same as this but without the addition of Ca^{2+} or pyruvate (zero-Ca^{2+} HEPES).

Preparation of 500 mL EFS solution (1.8 mM Ca^{2+} HEPES-BDM).

Add the components listed in the table below to 400 mL of ultra-pure H_2O and then make up to 500 mL:

Solid reagents		
	Mass (g)	Final concentration (mM)
Sodium chloride	3.80	130.00
Potassium chloride	0.185	5.00
Glucose	0.90	10.00
Sodium pyruvate	0.11	2.00
HEPES	1.19	10.00
2,3-Butanedione monoxime	1.50	30.00
Solutions		
	Volume (mL)	Final concentration (mM)
1 M Calcium chloride	0.90	1.80
1 M Magnesium chloride	0.60	1.20

CRITICAL Freshly prepare on the day of the experiment and keep chilled to 4 ºC in the fridge until ready for use. Unused buffer should be discarded at the end of the day.

EQUIPMENT SETUP

Imaging chamber
Use Super Glue to attach an aluminium clip to each plastic platform, allow to set. Using a pipette tip spread silicone grease between the plastic platform and coverslip, leaving ~5 mm between clips. Spread silicone grease along the entire edge of the coverslip. Sandwich each platform between the coverslip and chamber, leaving ~5 mm of clear coverslip between the clips (**Fig. 1a, 2**). The coverslip should be sealed to the chamber base. CRITICAL To enhance the ability to study multiple viable strips from a single biopsy we recommend building multiple imaging chambers before the arrival of the biopsy to the laboratory.

Microscope for high resolution imaging
This is required to assess cell structure and following immunohistochemistry. We use a linescan confocal microscope (Olympus FV1200) mounted on an inverted microscope (IX81, Olympus, Japan) to acquire 3 dimensional confocal stacks using a 40x (1.15 NA, 0.25 mm WD, Olympus) water immersion objective, obtaining z-stacks through the endocardial wall in 0.3 to 0.5 µm steps, digital zoom of 1- to 3-fold, and 1024 x 1024-pixel Kalman-averaged images. We use sequential excitation at 405 nm, 488 nm, 543 nm and/or 635 nm fluorescence and acquire images using Fluoview software (Olympus, Tokyo, Japan). At digital zoom 3, each pixel is ~0.1 μm^2.

Microscope for high speed imaging
A microscope capable of acquiring 512 x 512-pixel images at 30 Hz is necessary for Ca^{2+} imaging experiments. Either Nipkow (spinning) disk or resonant scanner confocal microscopes can be used. We image cells using a 40x long working distance (0.8 NA, 3.3 mm WD, Olympus) water immersion objective and sensitive camera (iXon 887 EMCCD camera, Andor Technology, UK)

coupled to a Nipkow spinning disk confocal head (CSU22, Yokogawa, Japan), mounted on an inverted microscope (IX70, Olympus, Japan). Following excitation at 488 nm, we use imaging software (Andor iQ software, version 3.5, Andor Bioimaging Division, UK) to acquire the fluorescence emission intensity (at 513-563 nm) from 4-10 cells within the slice, at a frame rate of between 30- to 60-Hz (crop the 512 x 512-pixel full chip to increase speed). Each pixel is ~0.4 μm^2.

PROCEDURE

Excision of the right atrial appendage (RAA)

1| Follow option A if using human or porcine RAA or option B if using rat RAA.

(A) Excision of human or porcine RAA ●TIMING 1 min

(ii) Using sterile conditions, perform sternotomy. After heparinization to achieve an activated clotting time of >400 s, remove the tip of the RAA following the positioning for a purse-string suture for subsequent establishment of cardiopulmonary bypass.

CAUTION The procedure must be carried out by senior clinicians/vetinarians in a controlled manner in an approved clinical surgical theater, under general anesthesia and full monitoring.

(iii) Immediately place the biopsy in ~40 mL chilled (4-8 ºC) zero-Ca^{2+} HEPES in a 50 mL falcon tube, place the tube in a 4-hole rack positioned between frozen thermal-packs in a clinical cool-bag ready for immediate transport.

▲**CRITICAL** The biopsy must be collected with care with no excessive handling and immediately placed in fully thawed and chilled (4-8 ºC) zero-Ca^{2+} HEPES.
▲**CRITICAL** The cool-bag must retain a temperature of 8-10 ºC for at least the entire duration of transport. Transfer to a fridge immediately on arrival, ideally within 4 h of excision. Avoid direct contact between the 50 mL tube and ice.
? TROUBLESHOOTING

■**PAUSE POINT** The porcine RAA remains viable for further studies if kept in the fridge (4-8 ºC) for up to 6 h. We cannot reliably state how long the human RAA remains stable due to the inherent large variability in patients' biopsies, and therefore recommend using them as soon as possible after arrival to the laboratory.

(B) Excision of rat heart ●TIMING 2 min

▲**CRITICAL** The following procedure minimizes the pain, suffering and distress of rats, while allowing the rapid excision of the heart after sacrifice. This is critical to obtain highly viable myocardial strips.

(i) Sacrifice the rat according to institutional and national regulatory guidelines. Our laboratory uses CO_2 followed by cervical dislocation.
(ii) Place the rodent in the supine position.
(iii) Locate the sternum. Hold the overlying skin with tweezers and make a small incision distally.
(iv) Once the sternum is exposed, hold it with tweezers. Make an incision along the entirety of the costal margin using scissors.
(v) Isolate the heart by cutting the large vascular connections (aorta, vena cava).

(vi) Place the heart into 40 mL 0.1 mM (low)-Ca^{2+} HEPES in a 50 mL falcon tube sitting in ice.

▲**CRITICAL** The heart should be removed within 1 min of sacrifice. The heart will stop beating when immersed in the chilled and buffered solution. Avoid allowing the solution to warm as the heart will start beating and cause irreversible damage.

■**PAUSE POINT** The rat heart remains viable for further studies if kept at 4-8 ºC for up to 1 h.

Preparation of the RAA myocardial strip ●**TIMING 5–10 min**

2| Follow option A if using human or porcine RAA or option B if using rat RAA. All strips should be ~5 mm lengths of muscle, keeping the strip width ~1.5 mm and thickness 150- to 300-µm to enable both diffusion of substrates and metabolites and to retain cellular integrity following stretching between clips.

▲**CRITICAL** Avoid overstretching the tissue biopsy during pinning and dissection. Avoid excessive manipulation of the biopsy. Curved Vannas micro-scissors enable a clean cut to isolate a strip from any shaped pectinate muscle bundle. Aim to keep the endocardial endothelial layer intact as the myocytes immediately below this layer will be imaged.
? TROUBLESHOOTING

(A) Preparation of human or porcine RAA strip

(i) On receipt of the RAA biopsy, remove the sample from the 50 mL tube and place into fridge-chilled low-Ca^{2+} HEPES solution to cover the RAA. Store in the fridge until ready for use.
(ii) Maintain the tissue at 4-8 ºC during dissection by placing a cold ice pack under the Petri dish.
(iii) Use insect pins to secure the corners of the RAA in the dissecting dish.
(iv) Make incisions along the borders of the RAA from the opening towards the apex using micro-scissors, avoiding the pectinate muscle bundles as possible.
(v) Open, flatten and secure the RAA using pins, ensuring the pectinate muscle bundles are clearly visible and accessible for dissection (**Fig. 2**).

▲**CRITICAL** See **Supplementary Video 1** to visualize Step 2A (ii-v).

(vi) Using curved Vannas micro-scissors, make a small incision at one end of a pectinate muscle and using the curvature of the scissors, carefully cut into the muscle to a maximum depth of ~300 µm and follow the column until sufficient length is acquired. Place each freshly dissected myocardial strip in cooled low-Ca^{2+} HEPES and store in the fridge until use.

▲**CRITICAL** See **Supplementary Video 2** to visualize Step 2A (vi).

(B) Preparation of rat RAA strip

(i) Fill the silicone-based Petri dish with fridge-chilled low-Ca^{2+} HEPES solution to cover the heart and store it in the fridge until ready for use.
(ii) Maintain the tissue at 4-8 ºC during dissection by placing a cold ice pack under the Petri dish.

(iii) Manipulate the heart using small tweezers to identify the RAA. While the RAA is facing towards you, use an insect pin to secure the apex of the heart into the silicone base of the Petri dish.

▲**CRITICAL** Make sure the RAA is not confused with the left atrial appendage. The latter has a thicker endocardial layer.

(iv) Isolate the RAA using small tweezers to hold the edge of the RAA and with straight scissors, make an incision inferior to the point where the RAA connects to the ventricles.

▲**CRITICAL** See **Supplementary Video 3** to visualize Step 2B (iv).

(v) Pin out the RAA to expose the pectinate muscle bundles.

▲**CRITICAL** See **Supplementary Video 4** to visualize Step 2B (v).

(vi) Use curved Vannas micro-scissors to cut lengths of muscle and place in cooled low-Ca^{2+} HEPES and store in the fridge until use. Up to 5 pectinate muscle strips can be dissected from each RAA.

▲**CRITICAL** See **Supplementary Video 5** to visualize Step 2B (vi).

■PAUSE POINT The exposed RAA remains viable for 5-6 h in the fridge, but we recommend using the pectinate muscle strips as soon as dissected.

Preparation for live cell imaging ●TIMING 1.5 h

3| The same procedure is used for all sources of RAA. Follow option A if using wheat germ agglutinin (WGA, excitation 543 nm laser) or option B for FluoVolt™ (excitation 488 nm laser) to label cell membranes. In both cases Hoechst 33342 (excitation 405 nm laser) is included to label nuclei. Follow option C if using Calbryte™-520 AM (excitation 488 nm laser) as a Ca^{2+} indicator and option D for loading Calbryte™-520 AM. Calbryte™-520 AM can alternatively be loaded after the strip has been mounted into the imaging chamber. See Box 1 for details of this alternative procedure that can be carried out in place of step 3D. See **Table 1** for details of membrane and nuclear stains we have successfully used.

(A) Use of WGA loading solution

(i) Add 15 µL of 10 mg/mL WGA Alexa Fluor 555 conjugate ([final] = 0.15 mg/mL) to 1 mL low-Ca^{2+} HEPES. Mix well, then add 1 µL of 10 µg/mL Hoechst 33342 ([final] = 16 µM).
(ii) Place 1 mL of loading solution prepared in step (i) in an Eppendorf tube, add 1- to 5- myocardial strips and cover to avoid light.
(iii) Rotate at 20 rpm for 20 min at room temperature (RT, 25 ºC).
(iv) Remove the loading solution and replace with RT low-Ca^{2+} HEPES solution.

▲**CRITICAL** Whilst acquiring z-stacks, use sequential laser excitation to avoid cross-over of excitation signals. Kalman averaging can be used to improve signal to noise, as necessary.

■PAUSE POINT Any residual 10 mg/mL WGA can be aliquoted, kept frozen and used for at least one year.

(B) Use of FluoVolt™ loading solution

(i) Using a 1.5 mL Eppendorf tube, add 3 µL of 1000x in DMSO (Component A) FluoVolt™ solution to 3 µL of 250 mg/mL Pluronic® in DMSO. Triturate until foaming.

▲**CRITICAL** The pluronic solution needs to be made fresh weekly and can be stored under anhydrous conditions. Heat the pluronic solution to 60 ºC and vortex well.

(ii) Add 1 mL 40 ºC low-Ca^{2+} HEPES and vortex well, making a 3x FluoVolt™ loading solution. Add 1 µL of 10 mg/mL Hoechst 33342 ([final] = 16 µM).

■**PAUSE POINT** The remaining FluoVolt™ 1000x in DMSO can be refrozen and used for at least one month. There is 5.25 µL DMSO/mL in the loading solution.

(iii) Place 1 mL of 3x FluoVolt™ loading solution in a 1.5 mL Eppendorf tube, add 1- to 5- myocardial strips and cover to avoid light.
(iv) Rotate at 20 rpm for 45 min at RT.
(v) Remove the loading solution and replace with RT low-Ca^{2+} HEPES solution.

(C) Use of Calbryte™-520 AM loading solution

▲**CRITICAL** Calcein AM and Cal-520® AM loading solutions can be prepared using a similar procedure.

(i) Add 22.9 µL DMSO to 50 µg vial of Calbryte™-520 AM ([final] = 2 mM). Triturate and vortex well.

(ii) Using a 1.5 mL Eppendorf tube, add 2.5 µL of 2 mM Calbryte™-520 AM to 2.5 µL of 200 mg/mL Pluronic® in DMSO. Triturate until foaming.

▲**CRITICAL STEP** The pluronic solution needs to be made fresh weekly and can be stored under anhydrous conditions. Heat the pluronic solution to 60 ºC and vortex well.

(iii) Add 1 mL 40 ºC low-Ca^{2+} HEPES and vortex well, making a 5 µM Calbryte™-520 AM loading solution. Allow solution to cool to RT.

■**PAUSE POINT** The residual 2 mM Calbryte™-520 AM in DMSO can be refrozen and used for at least one month. There is 4.5 µL DMSO/mL in the loading solution.

(D) Loading strips with Calbryte™-520 AM before mounting in the imaging chamber

▲**CRITICAL** Cal-520® AM can be loaded using a similar procedure.

(i) Add 1 mL of 5 µM CalBryte™-520 AM loading buffer into a 1.5 mL Eppendorf tube from Option C. Add 1- to 5- strips to the tube, cover to avoid light.
(ii) Rotate at 20 rpm for 1 h, at RT.
(iii) Remove the loading buffer and replace with RT low-Ca^{2+} HEPES solution. Leave to de-esterify for 20 mins.

Box 1: Loading strips with Calbryte™-520 AM after mounting in the imaging chamber

An ability to image strips that are already mounted during the loading period has the advantage that the loading time can be adjusted to when cells are clearly visible and are still fasciculating. In our experience the loading time could be reduced using this method. Furthermore, the endocardial endothelial cells are more likely to be damaged when the strip is placed in the rotator. The spontaneous Ca^{2+} event frequency using this method matched that of preloaded strips, prepared as described in step 3D of the main procedure. **Following acquisition of** spontaneous Ca^{2+} events (steps 1-5 below) the same strip can be used to establish the effect of electrical field stimulation (EFS) (steps 6-10 below).

▲**CRITICAL** Imaging chamber #1, #2, or #3 listed under EQUIPMENT can be used for electrical field stimulation. External electrodes can be held with micromanipulators for all three options. Imaging chambers #2 and #3 can be purchased with field stimulation options.

1| Fill the imaging chamber with chilled low-Ca^{2+} HEPES and mount a strip, as described in steps 4-7 of the main procedure, and place the chamber on the stage of an inverted microscope.

2| Remove the HEPES solution from the chamber and replace with Calbryte™-520 AM loading buffer, made as described in step 3C of the main procedure (use 1 mL or a volume that covers the RAA strip).

3| Incubate the RAA strip for 12-15 min at RT.

▲**CRITICAL** Monitor fluorescence intensity to avoid overloading. Adjust the timing until clear spontaneous Ca^{2+} events can be observed within myocytes.

4| Remove the loading buffer from the chamber and replace with RT low-Ca^{2+} HEPES solution. Leave to de-esterify for 20 mins.

5| Acquire spontaneous Ca^{2+} events at 30- to 60-Hz using Nipkow spinning disk confocal microscopy.

6| Place the chamber in the fridge and replace the low-Ca^{2+} HEPES with fresh cold low-Ca^{2+} HEPES solution.

7| After 10 mins, replace the low-Ca^{2+} HEPES with 1.0 mM-Ca^{2+} HEPES solution.

8| After 10 mins, replace the 1.0 mM-Ca^{2+} HEPES with 1.8 mM-Ca^{2+} HEPES solution containing 30 mM BDM (HEPES-BDM).

▲**CRITICAL** Raising Ca^{2+} concentration must be performed stepwise and in the fridge to avoid substantial cell damage at RT.
? TROUBLESHOOTING

9| After 10 min, remove the chamber from the fridge and warm to RT.

10| Record spontaneous Ca^{2+} events and EFS-evoked Ca^{2+} transients (CaTs) in 1.8 mM Ca^{2+} at RT using Nipkow spinning disk confocal microscopy at an image acquisition frequency of ~35 Hz. We have used 7-10 V pulses delivered for 5 ms at a frequency of 1 Hz. We also monitor the current between the electrodes each day to ensure effective EFS. Electronic signals for the commencement of each image frame and the stimulator pulse are delivered and acquired to allow offline alignment of stimuli and CaTs.

▲**CRITICAL** Ensure the strip is positioned between the electrode tips. Image the strip using a 4x objective and ensure the tips are aligned across the strip and that current flows through the strip.
▲**CRITICAL** To avoid movement artefacts and increased metabolic requirements during intense myocyte contraction, add 30 mM BDM to a HEPES buffered solution containing

1.8 mM Ca^{2+}, as described in a similar protocol.[6] See **Reagent setup** for details of the components required.
? TROUBLESHOOTING

END BOX

Mounting the RAA into a chamber ●TIMING 5 min

4| Fill the 2 mL imaging chamber with chilled low-Ca^{2+} HEPES solution. Transfer an isolated strip to the chamber by holding one end using fine tweezers.
? TROUBLESHOOTING

5| Ensure the RAA strip has the endocardial side facing towards the coverslip (using an inverted microscope).
? TROUBLESHOOTING

6| Using 2 sets of fine tweezers, place and secure the RAA strip between the clips. Suspend at least 200 µm from the bottom of the imaging chamber using muscle clips. This arrangement allows uniform sample superfusion, as previously described for microvasculature studies in rat ureter and vas deferens.[33,34]

7| Stretch the RAA strip until straight and tawt by gently manipulating the plastic platforms attached to the clips (**Fig. 1b, 2**).
? TROUBLESHOOTING
▲CRITICAL See **Supplementary Video 6** to visualize step 4-7.

8| Place the chamber on the stage of an inverted microscope (**Fig. 1b**).

▲CRITICAL Image the strip using transmitted light. If healthy, the majority of myocytes will fasciculate. If no fasciculation is observed, it is likely the strip is damaged and is not suitable for further study. When deciding which part of the myocardial strip to focus on, one should be aware that the density and orientation of cardiac myocytes varies considerably not only between biopsy sources but also within a given strip. The sarcomeres can be seen with transmitted light through the microscopy eyepieces; use these as a reference point. The data we show here is from regions of relatively uniform and aligned myocardial fibers immediately below the EECs.
? TROUBLESHOOTING

Imaging spontaneous Ca^{2+} events ●TIMING up to 2 h

9| Record spontaneous Ca^{2+} events using Nipkow spinning disk confocal microscopy at an image acquisition frequency of 30-60 Hz. CRITICAL STEP The three imaging chambers described all require the use of a 40x objective with working distance at least 300 µm, see EQUIPMENT.

Preparation for cellular viability tests ●TIMING 30 min
▲CRITICAL Cellular viability is measured in mounted strips following Ca^{2+} imaging, or if Ca^{2+} indicator dye is not used, calcein AM (1 µM, excitation 488 nm) can be loaded with the nuclear stains. Hoechst 33342 is cell permeant (for live and dead cell nuclei), whereas propidium iodide is not (for dead cell nuclei), and together with the AM dyes which de-esterify in live cells only, can be used to assess cellular viability. See **Table 1** for details of the nuclear stains we have successfully used.

10| Add 10 µg/mL Hoechst 33342 and 10 µM propidium iodide to the imaging chamber and incubate for 20 mins at RT. If required, also add 1 µM calcein-AM.

11| Replace with low-Ca^{2+} HEPES solution.

12| Transfer to a linescan confocal microscope and image at high resolution.

▲**CRITICAL** The Ca^{2+} indicator dye begins to leak out of cardiac myocytes during the incubation with nuclear dyes, so time is of the essence. Check for the ability to simultaneously image three wavelengths (405 nm, 488 nm, and 543 nm lasers).

Fixing and immunolabeling RAA myocardial strips ●**TIMING Fixing, 1 h; labeling, 20 h**
▲**CRITICAL** Immunolabeling is performed in mounted strips using each RAA tissue source. Tissue is fixed immediately following Ca^{2+} imaging and cell viability tests or on freshly mounted tissue. See **Table 1** for details of the antibodies used. The thickness of the cut myocardial strips can also be established at the completion of experiments.

13| Replace the HEPES-buffed solution over a stretched strip with 4% paraformaldehyde at RT.

14| After 1 h, wash with PBS 3x 5 min.

■**PAUSE POINT** After washing, fixed myocardial strips can be removed from the chamber and pinned into a petri dish filled with PBS and stored in the fridge for up to a week.

15| To immunostain RAA strips, follow option A. To measure the thickness of the cut myocardial strips, follow option B.

(A) Immunostaining of RAA strips

(i) Make blocking buffer (15 mL PBS + 75 µL Triton X-100 + 0.15 g BSA + 7.5 µL Tween-20). Permeabilise the strip with blocking buffer for 2 h at RT.
(ii) Incubate the strip with primary antibody in blocking buffer at 4 °C overnight.
(iii) Wash the strip with PBS 3x 5 min.
(iv) Incubate the strip with secondary antibody in blocking buffer containing nuclear stain (10 µg/mL Hoechst 33342) for 2 h at RT.
(v) Wash the strip with PBS 3x 5 min.
(vi) Place the strip onto a coverslip, add ~15 µL anti-fade mounting medium, place a second coverslip to seal the strip in medium, and image using linescan confocal microscopy.

▲**CRITICAL** Using two coverslips allows the tissue to be flipped over (as necessary) to ensure the desired imaging from the endocardial surface.

■**PAUSE POINT** Stained and mounted RAA myocardial strips can be kept for at least 2 weeks. However, be aware that secondary antibody fluorescence decreases with time.

(B) Establishing the thickness of the cut RAA strips

(i) Incubate strips with 1 µL of 10 µg/mL Hoechst 33342 ([final] = 16 µM) for 20 mins.
(ii) Wash once with PBS.
(iii) Remove from the clips, discarding the damaged ends, and carefully cut the strip into two or three strip-sections using a sharp knife.
(iv) With the cross-sectional myocardial surface facing the coverslip, use a 10x objective to image the edges of each strip-section and measure the distance between the

endocardial surface and cut surface towards the epicardium in three positions. The nuclear label clearly defines the edges.

(v) Average the three values for each strip-section. Examples of typical results obtained are in **Table 2**. The porcine atrial appendage myocardial strips cut in **Supplementary Video 2** are included in this analysis.

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 3**.

•TIMING

Total time required: ~4.5 h (excluding immunolabeling)
Reagent setup: 30-60 min
Equipment setup: 10-30 min
Step 1, excision of the heart: 2 min (if excision cannot take place in the laboratory, additional biopsy transport time of up to 4 h will be needed)
Step 2, preparation of the atrial strip: 5-10 min
Step 3, loading cellular and membrane dyes: 1.5 h (60 min for loading + 30 min for de-esterification (see Box 1 for timing if loading RAA strips after mounting in the imaging chamber)
Step 4, mounting the strip into a chamber: 5 min
Step 5, imaging spontaneous Ca^{2+} events: up to 2 h
Step 6, cell viability loading: 30 min
Step 7, fixation: 1 h (followed by incubation with antibodies: 20 h)

ANTICIPATED RESULTS

In this section, we present typical data from biopsies collected from patients without coronary artery disease (a subset had atrial fibrillation), healthy pigs and healthy rats. Each *N* represents data from an individual biopsy. All data are presented as mean ± standard error.

Live cell studies

The stretched myocardial strips offer the ability to assess structure using either live or fixed tissue. Membrane, cytoplasmic, and organelle dyes can be used for live cell imaging (**Fig. 2**). As the myocardial strips retain their endocardial surface, and do not require sectioning, they provide a technically undemanding and rapid method to routinely image live biopsy samples.

Ca^{2+} signaling

The results we show here were obtained using a Nipkow disk confocal setup. The laser intensity was set between 10-15% each day, sufficient to see the loaded cells at 30+ Hz, while minimizing cellular damage and photobleaching. The baseline fluorescence was usually ~1200 units in a 0-4095 unit scale, with background at ~1000 units. Values for fluorescence are background subtracted to generate F/F_0. The extent of photobleaching can be seen as a reduction in the F/F_0 baseline over time. We did not see any evidence for photobleaching (**Fig. 3 to Fig. 9**), and found that spontaneous Ca^{2+} events and EFS-evoked responses were consistent within an imaging field of view for at least 60 min. This enabled the same cells to be imaged before and after incubation with blockers.

Spontaneous Ca^{2+} events in human cardiomyocytes

Figure 3 shows typical data from human cardiomyocytes. It is clear that three types of spontaneous Ca^{2+} events, Ca^{2+} waves, Ca^{2+} sparks and Ca^{2+} spikes, can be seen and counted. The majority of human atrial cardiomyocytes, 79 ± 9%, displayed propagating Ca^{2+} waves; of these cells 33 ± 8% also generated elementary Ca^{2+} sparks and 25 ± 10% of cells generated rapid, simultaneous Ca^{2+} spikes (*N* = 12, circa 2 strips and 10 cells per biopsy; **Fig. 3**, **Supplementary Videos 7–8**). In the experiments, the cells did not tend to move (no fasciculations) during Ca^{2+} events. When movement was observed, it was associated with Ca^{2+} spikes, reflecting spontaneous action potential-driven CaTs in electrically-coupled cells. These Ca^{2+} spikes were not observed using porcine or rat biopsies. By imaging these multicellular preparations, it is possible to demonstrate the extent of coupling. It was also clear that a subset of Ca^{2+} spikes were autonomous, suggesting that specialized uncoupled cells generated their own action potentials. Across all samples, the frequency of Ca^{2+} waves, sparks and spikes at 25 ºC was 21.5 ± 3.9 events.min^{-1}, 5.1 ± 1.7 events.min^{-1} and 25.3 ± 8.6 events.min^{-1}, respectively (*N* = 12; **Fig. 3**). At 30 ºC, the frequency of Ca^{2+} waves increased to 57.0 ± 2.1 events.min^{-1} (*N* = 4), and in these four biopsies no Ca^{2+} sparks or spikes were observed.

Spontaneous Ca^{2+} activity in porcine cardiomyocytes

By showing an ability to resolve Ca^{2+} sparks and Ca^{2+} spikes in other preparations, it is possible to use the same approach to compare biopsy sources. **Figure 4** shows typical data from porcine cardiomyocytes. Spontaneous Ca^{2+} activity was characterised exclusively by global Ca^{2+} waves and localised Ca^{2+} sparks, neither spikes nor synchronized contraction of atrial strips were observed. All cardiomyocytes displayed asynchronous propagating Ca^{2+} waves (*N* = 5, 51 cells analysed), and 37 ± 19% of these cells also generated localized Ca^{2+} sparks. Fasciculation could be observed (**Supplementary Video 9**). The temperature-dependence of the frequency of Ca^{2+} waves and sparks can be established. At 25 ºC, it was 26.5 ± 8.6 events.min^{-1} and 20.2 ± 16.2 events.min^{-1}, respectively (*N* = 5; **Fig. 4**, **Supplementary Video 9**). At 30 ºC, the frequency of Ca^{2+} waves increased to 45.4 ± 6.2 events.min^{-1} (*N* = 5) and Ca^{2+} sparks 12.4 ± 8.0 events.min^{-1} (*N* = 5, in 2 of 5 biopsies).

Spontaneous Ca^{2+} activity in rat cardiomyocytes

Figure 5 shows typical data from rat cardiomyocytes. In this tissue, spontaneous Ca^{2+} activity was characterised almost exclusively by asynchronous Ca^{2+} waves; only 1 ± 1 % of cells generated Ca^{2+} sparks. The frequency of Ca^{2+} waves at 25 ºC was 138.1 ± 21.5 events.min^{-1} (*N* = 5, circa 10 cells per biopsy; **Fig. 5**, **Supplementary Video 10**), and at 30 ºC was 187.0 ± 10.1 events.min^{-1} (*N* = 11, circa 10 cells per biopsy). By using the same experimental conditions, this approach allows a comparison of the frequency of spontaneous Ca^{2+} events between species, which aligns with the resting heart rate and frequency of SPOCs observed; humans and pigs slower than rats.[47]

CaTs in rat cardiomyocytes

It was important to modify the composition of the bathing solution to prevent contraction (and associated hypoxia) in these experiments, and this alone has an effect and reduces the frequency of spontaneous Ca^{2+} events (**Fig. 6**). Nevertheless, clear Ca^{2+} events can still be observed in the un-paced myocardial strips (**Supplementary Video 11**), as shown in myocardial slices, multicellular ventricular papillary trabeculae, and Langendorff-perfused hearts with similar bathing solutions containing either BDM,[6,18] cytochalasin D[20] or cytochalasin D with blebbistatin[21] to mechanically arrest the hearts. At a frequency of 1 Hz, and at 25 ºC, electrical stimulation evoked rapid and reproducible CaTs, and during the train of stimulation (1, 3, or 10 pulses), no spontaneous Ca^{2+} activity was observed between CaTs. On cessation of EFS, and there was a short period of no Ca^{2+} events (delay after 1 pulse: 4.7 ± 0.9 s; after 10 pulses: 6.9 ± 2.0 s; *N* = 6, **Fig. 6**), before spontaneous Ca^{2+} events re-emerged at the same frequency post-EFS as pre-EFS (**Fig. 6**, **Supplementary Video 11**). It is possible to demonstrate the influx and release channels

underlying spontaneous Ca^{2+} events and CaTs by incubating the myocardial strips with selective blockers. The frequency of spontaneous Ca^{2+} events of 20.6 ± 2.8 events.min^{-1} (*N* = 10; in the presence of 1.8 mM Ca^{2+} and BDM), was reduced by the L-type voltage-gated Ca^{2+} channel blocker nifedipine (1 μM) to 14.0 ± 3.7 events.min^{-1} (*N* = 5); and abolished by the ryanodine receptor blocker ryanodine (1 μM; 0.0 ± 0.0 events.min^{-1}, *N* = 5). The peak CaTs were significantly reduced by either nifedipine or ryanodine, and abolished by their combination (**Fig. 6**; *N* = 5-10). Data using this approach can be compared to other approaches. For example, Ca^{2+} waves were reported in quiescent Langendorff-perfused whole rat or mouse hearts, but were either absent,[18] rare[20] or condition-dependent[21] during electrically-evoked sinus rhythm, consistent with our observations. By using this approach in combination with or comparison to whole hearts and isolated cells, components of specific research questions can be addressed.

Viability

On completion of experiments, live cell imaging should be used to assess cellular viability. The same membrane, cytoplasmic, and organelle dyes that are used for live cell imaging can be used. In addition to the Ca^{2+} indicator dye, which only becomes de-esterified in live cells, nuclei can be labeled with cell-permeant Hoechst 33342 (labels all cells) and cell-impermeant propidium iodide (to label dead cells). **Figure 7** shows typical data comparing the two methods we used to load cells. When the myocardial strips were loaded and rotated prior to mounting, it was found that the atrial myocytes were at least 90% viable (*N* = 10), and equally, very few dead myocytes were seen when the cells were loaded following mounting into the chamber (*N* = 6; **Fig. 7**). While developing the protocol, we established that the frequency of Ca^{2+} waves was lower at 25 ºC than 30 ºC, but the cells at both temperatures did not become overloaded when 0.1 mM Ca^{2+} was used in the bathing solution. However, when the extracellular [Ca^{2+}] was raised to 1.0 mM, cells became overloaded, and we could show that the proportion of cells with Ca^{2+} waves decreased (*N* = 4; **Fig. 7**). This trend continued as either temperature or extracellular [Ca^{2+}] was raised further, and we therefore chose 25 ºC and 0.1 mM Ca^{2+} to maintain dye within the cells and retain viability, respectively.

Further applications

Each of the protocols outlined can be used in multiple combinations with different tissue sources. Some examples include the labeling of extracellular structures for studies of fibrosis (collagen, **Fig. 7**; elastin **Fig. 8**), studies of hetero-cellular interactions by simultaneous myocyte and EEC Ca^{2+} imaging (**Fig. 8, Supplementary Video 12**), immunolabeling in confocal z-stacks through the tissue (**Fig. 8**), and the use of ventricular myocytes (**Fig. 9, Supplementary Videos 13 to 15**).

We anticipate this protocol will facilitate the study of the microcirculation within the myocardial strips and changes in response in models of disease.[48] The concept can also be extended to other muscular preparations, including skeletal muscle, and the microcirculation within them.

Data availability

The original data for the experiments shown in **Figs. 2–7**, and **Fig. 9** are available as Source Data with this protocol.

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AUTHOR CONTRIBUTIONS K.A.D. wrote the manuscript, collected and analyzed data and contributed to the optimization of the protocol. L.B. and Y.Y.H.N. contributed to writing the manuscript. L.B., Y.Y.H.N., E.W., L.W. collected and analyzed data and contributed to the optimization of the protocol. E.F. collected data. R.A. provided all human and porcine specimens and contributed to the manuscript. All authors proof-read the manuscript.

Competing interests

The authors declare that they have no competing financial and non-financial interests.

Additional information

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Supplementary information

Supplementary Video 1 Porcine RAA strip Prep #1
Cutting and pinning of porcine right atrial appendage

Supplementary Video 2 Porcine RAA strip Prep #2
Cutting of porcine right atrial appendage into myocardial strips

Supplementary Video 3 Rat RAA strip Prep #1
Isolation of rat right atrial appendage from whole heart

Supplementary Video 4 Rat RAA strip Prep #2
Cutting and pinning of rat right atrial appendage

Supplementary Video 5 Rat RAA strip Prep #3
Cutting of rat right atrial appendage into myocardial strips

Supplementary Video 6 Rat RAA strip Prep #4
Mounting of rat right atrial appendage myocardial strip into three types of imaging chambers (#1, #2 and #3)

Supplementary Video 7 Human RAA
Spontaneous Ca^{2+} waves in human right atrial appendage myocardial strip

Supplementary Video 8 Human RAA
Spontaneous Ca^{2+} sparks in human right atrial appendage myocardial strip

Supplementary Video 9 Porcine RAA
Spontaneous Ca^{2+} waves in porcine right atrial appendage myocardial strip

Supplementary Video 10 Rat RAA
Spontaneous Ca^{2+} waves in rat right atrial appendage myocardial strip

Supplementary Video 11 Rat RAA EFS
Electrically-evoked CaTs in rat right atrial appendage myocardial strip

Supplementary Video 12 Rat RAA Myo-EEC
Simultaneous imaging of cardiac and endocardial endothelial cell Ca^{2+} in rat right atrial appendage myocardial strip

Supplementary Video 13 Rat LV strip Prep
Isolation, cutting, pinning and dissection of rat ventricles followed by cutting of myocardial strips from rat left ventricle.

Supplementary Video 14 Rat LV
Spontaneous Ca^{2+} waves in rat left ventricle myocardial strip

Supplementary Video 15 Rat LV EFS
Electrically-evoked CaTs in rat left ventricle myocardial strip

Antibody/stain	How it is made	Dilution used	Manufacturer
Primary antibodies			
Sarcomeric alpha-actinin	Raised in mouse, anti-rabbit, 1 mg/mL	1 in 100, 10 µg/mL	Abcam
Von Willebrand Factor-FITC conjugate	Raised in sheep, anti-human, 10 mg/mL	1 in 400, 25 µg/mL	Abcam
TRPC6	Raised in guinea pigs, anti-mouse, 0.9 mg/mL	1 in 100, 9 µg/mL	Alomone
TRPV4	Raised in rabbit, anti-rat, ≥ 0.3 mg/mL	1 in 100, 3 µg/mL	Sigma
Secondary antibodies			
Alexa Fluor® 488	Raised in goat, anti-rabbit, 2 mg/mL	1 in 1000, 2 µg/mL	Invitrogen
Alexa Fluor® 555	Raised in goat, anti-guinea pig, 2 mg/mL	1 in 1000, 2 µg/mL	Invitrogen
Alexa Fluor® 647	Raised in goat, anti-mouse, 2 mg/mL	1 in 500, 4 µg/mL	Abcam
Membrane staining			
Wheat germ agglutinin-Alexa Fluor 555 conjugate	Stock 10 mg/mL	1 in 66.7, 0.15 mg/mL	Invitrogen
Nuclear staining			
Hoechst 33342	10 mg/mL	1 in 1,000	Invitrogen
Propidium iodide	Stock 10 mM	1 in 1,000	Sigma-Aldrich

TABLE 1. Antibodies and staining methods.

	Myocardial strip no.	Strip-section average (μm)	Strip average (μm)
Chamber #1	1	244 , 212	228
Chamber #3	2	160, 190	175
	3	178, 273, 197	216
	4	324, 164, 146	211
	5	147	147
	6	134, 202, 238	191
Mean ± SEM			195 ± 12, *n* = 6 strips, *N* = 1 biopsy

TABLE 2. Porcine atrial appendage myocardial strip thickness measurements.

Step	Problem	Possible reason	Solution
Equipment – micro-scissors	Difficulty dissecting myocardial strip	The blades are blunt or damaged	Sharpen the blades using blade sharpener or replace the micro-scissors
		Micro-scissors are dirty	Clean the micro-scissors with >70% ethanol
Step 1A	Non-viable RAA strip	Biopsy was collected too late during surgery	Ensure surgeon and nurse are aware of the importance of rapid and careful tissue collection
		Biopsy was placed in wrong transport solution	Ensure the nurse has prepared fully thawed transport solution sitting in the transport cool-bag, before surgery commences
		Temperature of transport buffer too high or too low	Measure the temperature of the transport buffer when it arrives. Replace the transport cool-bag, or alter the number of thermal-packs
Steps 2 and 8	Non-viable RAA strip	pH of the HEPES solution	pH the HEPES solution to 7.40 and use fresh tissue
		Damage during dissection	Discard the tissue. Ensure adequate training in manipulating and cutting the RAA from the edge
		Used straight micro-scissors to dissect strip	Use sharp curved micro-scissors to allow smooth and even cuts
Steps 2, 4-7	Non-viable RAA strip	Excessive manipulation of RAA strip	Use fine tweezers, only handle the edge of the strip and minimize contact with the strip. Avoid overstretching the strip
Steps 3D and 9, Box 1: Step 5	Ca^{2+}-indicator dye leaves cells	Temperature too high	At 30 ºC Cal-520 is not retained by the cells, use Calbryte-520
Box 1: Step 8	Significant cell death when introducing more Ca^{2+} to HEPES solution	Wrong temperature	Make all changes in Ca^{2+} concentration using fridge-chilled solutions, in a stepwise manner and in the fridge
Box 1: Step 10	Significant movement/ myocyte contraction	Excitation-contraction uncoupler was not added to the HEPES solution	Ensure that BDM has been added to the 1.8 mM Ca^{2+} HEPES solution
	No EFS-stimulated events	Poor position of electrodes	Position the electrodes so that the strip sits between them
		No current is passing between the electrodes	Use an oscilloscope to measure the current passing between the electrodes. The voltage can be adjusted accordingly
Step 4	Chamber leaking	Coverslip and the chamber are not fully sealed	Ensure that adequate silicone grease is used. Test the seal of the chamber prior to experiment
Step 5 and 8	Unable to image myocytes using transmitted light	Endocardial side is facing the wrong way	Ensure the endocardial side is facing the coverslip for an inversed microscope and vice versa

TABLE 3. Troubleshooting table.

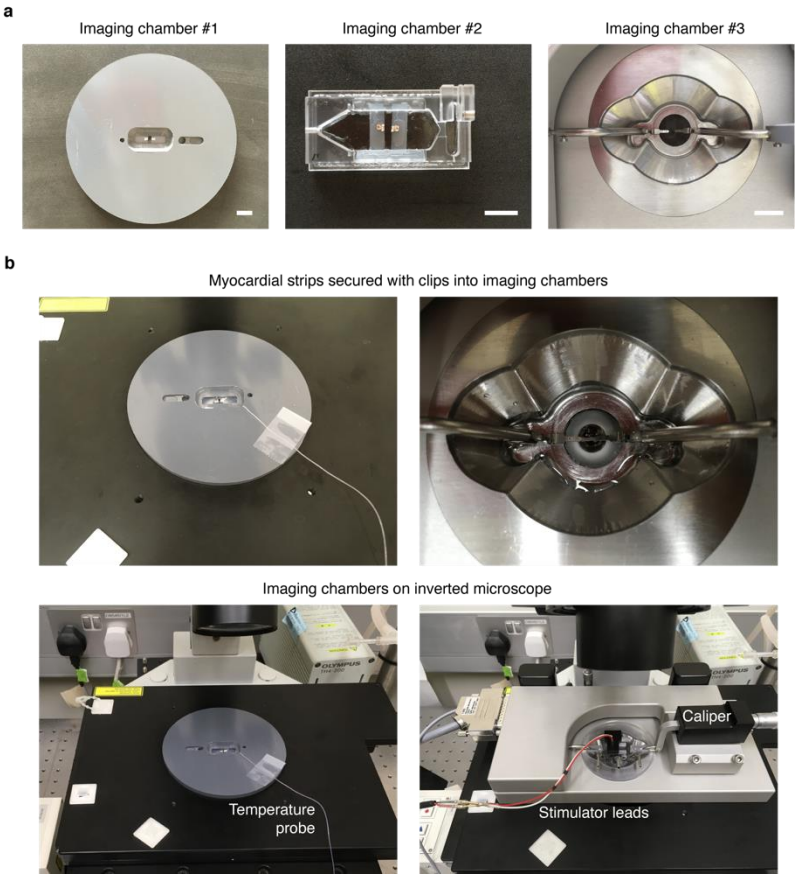

Fig. 1. Images of the three imaging chambers and mounted myocardial strips. a, Aluminium clips secure the myocardial strips in imaging chambers #1 and #2. Scale bars, 10 mm. **b**, Once mounted, the chambers are placed on the stage of an inverted microscope, and images captured through a long working distance 40x objective. Experiments are performed at 25 ºC, but all myocardial strip setups can be heated either via the chamber itself (#2 and #3) or with temperature-controlled superfusion (#1).

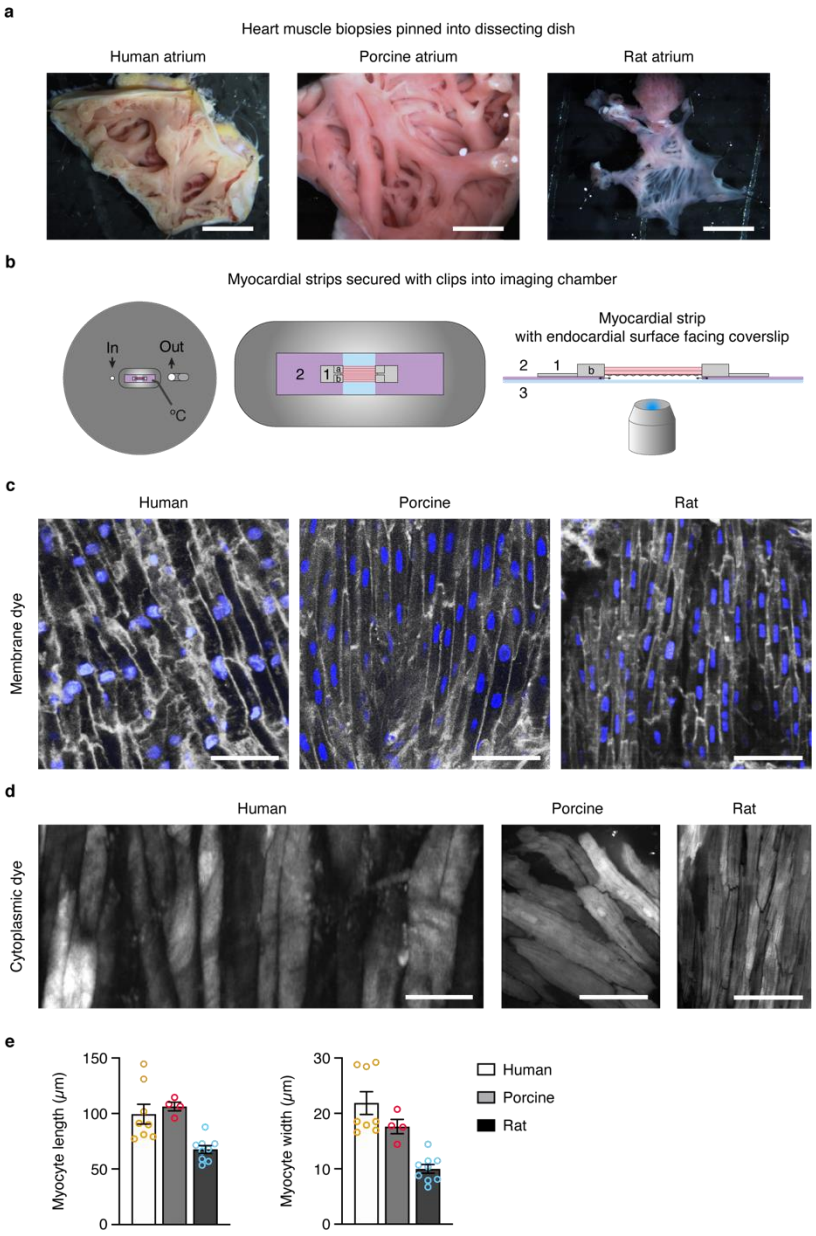

Fig. 2. Dissection, mounting and imaging myocardial strips. a, Heart muscle biopsies pinned ready for dissecting myocardial strips. Scale bars, 500 µm. **b**, Schematic of the custom-made imaging chamber (option #1 in EQUIPMENT), the distance between clips is 5 mm; rest to scale. Left panel, top view of the superfusion inflow and outflow and temperature probe. Middle panel, zoomed image indicates 1, aluminium clips, a,b folded over the strip; 2, plastic platforms; and the myocardial strip held at each end. Right panel, side view of myocardial strip held above 3, a coverslip; imaged using an inverted microscope, with the endocardial endothelial cells facing towards the coverslip. **c**, Confocal images of human cardiomyocytes loaded with wheat germ agglutinin (WGA) Alexa Fluor 555 conjugate membrane dye, porcine and rat cardiomyocytes labeled with FluoVolt™, and all nuclei stained with Hoechst 33342 in blue. Scale bars, 50 µm. **d**, Confocal images of human, porcine and rat cardiomyocytes loaded with Cal-520® AM or Calbryte™-520 AM. Scale bars, 50 µm. **e**, Mean length and width of cardiomyocytes from human (*N* = 8), pig (*N* = 4) and rat (*N* = 9). Each *N* represents at least 10 cells.

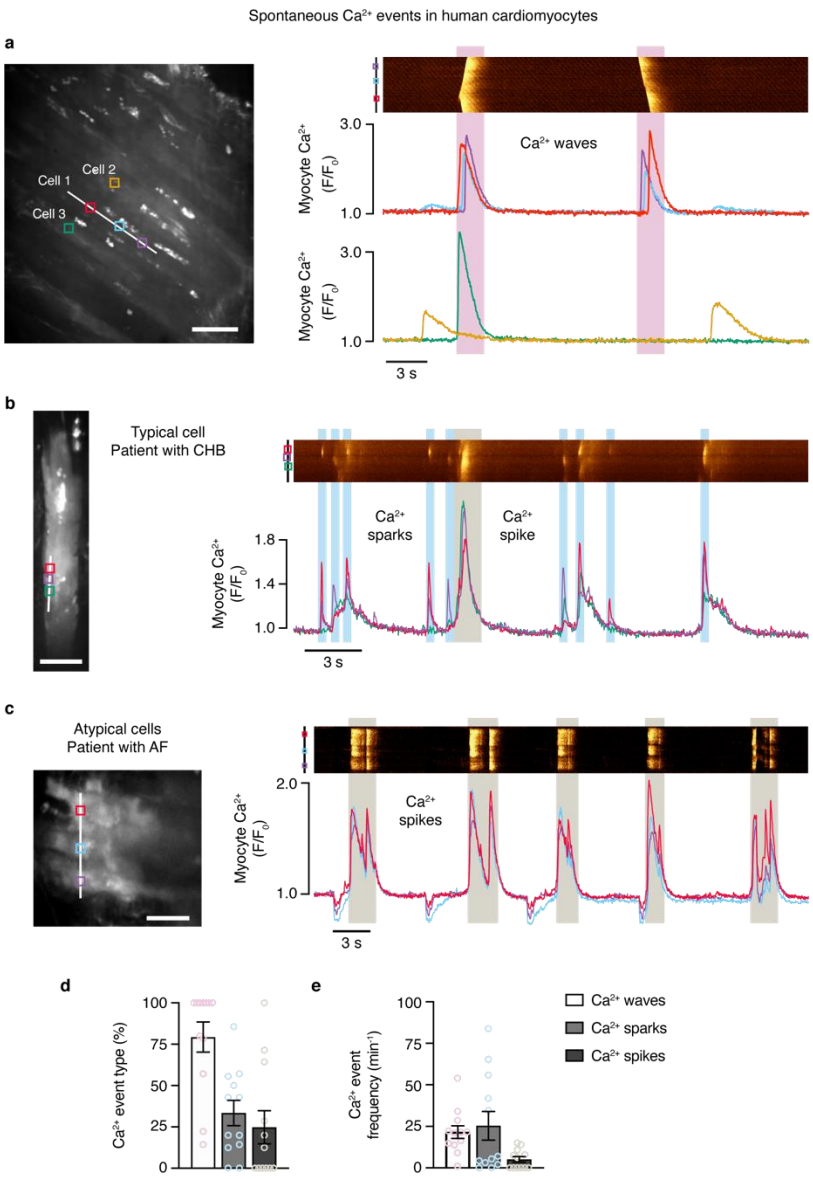

Fig. 3. Spontaneous Ca^{2+} events in human atrial cardiomyocytes. Human myocardial strips contain heterogeneous cells that can generate Ca^{2+} waves, Ca^{2+} sparks and Ca^{2+} spikes at 25 °C. **a**, In a typical cell from a patient with sinus rhythm (SR), the cells generate asynchronous spontaneous Ca^{2+} waves. See **Supplementary Video 7**. **b**, In a typical cell from a patient with complete heart block (CHB), there is a higher frequency of localized Ca^{2+} sparks. See **Supplementary Video 8**. **c**, In an atypical cell from a patient with AF, the cells respond simultaneously as though driven by action potentials. All cells were representative confocal images of human right atrial appendage cardiomyocytes loaded with Cal-520® AM Ca^{2+} indicator dye. Scale bars, 20 µm in all images. White lines and coloured regions of interests on images correspond to kymographs and relative fluorescence (F/F_0) traces, respectively, against time in seconds. F/F_0 was calculated by dividing the time-course of fluorescence intensity (F) by the average baseline fluorescence (F_0). **d**, Summary data showing the relative distribution, shown as a percentage, for each type of spontaneous Ca^{2+} event observed, averaged from all cells in a sample (N = 12). **e**, Summary data showing the frequency of each type of spontaneous Ca^{2+} event in each biopsy (N = 12). Each N represents the average of 1- to 10-cells per biopsy; subcellular responses were averaged in each cell.

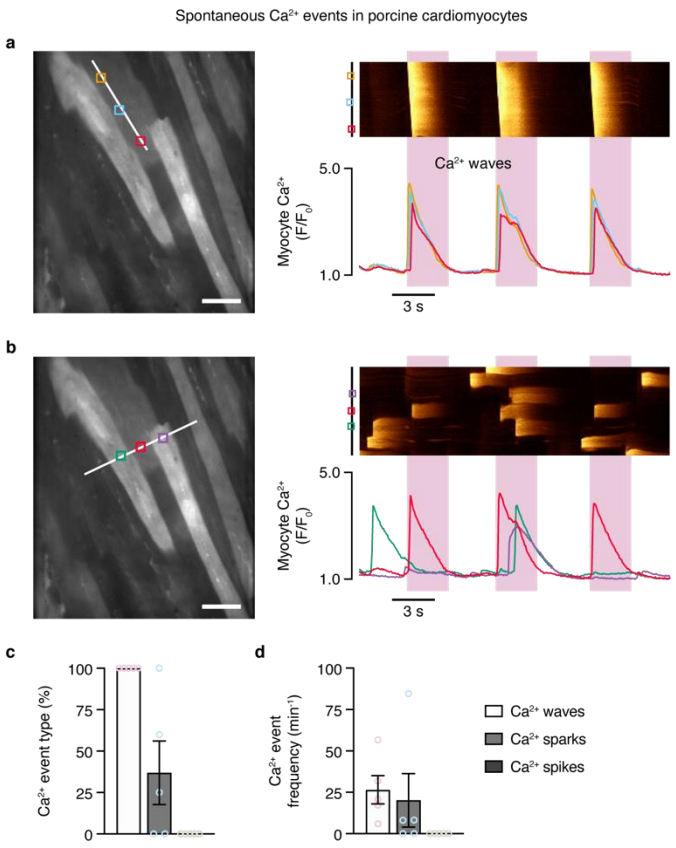

Fig. 4. Spontaneous Ca^{2+} events in porcine atrial cardiomyocytes. Porcine myocardial strips contain homogeneous cells that can generate Ca^{2+} waves and Ca^{2+} sparks, but no Ca^{2+} spikes were observed at 25 °C. **a**, **b**, Cardiomyocytes are loaded with Calbryte™-520 AM Ca^{2+} indicator dye. Scale bar, 20 µm. Spontaneous Ca^{2+} waves propagate along cells and are asynchronous between cells. White lines and coloured regions of interests on images correspond to kymographs and relative fluorescence (F/F_0) traces, respectively, against time in seconds. F/F_0 was calculated by dividing the time-course of fluorescence intensity (F) by the average baseline fluorescence (F_0). **c**, Summary data showing the relative distribution, shown as a percentage, for each type of spontaneous Ca^{2+} event observed, averaged from all cells in a sample (*N* = 5). **d**, Summary data showing the frequency of each type of spontaneous Ca^{2+} event in each biopsy (*N* = 5). This preparation fasciculated, see **Supplementary Video 9**. Each *N* represents the average of 5- to 10-cells per biopsy; subcellular responses were averaged in each cell.

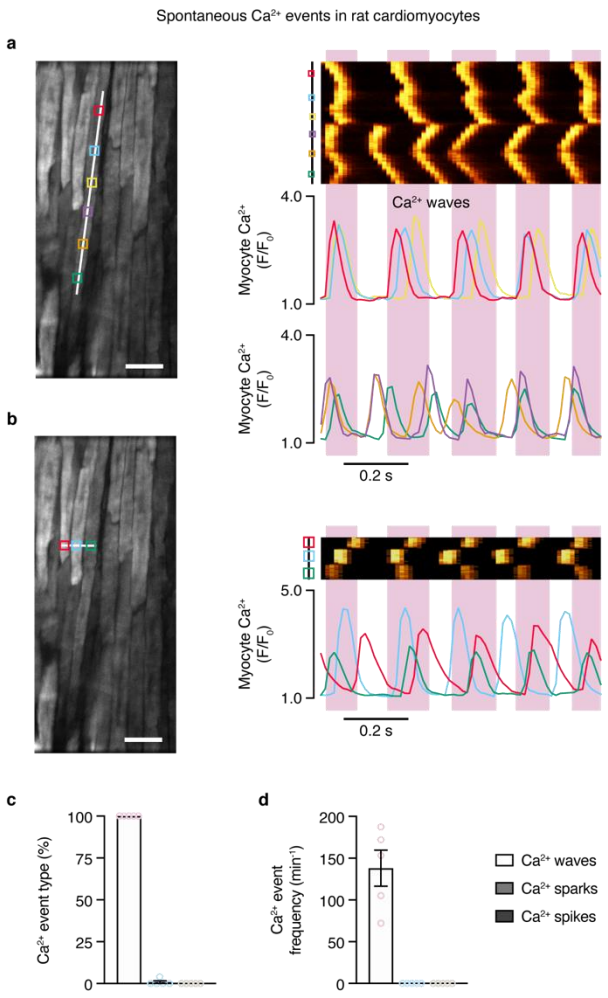

Fig. 5. Spontaneous Ca^{2+} events in rat atrial cardiomyocytes. Rat myocardial strips contain homogeneous cells that can generate Ca^{2+} waves, but very limited Ca^{2+} sparks and no Ca^{2+} spikes were observed at 25 °C. **a**, **b**, Cardiomyocytes are loaded with Calbryte™-520 AM Ca^{2+} indicator dye. Scale bars, 20 µm. Spontaneous Ca^{2+} waves propagate along cells, and are asynchronous between cells. White lines and coloured regions of interests on images correspond to kymographs and relative fluorescence (F/F_0) traces, respectively, against time in seconds. F/F_0 was calculated by dividing the time-course of fluorescence intensity (F) by the average baseline fluorescence (F_0). **c**, Summary data showing the relative distribution, shown as a percentage, for each type of spontaneous Ca^{2+} event observed, averaged from all cells in a sample (*N* = 5). **d**, Summary data showing the frequency of each type of spontaneous Ca^{2+} event in each biopsy (*N* = 5). See **Supplementary Video 10**. Each *N* represents the average of 5- to 10-cells per biopsy.

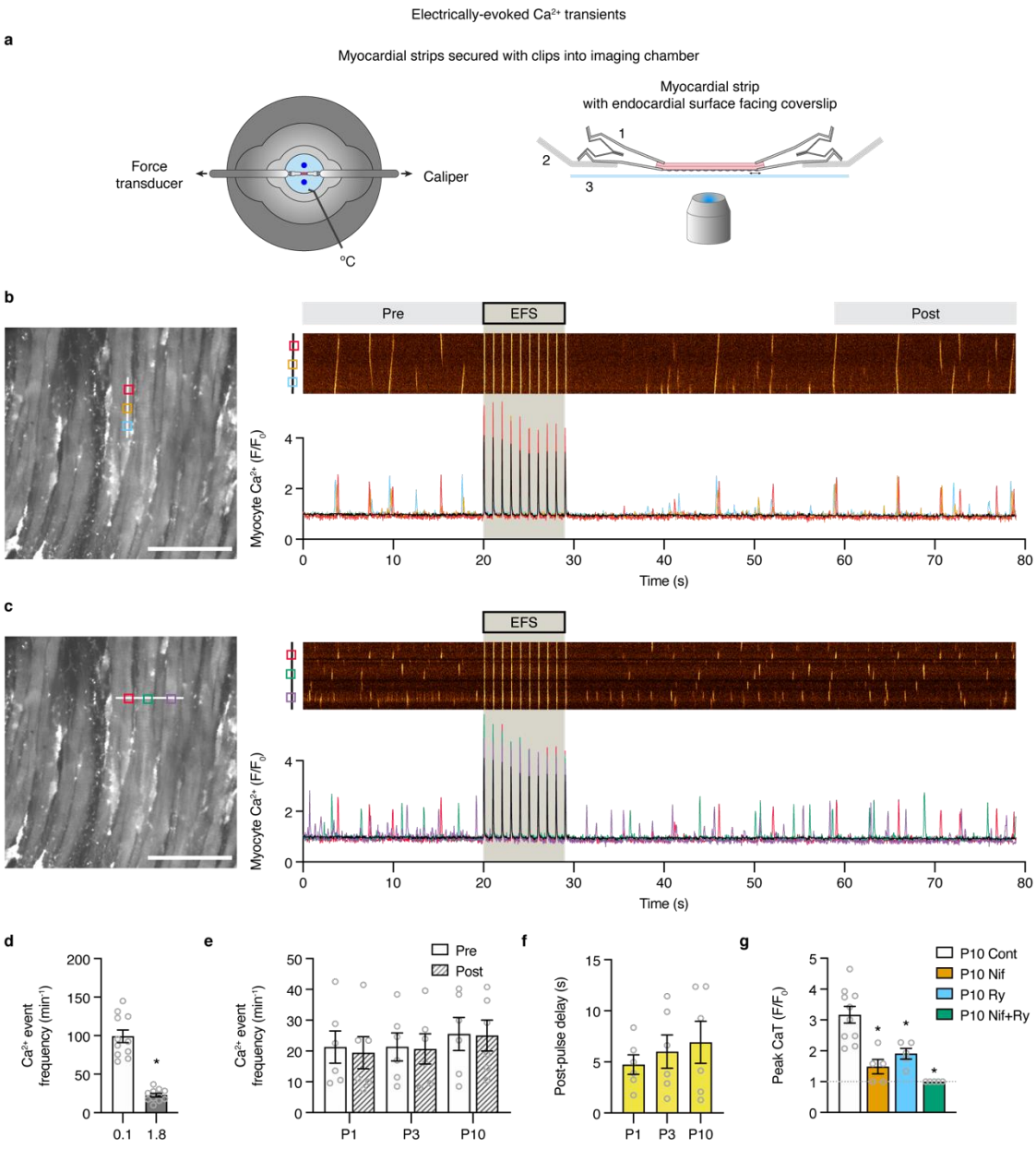

Fig. 6. Ca^{2+} transients (CaTs) in rat atrial cardiomyocytes. a, Schematic of the Danish Myo Technology imaging chamber (option #3 in EQUIPMENT). Left panel, top view of the chamber, stimulating electrodes (blue circles) and °C, temperature probe. Each clip (micro clamp, see EQUIPMENT) is attached to a side arm, one connected to a force transducer, the other to a caliper to stretch the strip. Right panel, zoomed side view image indicates 1, spring clips; 2, side arm; and the myocardial strip held above 3, a coverslip; imaged using an inverted microscope, with the endocardial endothelial cells facing towards the coverslip. **b**, **c** Cardiomyocytes are loaded with Calbryte™-520 AM Ca^{2+} indicator dye. Scale bars, 50 µm. **b**, Spontaneous Ca^{2+} waves propagate along cells, and **c**, are asynchronous between cells. Electrical field stimulation (EFS, 10 pulses at 1 Hz, 7-10 V, 5 ms pulse width) commenced at 20 s, to evoke repeated CaTs. Following cessation of EFS and a short delay, spontaneous Ca^{2+} events recommenced. See **Supplementary Video 11**. White lines and coloured regions of interests on images correspond to kymographs and relative fluorescence (F/F_0) traces, respectively, against time in seconds. The full field response is shown in black. F/F_0 was calculated by dividing the time-

course of fluorescence intensity (F) by the average baseline fluorescence (F_0). **d**, Summary data showing the frequency of spontaneous Ca^{2+} events in low-Ca^{2+} HEPES solution (0.1; *N* = 11) and 1.8 mM Ca^{2+} HEPES-BDM solution (1.8; *N* = 11). *, *P* < 0.05 compared to 0.1 mM Ca^{2+} (passed Shapiro-Wilk normality test, paired two-tailed t-test, *P* < 0.0001). **e**, Summary data showing the frequency of spontaneous Ca^{2+} events pre- and post-EFS, measured during the period indicated in **b**, with either 1 (P1), 3 (P3) or 10 (P10) pulses in the train of EFS (*N* = 6, paired data). **f,** Summary data showing the delay from ceasing EFS to the first spontaneous Ca^{2+} event (post-pulse delay) following P1, P3 or P10 EFS (*N* = 6, paired data). **g,** Peak CaT (*N* = 5-10) under control conditions (Cont), and in the presence of nifedipine (Nif, 1 µM), ryanodine (Ry, 1 µM) or their combination. F/F_0 was calculated by dividing the average of the 10 EFS-evoked fluorescence intensity peaks (F) by the average baseline fluorescence (F_0). *, *P* < 0.05 compared to P10 Cont: Nif, *P* = 0.0005; Ry, *P* = 0.0079; Nif+Ry, *P* < 0.0001; passed Shapiro-Wilk normality test, ANOVA with Tukey's post-comparison test. Each *N* represents the average of 4- to 6-cells per biopsy; subcellular responses were averaged in each cell.

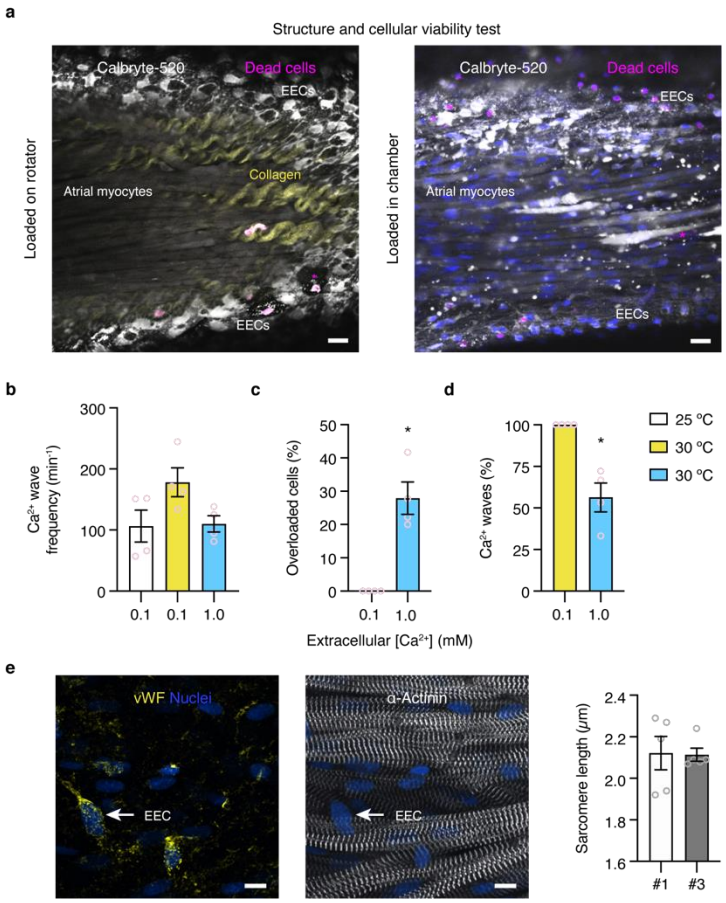

Fig. 7. Structure and viability of rat myocardial strips. a, Confocal images of rat myocardium loaded with Calbryte™-520 AM on the rotator for 60 min and in the imaging chamber for 15 min. In the left image, collagen was imaged at 870 nm, DAPI and Calbryte-520 at 790 nm using a pulsed Deep-See Mai Tai laser; right image using visible lasers. Scale bar, 20 μm. These images are representative of at least 5 biopsies. The two confocal modes are interchangeable for studies of cellular viability, with the advantage of deeper imaging using the 2-photon laser.[40] **b**, Summary data showing the effect of temperature and extracellular Ca^{2+} concentration on the frequency of Ca^{2+} waves in rat atrial myocytes, alongside **c**, the percentage of Ca^{2+} overloaded cells (N = 4, P = 0.0023 compared to 0.1 mM Ca^{2+}; unpaired two-tailed t-test), and **d**, the percentage of cells with repetitive Ca^{2+} waves (N = 4, P = 0.0023 compared to 0.1 mM Ca^{2+}; unpaired two-tailed t-test). *, $P < 0.05$. Each N represents the average of 4- to 6-cells per biopsy. **e**, Confocal images of a rat atrial appendage myocardial strip labeled for vWF and α-Actinin. The distance between 10 sarcomeres was measured in at least three cells per strip to provide summary data for sarcomere length (μm). N = 5 for myocardial strips fixed in either imaging chamber #1 or #3.

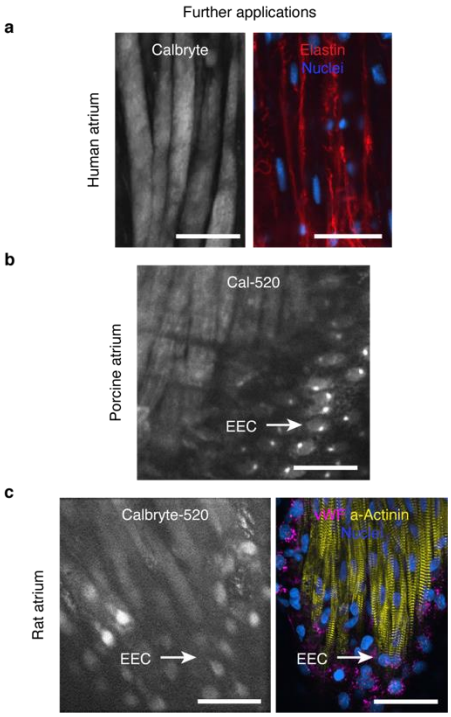

Fig. 8. Further applications. a, Human atrial myocytes can be loaded with Calbryte™-520 AM (instead of Cal-520® AM) to record spontaneous Ca^{2+} events. Live or fixed tissue can be imaged for collagen (**Fig. 7**), and elastin.[40] **b**, Porcine atrial myocytes and EECs can be simultaneously imaged in the same focal plane along the curvature of the myocardial strip. **c**, Spontaneous Ca^{2+} events in rat atrial myocytes can be imaged at the same time as adjacent EECs. See **Supplementary Video 12**. Tissue can be fixed for immunolabeling of both cell types, for example, EECs with von Willebrand Factor (vWF) and cardiac myocytes with α-actinin. Scale bars, 50 µm.

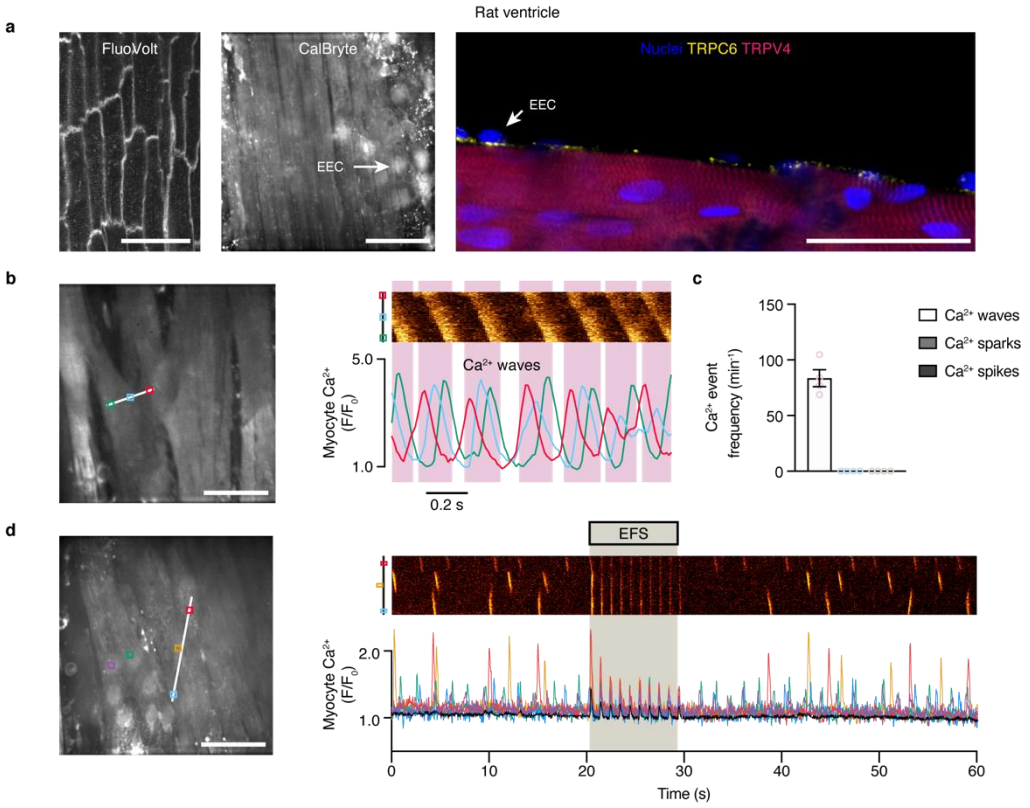

Fig. 9. Translation to rat left ventricle myocardial strips. See **Supplementary Video 13** for details of myocardial strip isolation. Strips are mounted as shown for atrial myocardial strips (see **Supplementary Video 6**). **a**, Membrane label with FluoVolt™ and Calbryte™-520 AM. EECs are clearly visible, and when imaged at the edge of the strip, TRPC6 channel expression is revealed (*N* = 1, for illustration). Scale bars, 50 µm. **b**, In cardiomyocytes loaded with Calbryte™-520, spontaneous Ca^{2+} waves are evident and appear to propagate between cells (see **Supplementary Video 14**). **c**, No Ca^{2+} sparks or spikes were observed (*N* = 3 rats, 4 strips, 5-cells per strip). **d**, In cardiomyocytes loaded with Calbryte™-520, CaTs in response to EFS overshadowed asynchronous spontaneous Ca^{2+} events (see **Supplementary Video 15**, representative of 2 experiments). **b**, **d**, White lines and coloured regions of interests on images correspond to kymographs and relative fluorescence (F/F_0) traces, respectively, against time in seconds. The full field response is shown in black. F/F_0 was calculated by dividing the time-course of fluorescence intensity (F) by the average baseline fluorescence (F_0). Scale bars, 50 µm.

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