SUPPLEMENTAL MATERIAL

Appendix A: Detailed Methods

Procedure for isolation of cardiac myocytes from adult mouse heart

Important: National and institutional guidelines and regulations must be consulted and adhered to before commencement of all animal work.

Please refer also to **Online Figure I** and **Online Videos I and II** for corresponding images and videos of the myocyte isolation procedure.

All chemicals were supplied by Sigma-Aldrich (Singapore) unless otherwise stated.

i) Pre-coating of culture surfaces

Tissue culture surfaces are coated with laminin, at a final concentration of 5 μg/ml in phosphate buffered saline (PBS), for at least 1 h at 37°C, or overnight at 4°C. Surfaces are best prepared fresh but may be sealed and stored at 4°C for up to 4 days. When using glass surfaces, extra volume may be required for complete coverage. Note that cells adhere less strongly to glass than plastic. Before plating cells, draw off laminin solution from wells, and wash 1x with PBS.

ii) Preparation of buffers and media

Media and buffers are prepared according to components detailed in Appendix D. Media, EDTA and Perfusion buffers, when sterilised using a 0.2 μm filter and handled under sterile conditions, are stable for up to two weeks at 4°C, protected from light.

Collagenase and protease XIV enzymes should be added to Collagenase buffer just before isolation. Room-temperature digestion is possible, however we generally perform enzymatic digestion steps at 37°C, in which case Collagenase buffer is warmed immediately before the isolation procedure using a clean water bath. All other buffers are brought to and used at room temperature.

Isolation of one heart requires roughly 30 ml EDTA buffer, 20 ml Perfusion buffer, up to 60 ml Collagenase buffer (or less if recycling, see Appendix B-v) and 10 ml Stop buffer.

iii) Preparation of equipment and surgical area

The surgical area is cleaned with 70% ethanol. EDTA, Perfusion, and Collagenase buffers are aliquoted into 2x, 1x and 5x 10 ml sterile syringes respectively, and sterile 27 G hypodermic needles are attached. We select 10 ml syringes largely due to ease of handling and steady control, but other sizes may be used if preferred. For 37°C digestion, collagenase syringes may be kept warm on a heated mat or in a clean wet/dry water bath, with hypodermic needles capped until use. 60 mm sterile petri dishes are prepared containing: 1x 10 ml EDTA, 1x 10 ml Perfusion, 1x 10 ml Collagenase and 1x 3ml Collagenase buffers. Clean surgical instruments: 1x skin forceps, 1x blunt-end scissors, 1x round-end forceps, 1x sharp-end scissors, 1x Reynolds forceps (haemostatic clamp) or equivalent, 1x sharp-end forceps, are sterilised with 70% ethanol and arranged as shown in Online Figure IA.

Isoflurane anaesthetic system apparatus is set up, with connections to a ventilation chamber and a nose-cone ventilator, which is positioned centrally on the surgery area. Mice are anaesthetised in the chamber with 100% O_2 at 0.5 l/min flow rate, containing isoflurane (atomiser dial at 4%, scale 1 to 5%). Once unconscious, mice are transferred to the surgery area, with anaesthesia maintained using the nose cone.

iv) Surgical procedure

Full anaesthesia is confirmed by reduced breathing rate and lack of toe-pinch reflex response. EDTA and Perfusion syringes are prepared by removal of needle caps, and ensuring no bubbles exist in the syringes or needle. The mouse chest is wiped with 70% ethanol and opened using skin forceps and blunt-end scissors just below the diaphragm, which is then cut through to expose the heart. Now using the round-end forceps, the left lung may be moved aside to reveal the descending aorta and inferior vena cava. Both are cut using the sharp-end scissors, at which point the heart is gently held using the round-end forceps, and 7 ml EDTA buffer is injected steadily within around 1 minute into the base of the right ventricle (RV), which can be identified by its darker colour. To clear as much blood as possible, the needle should not penetrate more than a few mm into the RV, and the angle of entry may be carefully varied during injection. Contractions will quickly cease and the heart will visibly lighten in colour.

v) Removal of heart

The ascending aorta is then clamped. Any haemostatic clamp will suffice, however, we favour the full-curved-ended Reynolds haemostatic forceps, which can reach around the heart and clamp the emerging aorta *in situ*. Clamping of the aorta in this manner does not require high precision, and inclusion of additional emerging vessels does not matter, although clamping the atrial appendages should be avoided. The clamped heart may then be removed by simple incision around the outside of the Reynolds forceps, and transferred to the 60 mm dish containing EDTA, where it should be almost completely submerged. Isoflurane and oxygen supply to the anaesthetic system may be switched off.

vi) Heart dissociation

Locate the left ventricle (LV), which is by far the larger of the ventricles, and forms a pointed apex at the base of the heart. Take the second EDTA syringe, check for no bubbles, and inject through the LV wall 2 or 3 mm above the apical point, again with the needle pushing no more than a few mm into the heart for best perfusion. At this time, very little pressure needs to be applied for the heart to inflate, and flow rate needs only be 1 ml per 2 or 3 minutes. A temptation is to over-apply, which can cause buffer to force into and perforate the left atrial appendage. This does not cause poor isolation results, and the researcher may proceed as normal, although such pressure is unnecessary. All that is required is to maintain full inflation, which is the best measure of adequate perfusion. The coronary circulation will be observed to clear, and areas of the heart surface will become pale.

After 6 minutes or application of all 10 ml EDTA buffer, whichever is first, the needle is removed and the heart is transferred to the dish of Perfusion buffer. In order to clear EDTA from the heart chambers and circulation, 3 ml Perfusion buffer is then injected into the LV via the same perforation left by the previous injection, where possible. Inexperienced users may find a magnification lens beneficial for identification of the original injection point. Again, the ideal pressure is the minimum needed to keep the heart fully inflated.

After 2 minutes or application of all 3 ml perfusion buffer, whichever is first, the heart is next transferred to the dish containing 10 ml Collagenase buffer, and the LV is injected sequentially through the same point with (typically pre-warmed) syringes of Collagenase buffer. As before, injection rate is just sufficient to keep the heart inflated. Initially this is typically around 2 ml/minute, but may increase as the procedure progresses. Following application of each syringe, 10 ml will need to be removed from the dish, to prevent overflow. This buffer may be stored and re-cycled, see Appendix B-v. The volume of Collagenase buffer required for complete digestion varies between hearts. Small, young, healthy hearts can digest in as little as 25 ml, while larger, older or fibrotic hearts may pass beyond 50 ml, necessitating the re-cycling of buffer. Signs of complete digestion include a noticeable reduction in resistance to injection pressure, loss of shape and rigidity, holes and/or extensive pale and fluffy appearance at the heart surface, and ejection of myocytes into the effluent buffer, which are just visible to the naked eye. The point of injection will often widen to the point where significant buffer appears to be flowing directly backwards, but the researcher may proceed as necessary.

Once satisfied that digestion is complete, the clamp is removed, and the heart may be separated into chambers or other specific regions as desired, using sharp scissors. The chosen region is then transferred to the unused 3 ml dish of Collagenase buffer, the purpose of which is to aid further dissociation of tissue without contamination from other regions of the heart (thus multiple dishes could be used here in order to isolate cells from multiple regions). The tissue is then gently teased apart into pieces roughly 1 mm x 1 mm, which will require very little force following a complete digestion. Dissociation is completed by gentle trituration for 2 min using a 1 ml pipette, with a wide-bore tip (purchased, or home-made using sterile scissors) to reduce shear stress.

5 ml Stop solution is then added to the cell-tissue suspension to inhibit further enzymatic reaction. The suspension may be gently pipetted for a further 2 minutes, and inspected under a microscope to confirm yields of rod-shaped myocytes. Myocytes may display

contraction at this stage due to mechanical stimulation, but should quickly acquiesce. The presence of large numbers of rounded, hypercontracted myocytes indicates a poor isolation, and demands troubleshooting, see Appendix C. The myocyte suspension is then transferred to a 50 ml centrifuge tube, which should be stored on its side at room temperature to reduce clumping of cells and prevent ischaemia. Cells may be stored with little loss of viability for up to 2 hours, in which time further isolations may be performed. However, such delays may not be suitable for all studies.

vii) Collection of cells by gravity settling

Subsequent processing of cells is always undertaken in a sterile Class II, type A laminar flow cabinet. Cell suspensions are first passed through a 100 µm pore-size strainer, in order to remove undigested tissue debris. The filter is washed through with a further 5 ml Stop buffer. Note that other protocols tend to choose meshes of between 200-500 µm pore size for this step, which can increase yield, but may occasionally allow passage of small, incompletely separated myocyte clusters. Cells are then allowed to settle by gravity for 20 min. Most myocytes will settle to a pellet, while most non-myocytes and cellular/extracellular debris remain in suspension. Thus, sequential gravity settling is a method to obtain a highly pure myocyte population, and avoids damage caused by centrifugation. Viable rod-shaped myocytes tend also to settle faster than round hypercontracted and dying myocytes, so enriching the pellet for viable rod-shaped cells. Division of cell suspension into two 15 ml tubes rather than one 50 ml tube can aid the formation of a pellet due to the more steeply angled base.

If cells are to be harvested immediately or used in experiments that do not require plating or physiological calcium levels, myocyte fractions are purified simply by three rounds of sequential gravity settling for 10 min in 4 ml fresh Perfusion buffer per 15 ml tube, retaining the myocyte-containing pellet each time. Cells may be analysed at any stage using a haemocytometer to gauge cell number and % viable (rod-shaped) cells. Myocytes are large and angular, and may not flow well under a haemocytometer coverslip, so better results are achieved by pipetting 10 ul cells directly onto the haemocytometer surface, and carefully placing the coverslip directly on top. Cells in at least 7 grids are counted and averaged before quantitation, to control for uneven cell distribution.

viii) Calcium re-introduction and culture of cells

Where myocytes are to be returned to physiological extracellular calcium levels and/or plated, it is important to do so in gradual increments, in order to avoid spontaneous contraction and achieve healthy populations of calcium-tolerant cells. This can be easily accomplished during gravity settling steps as above. Rather than resuspension of pellets in Perfusion buffer alone, pellets are resuspended sequentially in three calcium reintroduction buffers, made simply by mixing Perfusion buffer with increasing proportions of Culture media (Table 1). Buffers correspond to 0.34, 0.68 and 1.02 mmol/l Ca^{2+} respectively. The supernatant fractions, containing non-myocytes as well as rounded myocytes and some viable myocytes, are collected and combined from each round of gravity settling. Fibroblast

and Plating media can be warmed and equilibrated in a 37°C, 5% CO_2, humidified tissue culture incubator during this process.

Laminin solution is aspirated from the prepared culture surfaces (see above), which are then washed once with PBS. The final myocyte pellet is resuspended in 2 ml of room temperature Culture medium, and analysed using a haemocytometer as previously. Extra volume of pre-equilibrated Plating media is then added as appropriate, and cells are plated at application-specific densities: typically around 25 000 cells/ml, or 50 000 cells per well of a 6-well plate (or 35 mm dish), but this may be lowered four-fold or more for imaging studies. Cells are transferred to the tissue culture incubator and agitated gently in a side-to-side (not swirling) motion to ensure even distribution. Adhesion of rod-shaped myocytes occurs rapidly, within 20 minutes for most cells, in the serum-containing Plating medium. Adhesion is allowed to proceed for 1 h, during which time Culture medium may be pre-equilibrated in the incubator. Cells in the combined non-myocyte containing supernatant fraction can now be collected by centrifugation at 300 x *g* for 5 min, resuspended in pre-equilibrated Fibroblast media, plated on tissue culture surfaces, area ~23 cm^2 (0.5x 12-well plate) per LV and transferred to the culture incubator. Cell media is changed after 6-24 h culture, and every 48 h thereafter.

After 1 h, myocytes are washed once with pre-equilibrated Culture media, and then incubated in the Culture media, for the required experimental duration. Rounded myocytes do not adhere strongly and are removed by this process. Note that cultured myocytes must be handled with care. Avoid shocks, vibrations, and rapid aspiration/introduction of media. Always wash gently using warm culture media to reduce ionic fluctuations and change media one well at a time to avoid prolonged exposure to air, particularly if culturing on glass surfaces. Media is changed after every 48 h in culture. When fixing cells, best results are obtained by adding formaldehyde dissolved in Culture medium slowly to an equal volume of culture medium already in the well. Do not swirl or shake.

ix) Additional methods

Automated infusion pump setup

For testing of compatibility with automated infusion pumps, hearts were injected via a flexible linker (Safeed extension tube SF-ET 152EL22, Terumo Singapore Pte., Singapore) using an automated infusion pump (AL-1000, World precision instruments, USA), instead of manual syringes, to control the flow of digestion buffers at pre-programmed rates as indicated.

Transverse aortic constriction (TAC) model of pressure-overload induced hypertrophy

Constriction of the transverse thoracic aorta was performed on 6-week old male C57/BL6J mice as previously described[1] and in our lab[2]. Briefly, mice were anaesthetised, intubated, and placed on a ventilator. Midline sternotomy was performed to allow visualisation of the aortic arch. The transverse aortic arch was ligated around the aorta distal to the brachiocephalic artery with a prolene suture overlying a 27 G needle. The needle was immediately removed and the chest and overlying skin were closed. Sham-operated controls underwent a similar procedure without ligation. Mice were euthanised after 8 weeks, and myocytes were isolated using our described protocol, for transcriptional analysis.

Mouse tail fibroblast isolation and culture

Following heart removal, mouse tails were taken and skin was removed. Tails were cut into 1 mm pieces and digested in 20 ml Collagenase buffer for 45 min at 37°C with constant agitation. Enzyme activity was stopped by addition of 5 ml Stop buffer. Cell suspension was passed through a 100 µm filter. Cells were collected by centrifugation (300 x *g*, 5 min), re-suspended in Fibroblast growth media and plated on tissue-culture treated plastic, area ~23 cm^2 (0.5x 12-well plate) per tail, in a humidified tissue culture incubator. Media was changed after 24 h and every 48 h thereafter.

Control of myocyte culture media pH

In order to optimise media pH for myocyte culture, we took advantage of the different buffering capacities of Earles' buffered and Hank's buffered M199 (or MEM) varieties, at two different concentrations of ambient CO_2. The possible combinations (Hank's buffered, 5% CO_2; Hank's buffered, 2% CO_2; Earle's buffered, 5% CO_2; Earle's buffered, 2% CO_2) resulted in equilibration of media at four pH values; pH6.7, pH7.0, pH7.4 and pH7.9, respectively. Media was changed daily, using pre-equilibrated fresh media, to reduce fluctuations and ensure no exhaustion of buffering capacity.

Immunocytochemistry and fluorescence microscopy

Cells were plated on tissue-culture plastic (for standard imaging) or borosilicate glass-bottom 35 mm petri dishes (MatTek Corp., USA; for confocal imaging), fixed in 4% formaldehyde and incubated for 1 h at room temperature in blocking buffer (5% bovine serum albumin (BSA), 5% foetal bovine serum (FBS) and 0.1% saponin in PBS. Cells were incubated for 16 h at 4°C with primary antibody in dilution buffer (1% BSA, 0.1% saponin in PBS), then for 1 h at room temperature with fluorescent-conjugated secondary antibody and fluorescent phalloidin conjugates in dilution buffer, and a further 10 min with 250 ng/ml 4',6-diamidino-2-phenylindole (DAPI; Thermo Scientific) in dilution buffer. Specific staining was detected using standard fluorescent (Nikon Eclipse Ti) or confocal microscopy (Nikon $A1R^+$si Confocal) using NIS-Elements (Nikon) image software. Antibody details are shown in Supplemental Table 1.

RNA extraction, reverse-transcription PCR and quantitative real time PCR (QPCR)

Total RNA was prepared using TRIzol (Thermo Scientific) according to manufacturer's protocol. cDNA was synthesised using High Capacity cDNA Synthesis Kit (Thermo Scientific). QPCR analysis was conducted using gene-specific primers, SYBR Select Master Mix (Thermo Scientific) and Corbett Rotor-Gene 6000 apparatus and software. mRNA levels are expressed relative to *18S* normalisation gene controls. Oligonucleotide primers were designed using PrimerBLAST (NCBI) and are listed in Supplemental Table 2. Software for building the gene expression heat map was developed and generously made available by Dr Euan Ashley, School of Medicine, Stanford University (http://ashleylab.stanford.edu/tools_scripts.html)

Calcein AM / Ethidium homodimer (Live/Dead) staining

Staining of cultured myocytes was performed using the LIVE/DEAD Viability/Cytotoxicity Kit for mammalian cells (Thermo Scientific) according to manufacturer's instructions. Briefly, Hoechst 33342 (Thermo Scientific), calcein and ethidium dyes were diluted to final concentrations of 10 µg/ml, 1 µmol/l and 0.5 µmol/l respectively, where appropriate, in Culture medium made using phenol-red-free M199 (Thermo Scientific), and added to cells. Cells were incubated for 30 min at room temperature, washed gently with fresh phenol-red-free Culture medium, and imaged using standard fluorescence microscopy (Nikon Eclipse Ti).

Mitochondrial labelling

Cardiac myocytes were plated on borosilicate glass-bottom 35 mm petri dishes and administered with MitoTracker® Red CMXRos dye (Thermo Scientific) at 250 nmol/l final concentration in a minimal volume of Culture medium immediately after removal of Plating medium, according to manufacturer's instructions. Cells were incubated for 30 min at 37°C, fixed in 4% formaldehyde and analysed by confocal microscopy (Nikon A1R^{+}si Confocal).

Myocyte adrenergic stimulation and Western blot

Myocytes were plated in the absence of 2,3-butanedione monoxime (BDM), ITS (Insulin/transferrin/selenium supplement) and lipid and kept in culture for 4 h, before exposure to adrenergic stimulation at concentrations and times as indicated. Plates were then placed on ice, cells were washed with ice cold PBS, and lysed by addition of a urea-based lysis buffer (235 mmol/l Tris pH 6.8, 18.75% glycerol, 5.6% sodium dodecyl sulfate, 6 mol/l urea, 1 mmol/l dithiothreitol), containing Halt™ protease and phosphatase inhibitor cocktail (Thermo Scientific). Total cell lysates were prepared by scraping of wells and transfer to 1.5 ml tubes on ice, passage through a 27 G needle x 5, and incubation at 4°C for 30 min with nutation. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and Western blot using specific antibodies according to manufacturers' instructions, were then performed using standard techniques, with the exception that lysates were incubated at 60°C for 10 min in sample buffer, not boiled, to avoid protein carbamoylation. Signals were detected with ECL Western Blotting Substrate (Thermo Scientific) using a ChemiDoc MP system (Bio-Rad).

Imaging of spontaneous calcium transients

Fluo-4, AM (Thermo Scientific) was resuspended to 10 mmol/l in anhydrous dimethyl sulfoxide (DMSO), and further diluted to 5 mmol/l with 20% Pluronic R F-127 (Thermo Scientific) in DMSO, according to manufacturer's instructions. Dissolved Fluo-4, AM was applied to plated cardiac myocytes at 5 µmol/l final concentration, in a minimal volume of Culture media plus 0.1 mmol/l sulfinpyrazone, immediately after removal of Plating media. Cells were incubated for 30 min at room temperature, then washed and incubated a further 30 min at 37°C in calcium imaging media (Culture media, but using phenol-red-free M199 media (Thermo Scientific) and containing 0.1 mmol/l sulfinpyrazone). Cells were then washed again and maintained in 2,3-butanedione 2-monoxime (BDM)-free calcium imaging media, and imaged using standard phase-contrast and fluorescence (Nikon Eclipse Ti; 488 nm excitation) microscopy, with NIS-Elements (Nikon) image analysis software. Norepinephrine was applied to 10 µmol/l final concentration where indicated.

Intracellular calcium handling and sarcomere length measurements in paced cells

Measurement of intracellular calcium and sarcomere shortening was performed in freshly isolated left ventricular cardiomyocytes using an integrated contractility/ photometry system (IonOptix Corporation, US) as described previously[3,4,5]. Briefly, adult left ventricular cardiomyocytes were loaded with 1 µmol/L of Fura-2-AM (Thermo Scientific, USA) for 30 min, allowed to de-esterify for 20 min and perfused with a standard Tyrode's solution (containing (mmol/l) NaCl 130, KCl 5.4, HEPES 10, $MgCl_2$ 0.5, $CaCl_2$ 1.8, and glucose 10 (pH 7.4)) in an open-perfusion chamber mounted on the stage of an upright microscope (Olympus). Myocytes were stimulated at different frequencies (1-4 Hz) using an external stimulator (Grass Technologies, US). Dual excitation (at 360 and 380 nm; F1 and F0) was delivered using OptoLED light sources (Cairn Research, UK) and emission light was collected at 510 nm (sampling rate 1 kHz). Simultaneous changes in calcium transients and sarcomere length were recorded using IonOptix software. Parameters measured include calcium amplitude, diastolic calcium levels, calcium transient decay (τ) and % sarcomere length (SL) shortening. All measurements were performed at room temperature.

Measurements of left ventricular cardiomyocyte sodium currents (I_{Na})

Cardiac myocytes were plated on glass coverslips (11 mm diameter) and superfused at 3 $ml.min^{-1}$ at room temperature with an external solution containing (mmol/l): NaCl 10, $C_5H_{14}ClNO$ 130, HEPES 10, $CaCl_2$ 1.8, MgCl 1.2, $NiCl_2$ 2, glucose 10, pH 7.4 (CsOH). Whole cell voltage clamp recordings were obtained using borosilicate glass pipettes (tip resistance 1–2 MΩ). The pipette solution contained (mmol/l): CsCl 115, NaCl 5, HEPES 10, EGTA 10, MgATP 5, $MgCl_2$ 0.5 and TEA 10, pH 7.2 (CsOH). All recordings and analysis protocols were performed using an Axopatch 1D amplifier (Molecular Devices, USA) and a CED micro1401 driven by Signal v6 software (Cambridge Electronic Design, UK). Series resistance was compensated between 60 and 90%. Experiments were terminated if series resistance was greater than 10 MΩ or if it increased (>20%). Current signals were sampled at 50 kHz and low pass filtered at 20 kHz. To assess Na^+ current-voltage relationships, currents were elicited using 100 ms step depolarisations over a range of -95 mV to +10 mV, in 5 mV increments, from a holding potential of -100 mV. I/V curves were fitted using the modified Boltzmann equation:

$$I_{Na} = G_{max}(V_m - V_{rev})/(1+Exp[(_{V0.5}-V_m)/k]) \text{ (equation 1)}[6]$$

where I_{Na} is the current density at a given test potential (V_m), G_{max} is the peak conductance, V_{rev} is the reverse potential, $V_{0.5}$ is the membrane potential at 50% current activation and k is the slope constant that describes the steepness of the current activation[6]. Measurements of steady state inactivation of I_{Na} were made by applying pre-pulses ranging from −120 to −40 mV in 5 mV increments for 500 ms prior to the test potential (−30 mV for 100 ms). I_{Na} inactivation curves were fitted using the equation:

$$\text{Normalised } I_{Na} = 1-(1/(1+Exp[(V_{0.5}-V_m)/k])) \text{ (equation 2)}[6]$$

where V_m is the pre-pulse potential, $V_{0.5}$ is the pre-pulse potential at which I_{Na} is half maximally inactivated and k is the slope constant that describes the steepness of the inactivation curve.

Adenoviral transduction

Purified adenoviral vectors Ad5.Myocd, Ad5.MyoDN (MyoCΔ381 in Wang et al., 2001)[7] and Ad5.GFP were kind gifts from Dr Sanjay Sinha, University of Cambridge, United Kingdom; originally purchased from the Gene Transfer Vector Core, University of Iowa. Cells were administered with adenovirus at 5 x 10^6 pfu/ml in a minimal volume of Culture media, immediately after removal of Plating media. Media was changed to fresh Culture media after 8 h. Cells were then incubated for 24 h before phenylephrine treatment and subsequent analysis.

Flow Cytometry

Cells were collected by centrifugation and resuspended in FACS buffer (PBS (pH 7.2) containing 0.5% bovine serum albumin (BSA) and 2 mmol/l EDTA). Cell samples were divided and incubated with Hoechst 33342 (10 µg/ml, Thermo Scientific) and relevant antibodies as appropriate, in accordance with manufacturers' guidelines. Cells were washed once, collected and resuspended in fresh ice-cold FACS buffer, and analysed using an LSRFortessa X-20 flow cytometer (BD).

Statistical Analysis

Data are representative of at least two independent experiments and were conducted in biological triplicates, and presented as mean ± standard deviation, unless otherwise stated. Differences between group means were examined using two-tailed, unpaired Student's t-test or using One Way Analysis of Variance (ANOVA) with Dunnett's test, and were accepted as significant when $P<0.05$.

Appendix B: Notes

i) Anaesthetic

Induction of rapid-onset anaesthesia using isoflurane ventilation involves no injections and causes the mouse minimal stress. Injected, conventional anaesthetics such as pentobarbital and ketamine have a longer onset and significantly reduce respiration, increasing the risk of myocardial ischaemia[8]. Euthanasia by CO_2 inhalation causes ischaemia and is not appropriate for myocyte isolation techniques. We have cultured myocytes from mouse hearts following cervical dislocation, using the protocol described here. However, such methodology is not ideal due in part to the potential for blood coagulation in the heart between death and perfusion, and heparin administration would be recommended if this procedure is necessary.

ii) BDM

This protocol uses the myosin II ATPase inhibitor 2,3-butanedione monoxime (BDM) to reduce contractions and improve the yield of isolated cardiac myocytes[9]. It is necessary to remove BDM from cultures before experimental applications including contractility, calcium transient or electrophysiology measurements. However, after 1-2 h incubation in the absence of BDM, a number of myocytes become terminally hypercontracted and die. Alternatives to BDM in cultures have been suggested, including N-benzyl-p-toluene sulphonamide (BTS)[10], and blebbistatin[11].

iii) Magnesium

Magnesium inclusion in Perfusion buffer may have multiple functions, including the protection of myocyte membrane integrity by stabilising the outer glycocalyx lamina in the low calcium environment[12]. Different protocols may introduce magnesium as a chloride or a sulphate compound, the reasoning behind this is unclear[13]. Any effect on chloride ion channels is likely to be negligible when compared to the 100-fold higher concentration of chloride elicited by NaCl. We tested magnesium chloride and sulphate, and found both give similar results.

iv) Oxygenation and pH buffering

A number of protocols pre-oxygenate dissociation buffers using oxygen or carbogen (95% O_2, 5% CO_2). We found no advantage to pre-oxygenation in our experiments. Possibly, isolation of myocytes from the hearts of larger rodents may show benefits due to thicker myocardia and decreased passive diffusion rates. However, if choosing to oxygenate, it is essential to match the choice of gas to the buffering system of dissociation solutions. Hanks or HEPES buffered solutions such as used in this report require pure oxygen; pH will become acidic after carbogen equilibration and poor yields are retrieved. Conversely, bicarbonate buffered solutions require carbogen equilibration, and oxygen alone induces alkaline pH.

v) Enzymes and re-cycling

We utilise a collagenase 2 (B) : collagenase 4 (D) : Protease XIV mixture as described by Zhou et al.[14], with slightly increased Protease XIV. Our collagenase enzymes are supplied by Worthington Biochemical (Lakewood, USA) and exhibit high batch-to-batch reproducibility (collagenase 2, ~210 units/mg; collagenase 4, ~260 units/mg) such that re-optimisation has to date been unnecessary. Collagenase 2 is a less pure extract with more basal clostripain activity than collagenase 4, which can sometimes be advantageous, and in many cases, 2.5 mg/ml collagenase 2 alone is sufficient at 37°C to attain good yields of myocytes. However, using the described mixture as standard performs consistently, including in older or diseased heart isolations[15].

Collagenase may be recycled by collection and re-use of effluent buffer from the heart after each syringe injection is complete. Care must be taken to prevent needle-prick injuries. However, to prevent cellular cross-contamination, fresh collagenase buffer is prepared for each heart.

Appendix C: Isolation procedure troubleshooting

Problem	Possible Cause	Solution
Poor digestion, heart does not soften	*Old/degraded enzymes*	*Purchase/prepare new enzymes*
	New enzyme batch with low activity	*Optimise enzyme concentration*
	Bubbles in syringe	*Ensure removal of bubbles before injection*
	Incomplete clearance of blood from heart	*Increase volume and time for EDTA buffer injection to RV if necessary*
	Old/fibrotic heart	*Increase digestion time, use 37°C if not already, increase enzyme concentration*
	Accidental addition of EDTA to Collagenase buffer	*Prepare new buffers*
Complete digestion, heart softens, but low yield of viable rod-shaped cells	*Old/contaminated buffers/reagents*	*Prepare new buffers, filter sterilise. Purchase new reagents, particularly BDM or Taurine*
	Impure water	*Use only ultrapure >=18.2 MΩ.cm H_2O*
	Incorrect buffer preparation	*Check preparation, remake buffers. Calibrate pH meter and check pH*
	Overdigestion	*Rare. Reduce digestion time / enzyme concentration*
Good yield, but cells die while in Stop buffer	*Old/contaminated FBS*	*Use new FBS. Try new batch if still unsuccessful*
	Impure water	*Use only ultrapure >=18.2 MΩ.cm H_2O*

Appendix D. Buffer and media constituents

i) EDTA buffer

Compound	Molar mass (g/mol)	Final concentration (mmol/l)	g / litre required
NaCl	*58.44*	*130*	*7.5972*
KCl	*74.55*	*5*	*0.37275*
NaH_2PO_4	*119.98*	*0.5*	*0.05999*
HEPES	*238.3*	*10*	*2.383*
Glucose	*180.16*	*10*	*1.8016*
BDM	*101.1*	*10*	*1.011*
Taurine	*125.15*	*10*	*1.2515*
EDTA	*292.24*	*5*	*1.4612*

Make in 1 litre ultrapure 18.2 MΩ.cm H_2O. Adjust to pH 7.8 using NaOH. Sterile filter.

ii) Perfusion buffer

Compound	Molar mass (g/mol)	Final concentration (mmol/l)	g / litre required
NaCl	*58.44*	*130*	*7.5972*
KCl	*74.55*	*5*	*0.37275*
NaH_2PO_4	*119.98*	*0.5*	*0.05999*
HEPES	*238.3*	*10*	*2.383*
Glucose	*180.16*	*10*	*1.8016*
BDM	*101.1*	*10*	*1.011*
Taurine	*125.15*	*10*	*1.2515*
$MgCl_2$	*95.2*	*1*	*0.095*

Make in 1 litre ultrapure 18.2 MΩ.cm H_2O. Adjust to pH 7.8 using NaOH. Sterile filter.

iii) Collagenase buffer

Enzyme	Final concentration (mg/ml)
Collagenase 2	*0.5*
Collagenase 4	*0.5*
Protease XIV	*0.05*

Alternatively, 100x collagenase and 1000x protease XIV (=50 mg/ml) stocks may be prepared in ultrapure 18.2 MΩ.cm H_2O, filter-sterilised, and stored in aliquots at -80°C for at least 4 months. Prepare Collagenase buffer by dilution in Perfusion buffer. Make fresh immediately before isolation.

iv) Stop buffer

Stop buffer is made with Perfusion buffer containing 5% sterile FBS. Make fresh on day of isolation.

v) Media constituents

Note 1: 100x BDM stocks (= 1 mol/l) are prepared by dissolving 1.01 g BDM in 10 ml ultrapure 18.2 MΩ.cm H_2O, filter-sterilised, and stored in aliquots at -20°C.
Note 2: 50x bovine serum albumin (BSA) stocks (= 5% w/v) are prepared by dissolving 1 g BSA in 20 ml PBS, filter-sterilized, and stored at 4°C. Keep sterile.
Note 3: M199 and DMEM/F12 media used here are supplied with l-glutamine already included. Ensure l-glutamine addition if using different suppliers.
Note 4: Penicillin/Streptomycin (P/S) antibiotic addition is optional.

vi) Plating media

Compound	Stock concentration	Final concentration	ml / 100 ml media required
M199	*-*	*-*	*93*
FBS	*100%*	*5%*	*5*
BDM	*1 mol/l*	*10 mmol/l*	*1*
P/S	*100x*	*1x*	*1 (Optional)*

Sterile filter, and keep sterile.

vii) Culture media

Compound	Stock concentration	Final concentration	ml / 100 ml media required
M199	*-*	*-*	*96*
BSA	*5%*	*0.1%*	*2*
*ITS**	*100x*	*1x*	*1*
BDM	*1 mol/l*	*10 mmol/l*	*1*
CD lipid †	*100x*	*1x*	*1*
P/S	*100x*	*1x*	*1 (Optional)*

*ITS; Insulin, transferrin, selenium. †CD lipid; chemically defined lipid concentrate
Sterile filter, and keep sterile. Protect from light.

viii) Calcium reintroduction buffers

Solution	ml for Buffer 1	ml for Buffer 2	ml for Buffer 3
Perfusion Buffer	*15*	*10*	*5*
Culture Media	*5*	*10*	*15*

For total volume of 20 ml per calcium reintroduction buffer. Make fresh on day of isolation.

ix) Fibroblast growth media

Compound	Stock concentration	Final concentration	ml / 100 ml media required
DMEM/F12	*-*	*-*	*90*
FBS	*100%*	*10%*	*10*
P/S	*100x*	*1x*	*1 (Optional)*

Appendix E. Isolation procedure materials and equipment

i) Surgical equipment

Item Name	Company	Catalogue number
Skin forceps	*Roboz, USA*	*RS-5248*
Blunt-end scissors	*Roboz, USA*	*RS-5965*
Curved-end forceps	*Roboz, USA*	*RS-5137*
Sharp-end scissors	*Roboz, USA*	*RS-5840*
Reynolds full-curved hemostatic forceps	*Roboz, USA*	*RS-7211*
Straight-end forceps	*Roboz, USA*	*RS-5070*

ii) Isolation and culture equipment

Item Name	Company	Catalogue number
60 mm petri dishes	*VWR, Singapore*	*25384-092*
10 ml syringes	*BD Bioscience, Singapore*	*302143*
27 G x ½ inch hypodermic needles	*BD Bioscience, Singapore*	*305109*
Wide-bore 1000 µl tips	*Axygen, USA*	*TF-1005-WB-R-S*
100 µm cell strainers	*SPL Singapore*	*93100*
0.22 µm filters	*Merck Millipore, Ireland*	*SLGP033RS*
35 mm Glass-bottom culture dishes	*MatTek Corp., USA*	*P35G-0-14-C*

iii) Buffer Reagents

Item Name	Company	Catalogue number
NaCl	*1st Base, Singapore*	*BIO-1111*
KCl	*Sigma-Aldrich, Singapore*	*P9541*
NaH_2PO_4	*Sigma-Aldrich, Singapore*	*S8282*
HEPES	*1st Base, Singapore*	*BIO-1825*
Glucose	*Sigma-Aldrich, Singapore*	*G8270*
BDM	*Sigma-Aldrich, Singapore*	*B0753*
Taurine	*Sigma-Aldrich, Singapore*	*T8691*
EDTA	*Sigma-Aldrich, Singapore*	*EDS*
$MgCl_2$	*Sigma-Aldrich, Singapore*	*M8266*
Collagenase 2	*Worthington, USA*	*LS004176*
Collagenase 4	*Worthington, USA*	*LS004188*
Protease XIV	*Sigma-Aldrich, Singapore*	*P5147*
FBS	*Thermo Scientific, Singapore*	*10270106*

iv) Cell culture Reagents

Item Name	Company	Catalogue number
Laminin (murine)	*Thermo Scientific, Singapore*	*23017-15*
Phosphate buffered saline	*Lonza, USA*	*17-512F*
M199 Medium	*Sigma-Aldrich, Singapore*	*M4530*
DMEM/F12 Medium	*Thermo Scientific, Singapore*	*11320-033*
Bovine Serum Albumin	*Sigma-Aldrich, Singapore*	*A1470*
ITS supplement	*Sigma-Aldrich, Singapore*	*I3146*
Chemically defined lipid concentrate	*Thermo Scientific, Singapore*	*11905-031*
Penicillin-Streptomycin	*Thermo Scientific, Singapore*	*15070-063*

Supplemental Table 1. Antibody details

Antibody	Concentration	Source	Manufacturer (Catalogue number)
ACTA2	*1:1000*	*Mouse*	*Dako (M085129)*
ACTN2	*1:1000*	*Mouse*	*Sigma (A7811)*
CD31	*1:200*	*Rabbit*	*Abcam (ab28364)*
CD45-PE	*1:20*	*Rat*	*Miltenyi Biotec (130-102-781)*
CD146-PE	*1:20*	*Rat*	*Miltenyi Biotec (130-102-844)*
THY1 (CD90)	*1:100*	*Rat*	*Abcam (ab3105)*
TNNT2	*1:500*	*Mouse*	*Thermo (MS-295-P1)*
VIM	*1:500*	*Rabbit*	*Abcam (ab45939)*
[Actin: Phalloidin]	*1:200*	-	*Abcam (ab176757)*
[Isolectin B4]	*1:100*	-	*Vector Labs (DL-1207)*

Supplemental Table 2. Primer list

Gene	Primer Sequence
18S	TTGACGGAAGGGCACCACCAG
	GCACCACCACCCACGGAATCG
Acta1	CACTTCCTACCCTCGGCAC
	TCTAGTTTCAGAGGCTGGCG
Cdh11	CTTGTGAATGGGACTCGGAC
	TCAAAGGGCCACAAAGCACA
Col1a1	GCAACAGTCGCTTCACCTAC
	GTGGGAGGGAACCAGATTG
Col1a2	TCGGGCCTGCTGGTGTTCGTG
	TGGGCGCGGCTGTATGAGTTCTTC
Ddr2	TTCCCTGCCCAGCGAGTCCA
	ACCACTGCACCCTGACTCCTCC
Gata6	TTGCTCCGGTAACAGCAGTGGCT
	CACTGTTCTCGGGGTTGGCGT
Gja	CCAACAGCAGCAGACTTTGA
	CGTGGAGTAGGCTTGGACC
Hk2	AGAACCGTGGACTGGACAAC
	CGTCACATTTCGGAGCCAGA
Myh6	CTACAAGCGCCAGGCTGAG
	TGGAGAGGTTATTCCTCGTCG
Myh7	AGCATTCTCCTGCTGTTTCCTT
	TGAGCCTTGGATTCTCAAACG
Mxi1	TGGGACTGTAGCCGTCTGT
	GCATGGAGGGGAACGATGAG
Nppa	TCGGAGCCTACGAAGATCCA
	GTGGCAATGTGACCAAGCTG
Nppb	GTTTGGGCTGTAACGCACTG
	TTGTGGCAAGTTTGTGCTCC
Pdgfra	GAGCCTGAGCTTTGAGCGA
	AGGACGAATTCAGCTGCACA
S100a4	GCACTTCCTCTCTCTTGGTCTG
	TCACCCTCTTTGCCTGAGTA
Slc2a1	GCTTGTAGAGTGACGATCTGAGCTA
	CTCCTCCCACAGCCAACATGAG
Tbx2	TCCGCACCTATGTCTTCCCA
	ATCACGCTCCGGCTTACAG
Tbx18	CGAAAGGGTCTCCCGTACCT
	AGATCTTCACCCGCATTGCT
Tbx20	CCCCGCTGCCAGCCAGGCTCTA
	GTGCACCCGTGGCTGGTACTTATGC
Tcf21	GGCCAACGACAAGTACGAGA
	GCTGTAGTTCCACACAAGCG
Thy1	TGGGTGCAGCAACTGGAGGC
	CTCGGGACACCTGCAAGACTGA
Vim	GCCGAAAGCACCCTGCAGTCA
	GCCTGCAGCTCCTGGATCTCTTCA

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Online video legends

Online video I

Video summary of the procedure for isolation of cardiac myocytes from the adult mouse heart. The chest of an anaesthetised 8-week old male mouse is opened to expose the heart. the descending aorta and inferior vena cava are cut and the heart is flushed by injection of 7 ml EDTA buffer into the right ventricle. The haemostatic clamp is applied to the emerging aorta. The heart is removed by incision around this clamp and transferred to a 60 mm dish containing fresh EDTA buffer. EDTA buffer is shown being injected into the apical region of the left ventricle, at the minimum flow rate required to produce full inflation of the heart. Remaining blood exits the right ventricle via the previous injection site. The heart surface becomes pale. The heart is then shown following sequential injection of 10 ml EDTA buffer, 3 ml Perfusion buffer and 40 ml Collagenase buffer, at which point digestion is complete. Loss of shape and rigidity, enlarged perforation at the point of injection, secondary perforations and pale patches with striated appearance on the myocardial surface are clearly visible. The emerging vasculature and right atrium, left atrium and right ventricle are then removed to leave the left ventricle, which is transferred to a new dish containing 3 ml Digestion buffer and pulled gently, with little resistance, into pieces of roughly 1 mm^3 size, using forceps. Cellular dissociation is completed by gentle trituration using a 1000 µl pipette with wide-bore tip and enzyme activity is inhibited by addition of 5 ml Stop buffer. Videos were edited using Freemake Video Converter (www.freemake.com).

Online video II

Magnified view of the left ventricle during sequential injection of dissociation buffers. Complete flushing of blood from the coronary circulation is clearly visible following injection of EDTA buffer into the apical region of the left ventricle. Note, the heart is now orientated so as to be viewed from the posterior aspect, thus the left ventricle now appears at the left side of the video. Whitening of the myocardial surface is visible. Re-insertion of the needle into the same injection point is demonstrated. Injection of digestion buffer starts from 43 seconds onwards. By the video end, digestion of the heart is close to complete. The heart displays an enlarged perforation at the point of injection, and pale patches with striated appearance on the myocardial surface. Videos were edited using Freemake Video Converter.

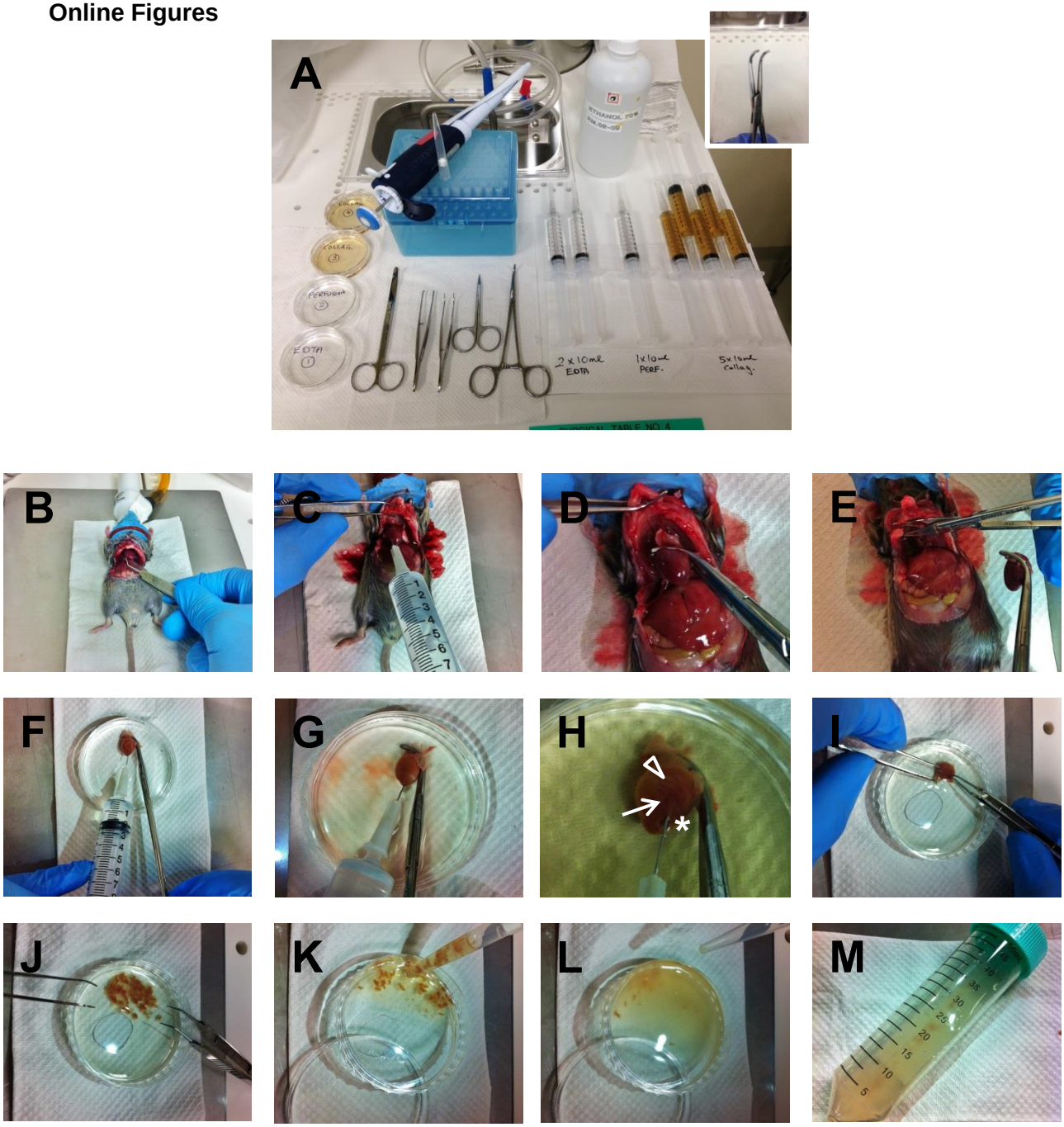
Online video III

3-dimensional rotating image showing complex organised sarcomeric structure in myocytes isolated from the adult mouse left ventricle. Myocytes were subjected to immunological staining with sarcomeric-alpha-actinin antibody (green), and DAPI (blue) counterstain, after isolation and 24 h culture. Confocal imaging was performed using a Nikon $A1R^+$si microscope. Z-stacks were arranged and the 3-dimensional video produced using NIS-Elements (Nikon) image software.

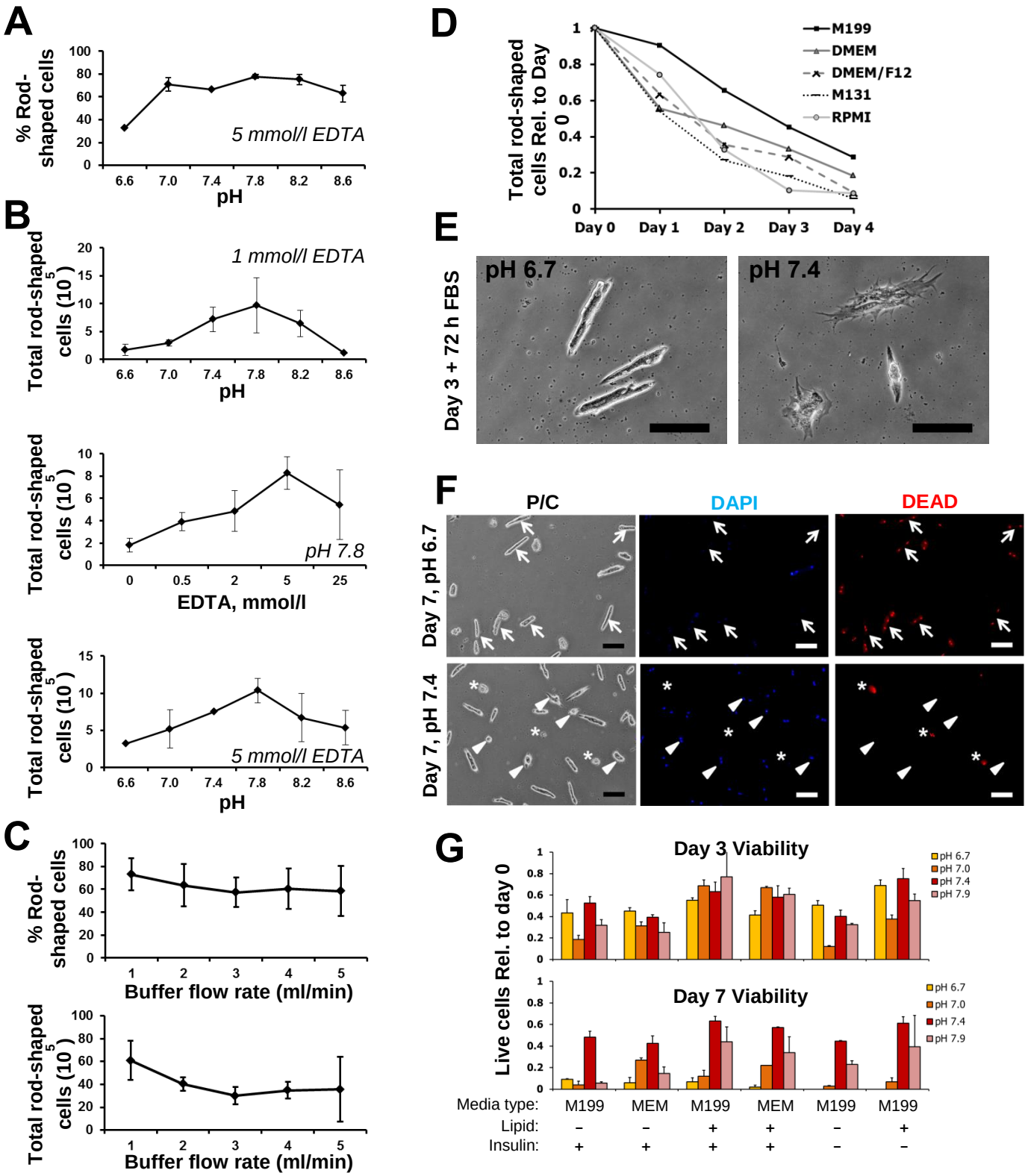
Online video IVA and IVB

Simultaneous spontaneous calcium transients and waves of partial contraction in myocytes isolated from the adult mouse left ventricle. Myocytes were loaded with the calcium-sensitive fluorophore Fluo-4 AM and imaged by phase contrast (A) and fluorescence (B) microscopy, using a Nikon Eclipse Ti microscope. Videos were captured using NIS-Elements (Nikon) image software and edited using Freemake Video Converter.

Online Figures

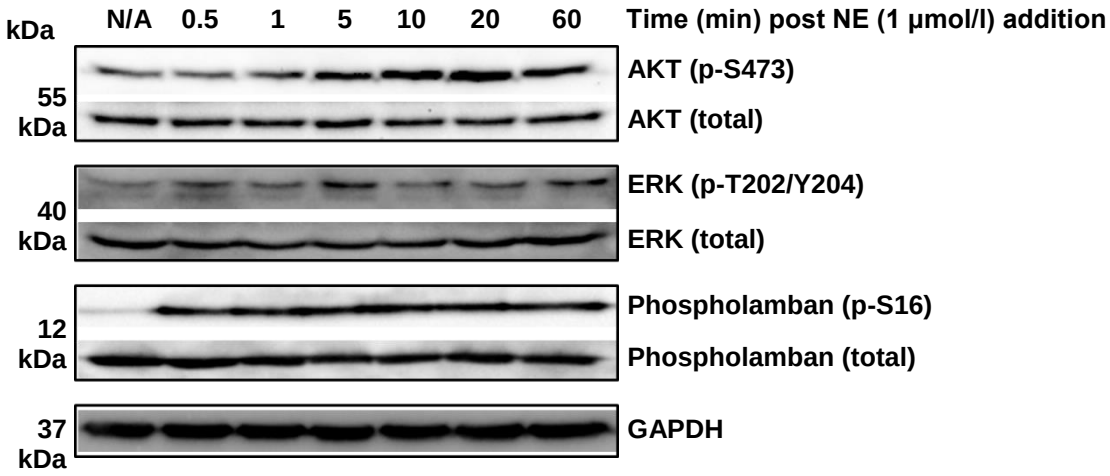


Online Figure I. Photographic images of the cardiac myocyte isolation procedure. A, The entire procedure requires only equipment and surgical instruments found readily in most animal surgical facilities. Inset image shows our preferred choice of full-curved-ended Reynolds haemostatic forceps to clamp the aorta. **B**, The chest cavity of the anaesthetised mouse is opened to expose the heart. Forceps point to the right ventricle, which has a darker colouration due to thinner wall and presence of deoxygenated blood. **C**, Injection of EDTA buffer into the right ventricle after cutting of descending aorta. **D**, Clamp application at emerging aorta. **E**, Removal of heart by incision around the clamped area. **F**, Injection of EDTA buffer into left ventricular apical region of the clamped heart. Remaining blood may exit via the right ventricular perforation and the heart surface becomes pale (**G**). **H**, Heart at completion of digestion, showing loss of shape and rigidity, enlarged perforation at point of injection (*), secondary perforations (arrow) and pale patches with striated appearance (triangle), as indicated. **I**, teasing apart of left ventricle myocardium to 1 mm pieces using forceps (**J**), before trituration using 1000 µl pipette with wide-bore tip (**K**). **L**, Complete tissue dissociation after trituration. **M**, After addition of Stop buffer, cell suspension is stored horizontally in a 50 ml tube at room temperature.

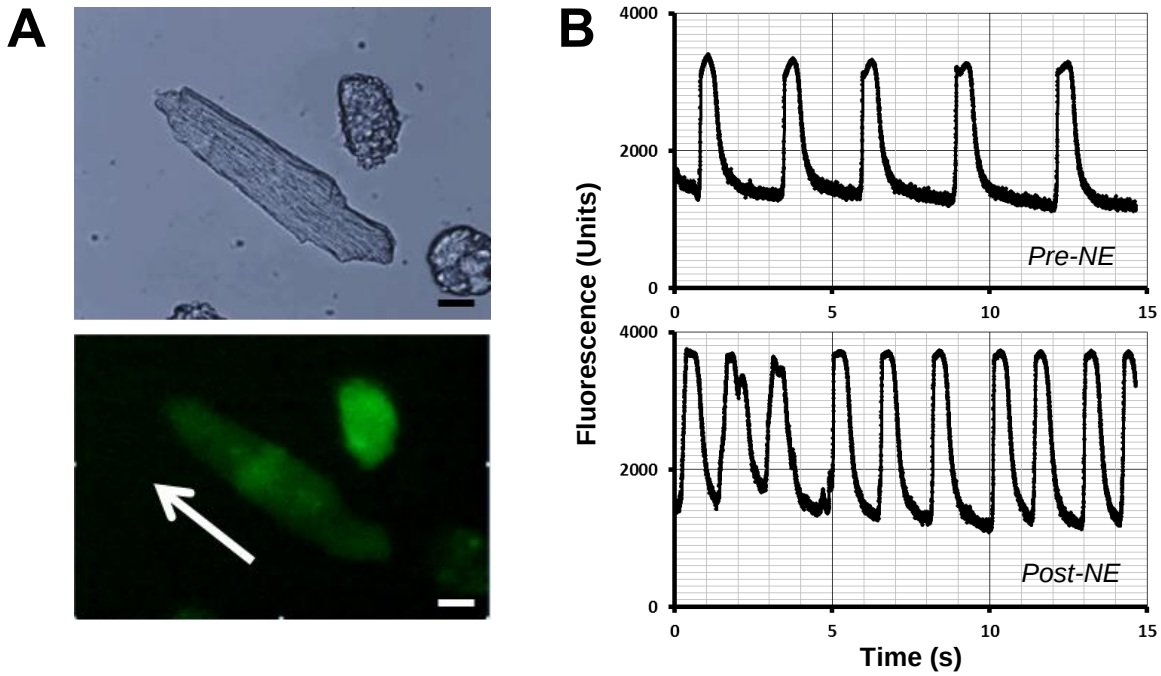


Online Figure II. Protocol optimisation and compatibility with automation. A, Confirmation that pH 7.8 remains optimal for isolation of rod-shaped myocytes, when using 5 mmol/l EDTA in EDTA buffer. **B**, Conditions producing the highest proportions of viable rod-shaped cardiac myocytes correspond to those yielding the highest total numbers of viable myocytes. **C**, The myocyte isolation protocol is compatible with automated pump infusion systems in place of manual injection. Mice were anaesthetised, right ventricles were manually flushed, and aortas were clamped as usual. Subsequent buffers were administered by injection into the left ventricle using an automated infusion setup, at flow rates varying from 1 to 5 ml/min, as indicated. Downstream processing and calculation of % rod-shaped myocytes were performed as normal. *Continued overleaf.*

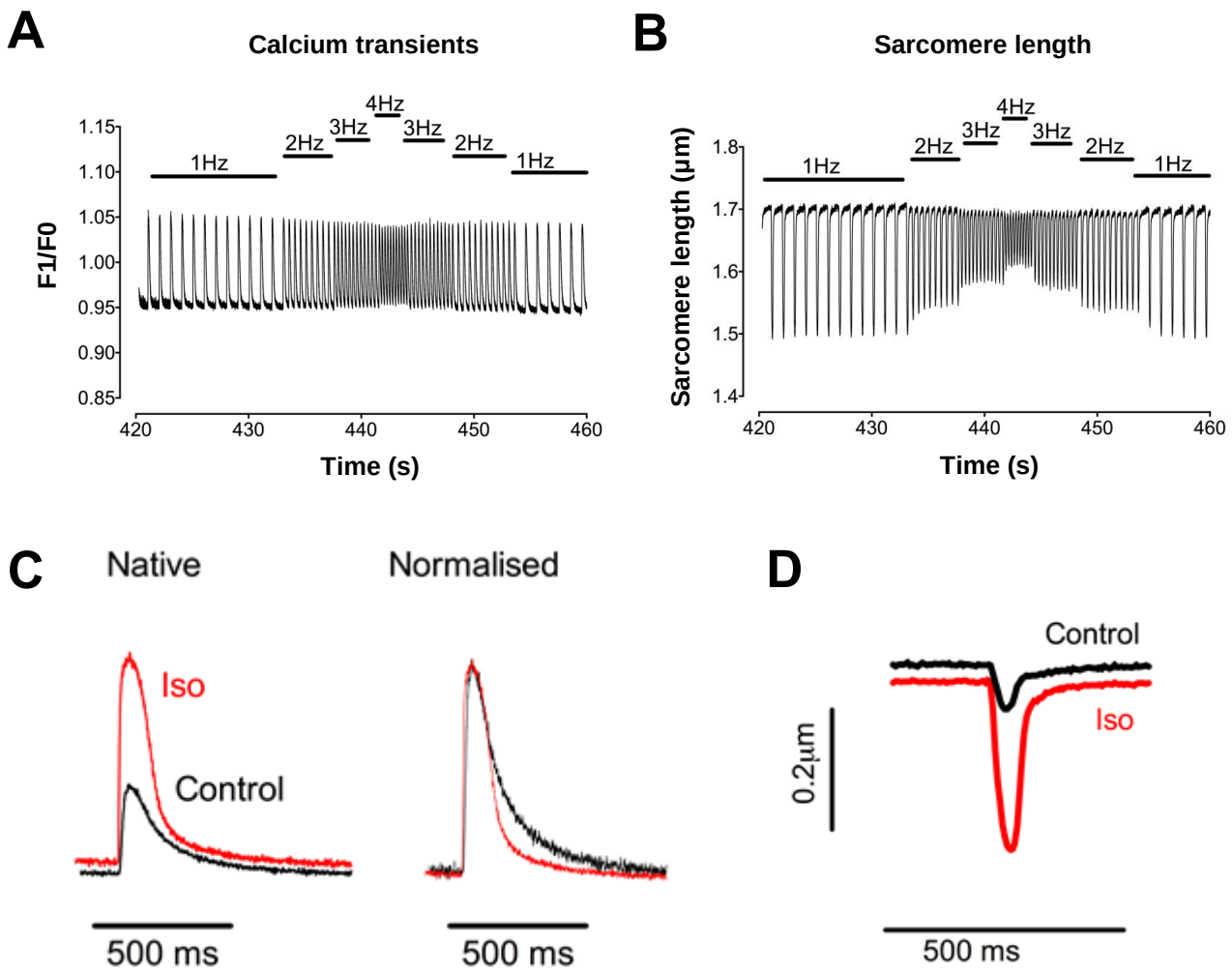
Online Figure II (Cont.) D, Initial testing of various basal media for maintenance of cultured myocytes. Culture media prepared using M199, DMEM, DMEM/F12, M131, or RPMI, all without addition of lipid, were tested for ability to maintain rod shaped myocytes in culture over a period of 4 days. Cell counts relative to day 0 were measured microscopically each day. **E**, Myocytes cultured for 3 days at reduced pH are resistant to remodelling in response to 3 day subsequent incubation in the presence of 10% FBS. **F**, Rod-shaped morphology and reduced remodelling do not equate well to myocyte viability in extended culture at reduced pH. Myocytes were maintained in culture for 7 days at pH 6.7 (top row) or pH 7.4 (bottom row) and stained with ethidium homodimer (EtH; red) dead-cell marker and Hoechst-33342 nuclear counterstain. White arrows indicate rod shaped cells at pH 6.7 staining positive for EtH. Conversely, EtH staining at pH 7.4 was reduced and limited to rounded cells (white asterisks), while numerous cells with rounded/remodelled morphology remained negative for EtH staining (white triangles). **G**, Bar chart representations of myocyte viability as determined by exclusion of EtH dye, after 3 days and 7 days in culture, in a range of pH conditions, with or without specific media supplements as indicated. M199 can be substituted with MEM, the preferred media base of some labs, with little difference under conditions tested. All graphical data show mean ± standard deviation, n=3 independent experiments, except **D** and **G**; n=2 independent experiments in biological triplicate.



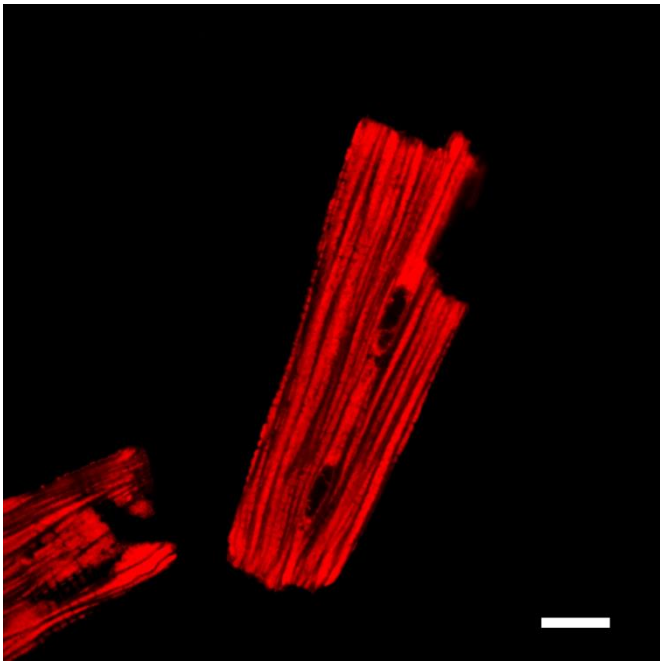
Online Figure III. Isolated myocyte preparations are suitable for biochemical signalling experiments. Myocytes were incubated in the presence of 1 µmol/l norepinephrine (NE), 4 hours after plating, for varying times as indicated. Lysates were subsequently analysed by Western blotting with specific antibodies to detect phosphorylation of protein kinase B (AKT), phospholamban (PL) and extracellular signal-related kinase (ERK). GAPDH was used as an additional loading control.



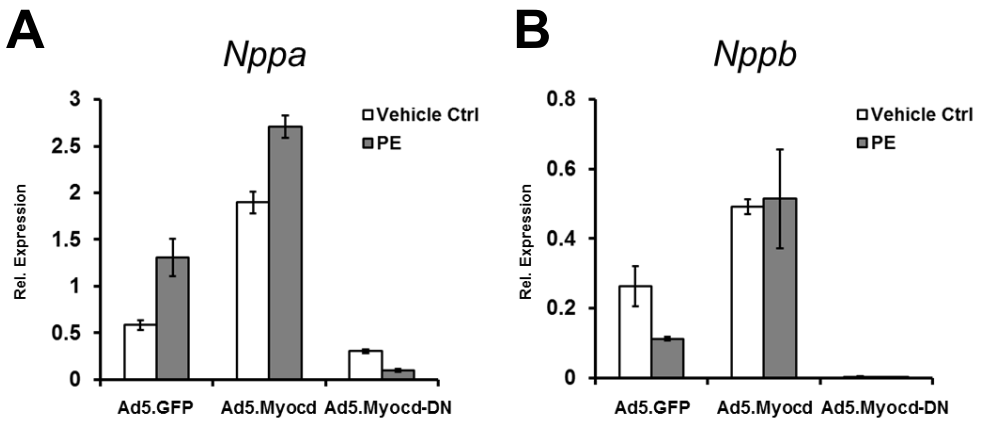
Online Figure IV. Visualisation of spontaneous calcium transients in isolated, plated myocytes. Myocytes were loaded with Fluo-4 AM and imaged. White arrow indicates direction of calcium wave propagation. Scale bars are 10 μm. **E**, Representative quantification of calcium transients in a single cell, before (top) and 5 min after (bottom) addition of 10 μmol/l norepinephrine (NE).



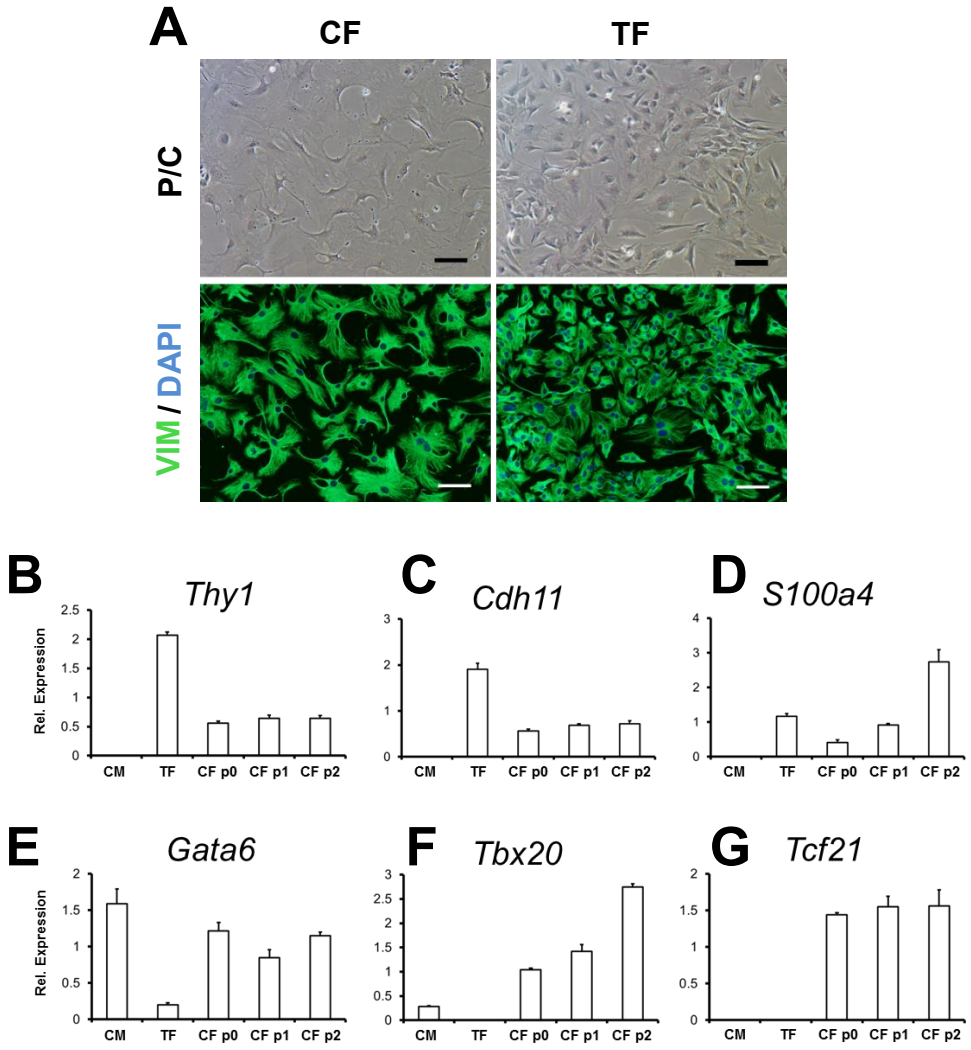
Online Figure V. Isolated cardiomyocytes respond to changes in stimulation frequency and isoproterenol (ISO). Myocytes were loaded with fura2-AM and paced at 2 Hz in the presence or absence of ISO. Calcium transients and sarcomere length shortening were measured in single myocytes using the integrated photometry/contractility system (Ionoptix). Representative traces show changes in (**A**) calcium transients and (**B**) sarcomere length following pacing at varying frequencies. **C**, Representative trace of calcium transient pre- (black) and post- (red) addition of ISO (1 µmol/l). Left panel displays an increase in transient amplitude. Right panel shows that when normalised, transient decay time is faster in the presence of ISO. **D**, Representative raw traces of sarcomere length pre- (black) and post- (red) addition of ISO (1 µmol/l).



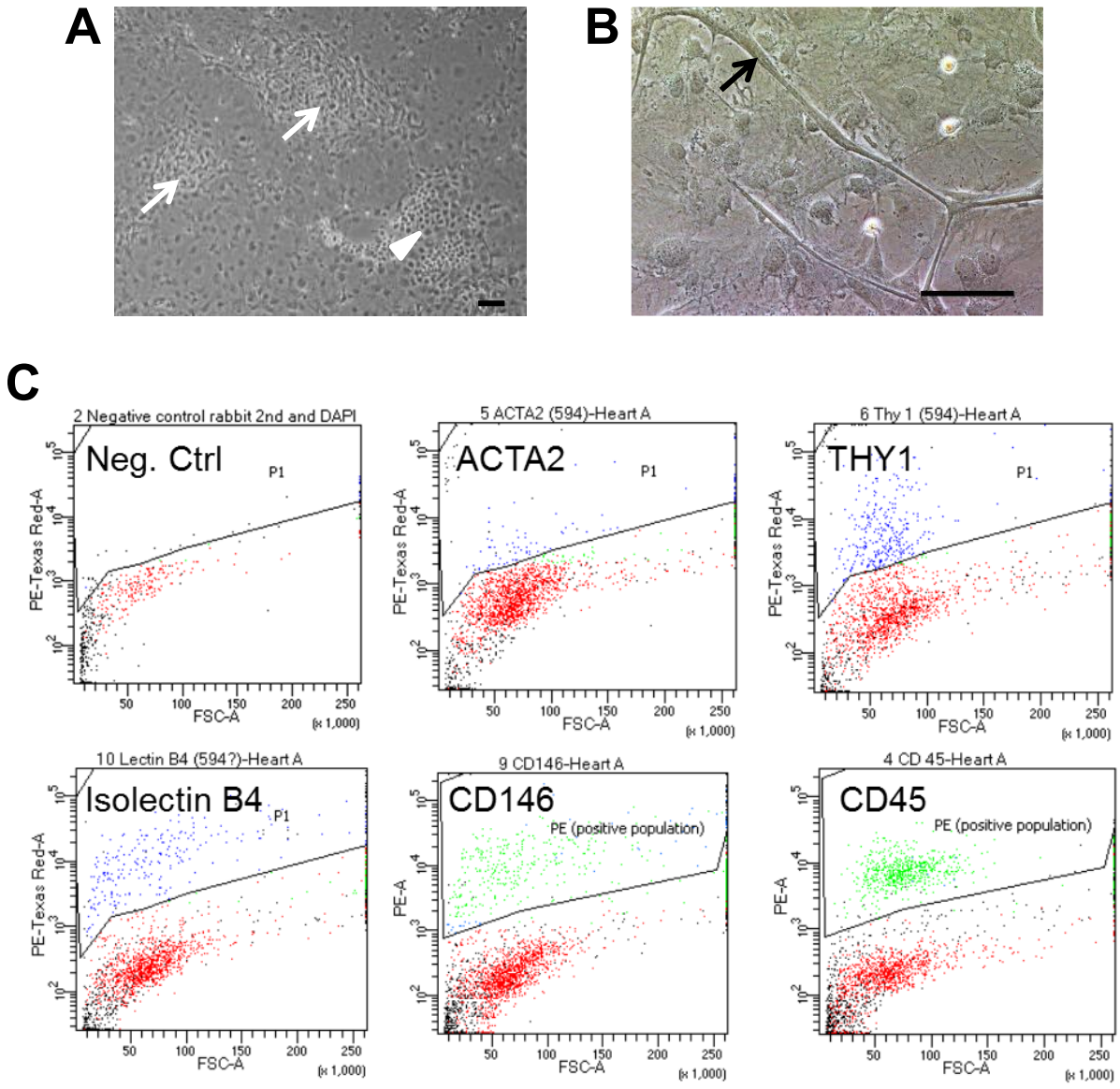
Online Figure VI. Visualisation of active mitochondria in cultured myocytes. Cells were loaded with MitoTracker Red, fixed and imaged using confocal microscopy. Scale bar is 10 μm.



Online Figure VII. Cultured cardiac myocytes are amenable to adenoviral transduction for exogenous gene expression. Cultured cardiac myocytes were transduced with adenovirus as described in *Methods*. Transduction with adenoviral vectors containing the myogenic transcriptional activator myocardin (Ad5.Myocd) caused marked upregulation of known target genes *Nppa* (**A**) and *Nppb* (**B**) compared to transduction with GFP control virus (Ad5.GFP). Conversely, transduction with dominant-negative myocardin constructs (Ad5.Myocd-DN) strongly suppressed their expression. 4 h incubation with 25 μmol/l phenylephrine (PE) stimulated *Nppa* although not *Nppb* expression, and this increase was completely abrogated in the presence of dominant-negative myocardin. Gene expression is relative to *18S* housekeeping control. Data show mean ± standard deviation, n=2 independent experiments in biological triplicate.



Online Figure VIII. Cultured cardiac fibroblasts exhibit characteristic morphological and transcriptional differences when compared to tail fibroblasts. A, Cultured cardiac fibroblasts (CF) and tail fibroblasts (TF) display pronounced morphological differences. Cells were cultured for 3 days, stained using vimentin antibody (VIM, green) and DAPI, and imaged using phase contrast (P/C) and fluorescent microscopy. Scale bars are 100 μm. **B**-**G**, Expression of three selected canonical fibroblast-related genes (**D**-**F**) and cardiogenic-related genes (**G**-**H**) in cultured cardiac myocytes (CM), CF and TF, after 3 days culture, and CF after one (p1) or two (p2) passages in culture. Expression is relative to *18S* housekeeping gene expression. Data show mean ± standard deviation, n=2 independent experiments in biological triplicate.



Online Figure IX. Cultured cardiac fibroblasts represent a heterogeneous population. A, Cardiac fibroblast (CF) cultures contain regions with cells exhibiting dissimilar (white arrows) and sometimes epithelial-like (white triangle) morphologies. **B**, Post-confluent cultures developed distinctive filamentous cellular networks overlying the fibroblast monolayer (black arrow). **C**, Flow cytometry profiles of non-myocyte fraction cells stained for putative cell markers: ACTA2 (smooth muscle), THY1 (cardiac fibroblast), CD146 and GSL-Isolectin-B4 (endothelial), CD45 (immunocyte). Positive populations are located in gate P1. All scale bars are 100 μm.