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NEW METHODS

A Simplified, Langendorff-Free Method for Concomitant Isolation of Viable Cardiac Myocytes and Non-Myocytes from the Adult Mouse Heart

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***Running title*:** Simplified Isolation of Adult Mouse Cardiac Myocytes

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ABSTRACT

***Rationale*:** Cardiovascular disease represents a global pandemic. The advent of and recent advances in mouse genomics, epigenomics and transgenics offer ever greater potential for powerful avenues of research. However, progress is often constrained by unique complexities associated with the isolation of viable myocytes from the adult mouse heart. Current protocols rely on retrograde aortic perfusion using specialised Langendorff apparatus, which poses considerable logistical and technical barriers to researchers, and demands extensive training investment.

***Objective*:** To identify and optimise a convenient, alternative approach, allowing the robust isolation and culture of adult mouse cardiac myocytes using only common surgical and laboratory equipment.

***Methods and Results*:** Cardiac myocytes were isolated with yields comparable to those in published Langendorff-based methods, using direct needle perfusion of the LV ex vivo and without requirement for heparin injection. Isolated myocytes can be cultured antibiotic-free, with retained organised contractile and mitochondrial morphology, transcriptional signatures, calcium handling, responses to hypoxia, neurohormonal stimulation and electrical pacing, and are amenable to patch clamp and adenoviral gene transfer techniques. Furthermore, the methodology permits concurrent isolation, separation and co-culture of myocyte and non-myocyte cardiac populations.

***Conclusions*:** We present a novel, simplified method, demonstrating concomitant isolation of viable cardiac myocytes and non-myocytes from the same adult mouse heart. We anticipate that this new approach will expand and accelerate innovative research in the field of cardiac biology.

Keywords:
Cardiac myocyte, cell isolation, cell culture, cardiac fibroblast, Langendorff.

Nonstandard Abbreviations and Acronyms:

BDM	2,3-butanedione monoxime
BSA	Bovine serum albumen
CF	Cardiac fibroblast
CM	Cardiac myocyte
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle Medium
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
EtH	Ethidium homodimer
FBS	Foetal bovine serum
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
I_{Na}	Sodium current
ISO	Isoproterenol
LV	Left ventricle
M199	Medium 199
NE	Norepinephrine
PBS	Phosphate buffered saline
(Q)PCR	(Real-time, quantitative) polymerase chain reaction
RV	Right ventricle
TAC	Transverse aortic constriction
TF	Tail fibroblast

INTRODUCTION

Cardiovascular disease constitutes a global pandemic[1,2]. Incidence of heart failure is increasing despite improvement in the understanding and management of disease, and prognosis remains poor[3]. Cardiac myocytes (CMs), the contractile cells of the heart, are the traditional focus of extensive research in cardiac biology. CMs coordinate rhythmic beating and integrate multiple hormonal, neural, electrical, mechanical and exosome-mediated signals through a variety of cell-surface and nuclear receptors[4,5]. Physiological adaptive responses may rapidly become pathological. A deeper mechanistic understanding is therefore imperative to the development of novel intervention strategies.

Myocytes in the intact adult myocardium exist in close association with neighbouring cells and extracellular matrix, and are highly sensitive to mechanical perturbations, enzymatic damage, hypoxia, nutrient bioavailability, pH and ionic fluctuations. Mounting of excised hearts on Langendorff apparatus and retrograde aortic perfusion with enzyme-containing buffers was conceived over 45 years ago[6,7] and remains the centrepiece of every modern established protocol to date for the isolation and study of adult rodent CMs[4,8,9,10,11,12,13,14,15,16,17].

However, the necessity for commercial or custom-made apparatus, and considerable expertise therewith, represents a significant financial, logistical and technical barrier for groups wishing to engage in research using isolated adult CMs. Also, Langendorff-based approaches suffer issues with sterility, and typically require pre-injection of animals with anti-coagulants such as heparin, which is detrimental to downstream polymerase chain reaction (PCR)-based analyses[18,19]. Furthermore, mouse CMs are exceptionally delicate, and successful mounting of the small mouse aorta onto Langendorff apparatus is particularly challenging. These issues risk precluding the full potential for cardiac research of recent advances in mouse genomics, epigenomics, transgenics and gene therapy[20,21,22,23].

We present here a novel alternative approach to the challenge of myocyte isolation and culture from the myocardia of adult mouse hearts. We introduce three key modifications to standard protocols. First, hearts are rapidly perfused with a high-EDTA buffer to inhibit contraction, coagulation, and to destabilise extra-cellular connections. Second, pH of buffers is adjusted to an optimal 7.8. Third, all buffers are introduced by intra-ventricular injection, with deep myocardial perfusion via the coronary vasculature induced by clamping of the aorta (Figure 1). The procedure is simple, flexible, does not require heparin injection, may be performed wholly in a sterile laminar flow cabinet and uses surgical tools and equipment found readily in most animal laboratory facilities.

Reproducible yields are achieved in line with published Langendorff procedures of up to 1 million myocytes per left ventricle and 81±6% viable, calcium-tolerant, rod shaped cells. Isolated CMs can be cultured antibiotic-free, retain organised morphology and functionality, and are amenable to patch clamping and adenoviral gene transfer. The procedure is compatible with automated pump infusion systems, and furthermore, permits the concurrent isolation, culture and co-culture of mouse non-myocyte resident cardiac populations, from the same regions, in the same heart. We anticipate that this new approach will expand and accelerate innovative research in the field of cardiac biology.

METHODS

Isolation and culture of cardiac myocytes and non-myocytes from adult mouse heart.
A schematic overview of the myocyte isolation procedure is shown in Figure 2. An expanded description of the procedure, accompanied with images and video, and complete materials list, is available in the Online Data Supplement, alongside full details of additional methods applied in this study (Appendix A-ix). All animal work was undertaken in accordance with Singapore National Advisory Committee for Laboratory Animal Research (NACLAR) guidelines. Relevant national and institutional guidelines and regulations must be consulted before commencement of all animal work.

Buffers and media were prepared as detailed in Appendix D. EDTA, Perfusion, and Collagenase buffers were apportioned into sterile 10 ml syringes, and sterile 27 G hypodermic needles were attached (Online Figure IA).

C57/BL6J mice aged 8-12 weeks were anaesthetised and the chest was opened to expose the heart. Descending aorta was cut and the heart was immediately flushed by injection of 7 ml EDTA buffer into the right ventricle. Ascending aorta was clamped using Reynolds forceps and the heart was transferred to a 60 mm dish containing fresh EDTA buffer. Digestion was achieved by sequential injection of 10 ml EDTA buffer, 3 ml Perfusion buffer and 30-50 ml Collagenase buffer into the left ventricle (LV). Constituent chambers (atria, LV, RV) were then separated and gently pulled into 1 mm pieces using forceps. Cellular dissociation was completed by gentle trituration and enzyme activity was inhibited by addition of 5 ml Stop buffer.

Cell suspension was passed through a 100 μm filter, and cells underwent four sequential rounds of gravity settling, using three intermediate calcium re-introduction buffers to gradually restore calcium concentration to physiological levels. The cell pellet in each round was enriched for myocytes, and ultimately formed a highly pure myocyte fraction, while the supernatant from each round was combined to produce a fraction containing non-myocyte cardiac populations.

Cardiac myocyte yields and % viable rod-shaped cells were quantified using a haemocytometer. Where required, the cardiac myocytes were re-suspended in pre-warmed Plating media and plated at an application-dependent density, onto laminin (5 μg/ml) pre-coated tissue-culture plastic or glass coverslips, in a humidified tissue culture incubator (37°C, 5% CO_2). After 1 h, and every 48 h thereafter, media was changed to fresh, pre-warmed Culture media.

The cardiac non-myocyte fraction was centrifuged (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 LV, in a humidified tissue culture incubator. Media was changed after 24 h and every 48 h thereafter.

RESULTS

Langendorff-free isolation of cardiac myocytes from the adult mouse heart.

Cannulation and retrograde perfusion of the aorta, to force dissociation buffers deep into the myocardium via the coronary vasculature, is the cornerstone of all widely adopted protocols for myocyte isolation from the adult mouse heart. We hypothesised that the same hydrodynamic effect could be achieved in a much simplified manner, by clamping the aorta, and injecting buffers directly into the left ventricle (Figure 1). We further supposed that due to the increased ease and speed of this procedure, pre-injection of mice with anti-coagulants such as heparin could be avoided and replaced by pre-clearing of heart chambers using the divalent cation chelating agent EDTA. Thus, the basis of methodology outlined in (Figure 2) was formed.

For each experiment, crude digestion product was monitored, and total cell number and % viable rod-shaped myocytes were subsequently quantified using a haemocytometer (Figure 3A). Following calcium reintroduction, myocytes remained quiescent and adhered to laminin-coated culture surfaces, displaying characteristic angular morphology and clearly organised sarcomeric striation patterns (Figure 3B and 3C). Optimal rod-shaped yields were most reliably achieved at dissociation buffer pH 7.8, and using a high pre-clearing buffer EDTA concentration of 5 mmol/l (Figure 3D, 3E, and Online Figure IIA and IIB). With these conditions, the protocol reproducibly yields up to 1 million myocytes per LV and 81±6% viable, rod shaped cells. This is in line with yields reported previously in established Langendorff-based protocols[9,10,12,15]. Furthermore, the protocol was found to be compatible with automated infusion pumps for controlled delivery of injected digestion buffers. Flow rates of 1-5 ml/min all produced average yields of over 60% rod shaped cells, demonstrating robustness of methodology, while the highest total cell yields were achieved at 1 ml/min (Online Figure IIC).

Morphology and culture of isolated LV cardiac myocytes.

We next explored the optimal conditions for maintaining viable cardiac myocytes in culture. Several varieties of basal media were initially tested as components of Culture media for their ability to support the preserved morphology of plated LV myocytes over a period of 4 days, and Medium 199 (M199) was selected, in line with some previous reports[14] (Online Figure IID). pH is a variable known to critically influence behaviour of myocytes in culture, with some labs opting for neutral or even slightly acidic pH6.9-7.0[9,10,12]. Experiments were therefore performed to test cell morphology and viability in a carefully controlled pH range of 6.7-7.9, as described in Methods (Online Appendix A-ix). Indeed, we observed that myocytes cultured at reduced pH best retained their rod-shaped morphology and were resistant to remodelling even in the presence of 10% FBS (Online Figure IID). However, we also discovered that while rod-shaped morphology is traditionally equated with cell viability, this is not necessarily the case, particularly after extended time in culture. Application of the nuclear ethidium homodimer (EtH) stain, which is excluded from viable cells, to myocytes after 7 days culture at pH 6.7 revealed that large numbers of rod-shaped cells were in fact non-viable (Online Figure IID). This was not the case for myocytes cultured at pH7.4, and furthermore, many of the cells that had not retained their rod-shape at pH7.4, nonetheless remained viable. We therefore highlight an important distinction between remodelling and viability, and suggest that while culturing at reduced pH can suppress myocyte remodelling and retain differentiated morphological characteristics, such conditions are in fact detrimental to cell viability in long-term culture. With this in consideration, we proceeded to separately quantify both rod shaped morphology and viability over 7 days in culture with a pH range of 6.7-7.9. Indeed, while there was little improvement in cell morphology (Figure 3F), there was a marked preservation of cell viability at pH7.4 beyond 3 days in culture (Figure 3G).

Adult myocytes exhibit a metabolic preference for fatty acid oxidation as an energy source[24]. We therefore tested media supplementation with a defined lipid mixture, and observed further improvement in viability to the extent that 60% of initially adhered myocytes remained viable after 7 days in culture (Figure 3H and 3I). Given concerns that lipid and insulin constituents of Culture medium could interfere with metabolic assays, it is noteworthy that cell viability without either of these additives remained above 40% (Online Figure IIG). Interestingly, insulin supplementation demonstrated clear improvements in viability only in lower pH range cultures. However, inclusion may still elicit functional benefits at pH7.4[25]. Media supplementation with 5 mmol/l taurine, creatine, adenosine and inosine[26,27,28,29] were additionally tested but had little beneficial effect (data not shown).

Progressive, active remodelling of adult cardiac myocytes in culture is well-documented[14,30,31]. Concordantly, the angular morphology and ordered sarcomeric arrangements of plated myocytes remained largely intact after 24 h culture (Figure 3J and Online Video III), but cell edges began to round after 2-3 days, and organised sarcomeric patterning was typically lost by day 7. Continued culture produced cells with distinctive emerging pseudopodia, and beyond 8 days, some cells appeared to re-establish organised contractile apparatus, beat spontaneously, or form contacts with neighbouring cells and contract in synchrony. This phenomenon was accelerated by addition of 10% FBS and removal of 2,3-butanedione monoxime (BDM), and bears resemblance to dated "re-differentiation" techniques[8,32].

Cultured myocytes are intact, retain transcriptional and functional characteristics and are amenable to investigative techniques.

The process of isolating adult cardiac myocytes carries an inherent risk of causing cellular damage, or activation of stress response pathways that could potentially confound in vivo or downstream transcriptional profiles. To test the integrity of the plasma membranes of freshly plated myocytes, a "live/dead" dual viability stain was employed. Myocytes were clearly able to de-esterify and retain calcein AM (green) fluorescent dye, while excluding EtH (red) nuclear stain, demonstrating intact, viable cells (Figure 4A). Peroxide-induced cell death led to loss of calcein retention, and gain of EtH staining.
Pressure overload of adult hearts induces myocyte hypertrophy, which is associated with increased expression of markers including natriuretic peptides ANP (*Nppa*), BNP (*Nppb*), and the skeletal isoform of alpha-actin (*Acta1*)[33]. To examine the conservation of these stress-associated transcriptional signatures, myocytes were isolated from transverse aortic constriction (TAC)-operated mouse hearts, 8 weeks post-operation, alongside sham-operated controls. Yields of viable rod-shaped myocytes dropped to 65±15% from TAC-operated hearts (data not shown), likely due to the pathological hypertrophic phenotype[34], although this is still well within the limits of successful myocyte isolations in established protocols. Despite this, QPCR analysis of myocytes isolated from both left and right ventricles revealed the preservation of increased transcriptional stress signatures in TAC-operated hearts, versus sham-operated controls (Figure 4B).

Transcriptional stress response, calcium handling, contractility, electrical potential and amenability to adenoviral transduction were next tested in freshly plated cells from healthy hearts to confirm applicability of isolated myocytes to scientific investigation. Hypoxic stress was induced by 24 h incubation in a chamber under a controlled nitrogen atmosphere containing 5% CO_2 and 0.2% O_2. Cells were then analysed for expression of hypoxia-responsive genes. Significant increases in expression *Nppa*, *Nppb*, foetal isoform myosin heavy chain (*Myh7*), glucose transporter (*Slc2a1*) and metabolism-related hexokinase (*Hk2*) genes, but not mitochondrial biogenesis related MAX interactor 1 (*Mxi1*), were strongly upregulated following hypoxic exposure[33,35] (Figure 4C).

To demonstrate the suitability of preparations for biochemical signalling experiments, plated myocytes were challenged with varying doses of adrenergic activators norepinephrine (NE) and isoproterenol (ISO). 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). Both stimuli elicited dose-dependent increases in AKT and PL phosphorylation (Figure 4D). AKT phosphorylation was also observed to increase with increased NE incubation time up to 20 min, while PL phosphorylation saturated within 1 min, and ERK exhibited somewhat fluctuating increases in phosphorylation over time (Online Figure III).

Following removal of BDM from Culture medium, plated myocytes exhibited spontaneous calcium transients, which could be visualised using the calcium-sensitive fluorophore Fluo-4 AM. Signals typically emanated from one or less frequently both termini, and moved steadily across the cell longitudinal axis, coinciding with waves of partial contraction (Online Figure IVA and Online Video IVA and IVB). Addition of NE elicited increased rates of spontaneous calcium transient initiation, propagation and cell contraction in individual cells, further indicating intact adrenergic signalling and response mechanisms in plated cells[36] (Online Figure IVB).

Additional experiments were performed to quantify calcium handling properties and adrenergic responses in freshly isolated myocytes. Electrically paced cells exhibited characteristic frequency-dependent changes in calcium handling (Online Figure VA) and sarcomere shortening (Online Figure VB), including reduced sarcomere shortening at higher pacing frequency, as expected[37]. Importantly, myocytes responded to adrenergic stimulation in a dose dependent manner, in accordance with previous studies[38]. Administration of ISO amplified both calcium transients and sarcomere length shortening (Figure 4E). Specifically, increasing doses of ISO stimulated increased calcium transient amplitude (Figure 4F) decreased calcium decay constant (τ) (Figure 4G) and increased sarcomere length shortening (Figure 4H). Representative individual pre- and post- ISO calcium transient and sarcomere length traces are shown in Online Figure VC and VD. A normalised calcium transient trace is shown to highlight the reduced transient decay time in the presence of ISO.

To confirm the amenability of isolated cardiac myocytes to patch clamp studies, sodium current (I_{Na}) was quantified in freshly isolated myocytes. Measurements of whole cell I_{Na} from single myocytes under voltage clamp mode could be readily evoked in all cells tested, with the peak I_{Na} current density measuring -36±3 pA/pF at a test potential of -40 mV. I_{Na} displayed robust voltage dependence with a mean $V_{0.5}$ of -52±1 mV and reverse potential of 8±2 mV (Figure 5A and 5B). These values are consistent with previous reports of rodent ventricular I_{Na} measured under similar conditions[39,40]. The steady state inactivation (availability) of I_{Na} exhibited voltage dependency with a mean $V_{0.5}$ of -86±1 mV (Figure 5C and 5D), again in keeping with previously recorded data[39,40]. Thus, the magnitude of I_{Na} and the activation and inactivation kinetics of I_{Na} are measurably preserved using this isolation technique.

Healthy adult cardiac myocytes contain dense, highly organised networks of mitochondria running parallel to sarcomeres in the cell longitudinal axis, which have key roles in cell bioenergetics, and in injury or disease[24,32,41,42]. To visualise active mitochondria in cultured myocytes, cells were loaded with membrane-potential-dependant MitoTracker Red CMXRos dye, and analysed by confocal microscopy. The characteristic mitochondrial network patterning of healthy myocytes was confirmed in our cells (Online Figure VI).

Experiments using cultured cells often require the manipulation of endogenous or exogenous nucleic acids. Adenoviral vectors are an effective tool for introduction of expression constructs into cardiac myocytes[9,13,14,43]. To test myocyte transduction capability in the current procedure, cells were treated with adenoviral expression constructs encoding the myogenic transcriptional coactivator myocardin (Ad5.Myocd), dominant-negative Myocd-DN (Ad5.Myocd-DN), or GFP control (Ad5.GFP). Myocd-DN encodes a truncated form of myocardin that competes with endogenous myocardin for binding at target gene promoters, but lacks a C-terminal transcription activating domain[44]. Subsequent analysis of gene expression demonstrated that Ad5.Myocd treatment caused significant 4-fold and 2-fold upregulation of

myocardin target genes *Nppa* and *Nppb*[45] respectively, whereas expression was strongly suppressed by treatment with Ad5.Myocd-DN. Phenylephrine treatment further increased *Nppa* although not *Nppb* expression in control and Ad5.Myocd-treated myocytes, but this increase was abrogated in Ad5.Myocd-DN-treated myocytes (Online Figure VII). Thus, the current protocol is well-suited for studies involving adenoviral-mediated gene transfer, expression and responses in cultured adult mouse cardiac myocytes.

Concurrent isolation and culture of cardiac myocytes and fibroblasts from a single mouse heart.

The mammalian adult heart contains substantial populations of non-myocyte cells, with emerging roles in cardiac physiology, pathology and regenerative capacity[46,47,48]. Cardiac fibroblasts (CF) represent a sizeable albeit ill-defined population, with critical functions during health and disease[49,50]. There are currently no peer-reviewed published protocols describing the concomitant isolation, culture and study of myocytes and fibroblasts from the same adult mouse heart.

Traditional protocols for CF culture involve a simple enzymatic digestion of the heart, centrifugation of crude product and plating in serum-containing media[50]. We set out to test whether CFs could be cultured in a similar manner from the current non-myocyte supernatant fractions of LV digestion products. For comparison, fibroblasts were isolated from mouse tails (TF) in parallel.

Cells were observed to attach and proliferate to near confluency within 4-5 days, and were confirmed positive for the fibroblast marker vimentin (VIM) by immunocytochemical staining. The absence of adhered CMs was confirmed by negative TNNT staining, and conversely, absence of contaminating fibroblasts in plated CM samples was confirmed by negative VIM staining, indicating complete separation of the two cellular fractions (Figure 6A).

Cultured cardiac fibroblasts recapitulate characteristics of cardiac fibroblasts from traditional protocols.

Cultured CFs displayed extensive morphological differences when compared to TFs, with increased cell spreading, cytoplasmic protrusions and a distinctive asymmetrical "looping" shape that were particularly pronounced at sub-confluent cell densities (Online Figure VIIIA). It has been reported that CFs express a cardiogenic transcriptional network[50]. Accordingly, freshly cultured CMs, TFs, and CFs (p0), and CFs after 1 (p1) and 2 (p2) passages, were harvested for analysis of gene expression. QPCR data is summarised graphically as a heat-map in Figure 6B. Expression of three selected canonical fibroblast and cardiogenic genes are also represented in standard format (Online Figure VIIIB-VIIIG). Detection of CM-associated genes was largely limited to myocytes. Freshly isolated CFs tended to express lower levels of canonical fibroblast marks than TFs, other than collagen synthesis genes *Col1a1* and *Col1a2*. Conversely, CFs showed markedly higher expression of cardiogenic-associated genes than TFs, often higher also than CMs. These results are consistent with previous findings[55], and pave the way for easily achievable co-culture experiments to study cell-cell interactions in vitro (Figure 6C). Furthermore, the cultured CFs were able to activate a transition to myofibroblasts, marked by increased production of smooth muscle alpha actin (ACTA2) in response to transforming growth factor beta (Tgfb) stimulation (Figure 6D), and this potential was retained for at least two passages (Figure 6E). Therefore, evidence supports the utility of the current protocol for the isolation, culture and study of cardiac fibroblasts, in addition to cardiac myocytes.

Isolated cardiac non-myocytes represent a heterogeneous population.

While cultured CF reliably recapitulated characteristic observations from previous studies, close visual inspection revealed areas within cultures displaying unique morphological features (Online Figure IXA), sometimes resembling endothelial networks (Online Figure IXB). Analysis of the non-myocyte-containing fraction by flow cytometry using specific antibodies confirmed the presence of smooth muscle

cells (ACTA2+), fibroblasts (THY1+), endothelial cells (CD146+, or positive for *Griffonia simplicifolia* isolectin-B4 staining), and immune-related cells (CD45+) (Figure 7A, and Online Figure IXC).

Plated cultures were tested for the presence of endothelial cells by immunocytochemical staining against CD31. In sub-confluent cultures, positive staining was detected, but limited to small, infrequent clusters (Figure 7B). However, in post-confluent cultures, CD31-positive cells marked the distinctive networks observed previously, which stained strongly for actin and negative for the fibroblast marker vimentin, leading to their positive identification as endothelial cell networks (Figure 7C and 7D). Although rarely discussed in literature, it seems likely that cardiac fibroblasts obtained by traditional methods would comprise a similarly heterogeneous population, which may have passed undetected, particularly when limiting studies to sub-confluent cultures[50]. However, the identification of such cells raises the tantalising prospect for utilisation of the current protocol in simplified concurrent isolation of not only myocytes and fibroblasts, but also endothelial cells, and the potential array of diverse cardiac-resident non-myocyte populations that continue to be investigated and discovered.

DISCUSSION

Isolated adult CMs have proven an ideal model for valuable insights into diverse aspects of cardiac physiology and pathobiology, from contractility, calcium handling and electrophysiology, to signalling, bioenergetics, drug testing, single cell transcriptomics and apoptosis[16,24,51,52,53,54,55,56]. However, progress using current protocols is often constrained by technical and logistical difficulties associated with the Langendorff-based isolation and maintenance of high yields of viable adult CMs. In this report, we present a novel, convenient approach to isolate viable, calcium-tolerant myocytes from the adult mouse heart, using only standard surgical tools and equipment, and without the prerequisite of heparinisation. Yields of total and viable myocytes are consistent with and sometimes exceed those reported in previous Langendorff-based procedures[9,10,12,15]. Furthermore, we demonstrate the concomitant isolation and culture of myocytes and non-myocytes from the same mouse heart.

The described protocol builds upon decades of international research, with buffer recipes and culture techniques adapted from work in a number of excellent papers and reviews[4,8,9,10,11,12,14,15]. We introduce three key modifications to standard protocols: pre-perfusion with high-EDTA buffer, pH correction, and most notably, simple intra-ventricular injection of all dissociation buffers.

The divalent cation-chelator EDTA was first tested as a substitute for heparinisation, amid some concern that EDTA may be damaging to myocytes[57,58]. However, a common theme of adult CM isolation procedures is the importance of initial perfusion using calcium-free buffers[59], with some reports indeed utilising low micro-molar concentrations of EDTA, or the higher-calcium-affinity analogue ethylene glycol tetraacetic acid (EGTA)[8,13,30,55]. It appears that initial chelation of divalent cations using EDTA may impart multiple benefits, including the inhibition of blood coagulation, inhibition of CM cell contractions, and loosening of intercellular connections[7,60].

Previous CM isolation methods adopt a physiological pH of between 7.0-7.4 for dissociation buffers, generally without presenting supporting evidence. It is unclear why a pH of 7.8 appears optimal for the current protocol. Possibly, higher pH off-sets acidification in cases of myocardial lactate production, increases EDTA affinity for divalent cations, or, interestingly, improves glucose utilisation via increased phosphofructokinase activity, which functions at an optimum pH of 8.0[61].

Introduction of dissociation buffers by intra-ventricular injection negates the requirement for Langendorff perfusion apparatus, simplifies, and easily facilitates the option for conducting the entire

procedure in a sterile laminar flow cabinet. The heart is perfused immediately at euthanasia, and precise identification of the mouse aorta is not necessary for clamp application, substantially reducing the potential for errors, blood coagulation, ischaemia and incorrect mounting of the heart, encountered when using traditional retrograde perfusion techniques. Some previous protocols emphasise the critical importance of maintaining the perfused heart at 37°C[55]. Digestion is certainly faster at 37°C, but we do not find temperature to be a key variable affecting the number or viability of isolated myocytes, and have successfully conducted isolation procedures at room temperature. The protocol is compatible with automated pump injection systems, for precise control of buffer pressure or flow rates. However, standard injection using disposable syringes is sufficient, and may help ensure sterility and a higher degree of control over perfusion for individual hearts, given that marked biological variability occurs between mice even within littermate groups. Sterile procedure and antibiotic-free culture may be particularly useful for studies involving CM calcium handling or electrophysiology, given the influence of streptomycin and analogues on certain ion channel functions[62].

Cultured myocytes retained characteristic morphology, transcriptional signatures and functionality, with organised sarcomeric contractile apparatus and mitochondrial networks, calcium handling, responses to neurohormonal, electrical and hypoxic stimuli, and amenability to patch clamping and adenoviral-mediated gene transfer. Progressive remodelling was observed with extended periods in culture, as noted in previous reports[14,30,31]. Various strategies to prevent or decelerate this process have been proposed, including addition of the bioactive molecules N-benzyl-p-toluene sulphonamide (BTS)[63], blebbistatin[64] or cytochalasin D[30,65]. BTS and blebbistatin are also suggested as specific myosin II ATPase inhibitors during myocyte culture to replace BDM, which has received some criticism for off-target bioenergetic, phosphatase and calcium regulatory effects[64,66,67,68]. Although not tested here, such compounds may easily be incorporated into the current protocol for application-specific purposes.

Non-myocyte cardiac populations are rapidly gaining recognition as key participants in heart biology and pathophysiology[46,47,48]. Cardiac fibroblasts were successfully cultured from the non-myocyte fraction of ventricular digestion product and closely recapitulated previously reported morphology and cardiogenic-like transcriptional profiles[50]. The presence of endothelial populations was also detected in cultures. It is unclear whether such populations exist in the fibroblast cultures of other reports. It is also likely possible, and through personal communication a practice in some labs, to equally isolate non-myocytes in the same manner from adult mouse heart digestion products after Langendorff-based protocols, although this is not to our knowledge published in any peer-reviewed literature. In either case, the current report raises the exciting prospect of simplified, simultaneous isolation and profiling of a range of non-myocyte populations, alongside viable cardiac myocytes, from the same regions, in the same mouse heart. Taken together, the described method offers a novel, convenient approach to the isolation and study of mouse cardiac myocytes, removing technical and logistical obstacles posed by previous Langendorff-based techniques and opening the door to new, critical and exciting research into both myocyte and non-myocyte populations in the adult mouse heart.

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DISCLOSURES
None.

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FIGURE LEGENDS

Figure 1. A new method to isolate cardiac myocytes from the adult mouse heart. Schematic diagrams illustrating the principle of the approach, in which traditional Langendorff-based retrograde aortic perfusion is replaced by simple injection of dissociation buffers into the left ventricle (**A**). Application of a haemostatic aortic clamp forces passage of buffers (blue arrows; **B**) through the coronary circulation (red), ensuring deep perfusion of the myocardium.

Figure 2. Summary of the cardiac myocyte isolation protocol. A detailed, extended description of methodology, with images and video, is available in the Online Data Supplement.

Figure 3. Protocol optimisation and isolation of high yields of viable cardiac myocytes. A-C, Representative images of adult mouse left ventricular digestion products, before plating (**A**), and after 1 h culture (**B** and **C**), showing yields of 80% rod-shaped, viable myocytes, with organised sarcomeric striations. Scale bars are 100 μm. **D**, Optimisation of dissociation buffer pH. Highest viable yields were obtained at pH 7.8. EDTA concentration was 1 mmol/l. **E**, Optimisation of EDTA concentration. Highest yields were obtained at 5 mmol/l EDTA. Buffer pH was 7.8. Data show mean ± standard deviation (s.d), n=3 independent experiments. **F-I**, Quantification of myocyte rod-shape morphology (**F** and **H**) and viability (exclusion of ethidium homodimer stain, **G** and **I**) over a timecourse of 7 days in culture at specified pH range (**F** and **G**) and with or without lipid supplementation at optimal pH 7.4 (**H** and **I**), as indicated. Data show mean ± s.d, n=2 independent experiments in biological triplicate. **J**, Immunological staining and confocal imaging of myocytes with sarcomeric-alpha-actinin antibody (ACTN2; green) and DAPI, after increasing time in culture, as indicated. Loss of sarcomeric organisation was observed after 8 days culture. 10% FBS was included in cultures from day 8 onwards, and BDM removed. Extended culture resulted in re-establishment of sarcomeric structures, formation of cell-cell contacts and synchronised, spontaneous contractility. Scale bars are 50 μm.

Figure 4. Cultured cardiac myocytes retain transcriptional and functional characteristics, and are amenable to investigation. A, Isolated myocytes are viable with intact plasma membranes. Freshly plated myocytes retain calcein (green) but exclude ethidium (EtH, red). Addition of 10 μmol/l butyl-peroxide (TBH) led to calcein escape and entry of EtH. Nuclear counterstain, Hoechst-33342. Scale bars are 100 μm. **B**, Myocytes from both ventricles retain characteristic transcriptional signatures after isolation from a pressure-overload model of hypertrophy (TAC), compared to sham-operated controls. Expression was quantified by QPCR, relative to *18S*. Data show mean ± s.d, n=3 mice per group. *$P<0.05$, **$P<0.01$, Student's t-test. **C**, Hypoxia-regulated genes *Nppa*, *Nppa, Myh7, Slc2a1* and *Hk2,* but not *Mxi1*, were significantly upregulated in cultured myocytes after 24 h hypoxic exposure. Data show mean ± s.d, n=3 independent experiments, expression relative to *18S*. **$P<0.01$, ***$P<0.001$, Student's t-test. *C***D-H**, Myocytes are responsive to adrenergic stimulation in a dose-dependent manner. **D**, Western blot to demonstrate phosphorylation of AKT and phospholamban (PL), 20 min after addition of norepinephrine (NE) or isoproterenol (ISO), as indicated. **E-H**, Myocytes were loaded with fura2-AM and paced at 2 Hz in the presence of ISO as indicated. Calcium transients and sarcomere length shortening were measured using the integrated photometry/contractility system (Ionoptix). **E**, Representative raw traces of calcium transients (upper panel) and sarcomere length (lower panel) recorded from a single cell, ISO added as indicated. Calcium transient amplitude (**F**), calcium transient decay (□□□**G**□, and % sarcomere length (SL) shortening (**H**) were subsequently quantified in response to ISO addition as indicated. Data show mean ± standard error, n≥9 cells from 3 hearts, *$P<0.05$, one way ANOVA followed by Dunnett's multiple comparisons test.

Figure 5. Isolated myocytes display normal sodium currents (I_{Na}). I_{Na} were measured in freshly isolated left ventricular cardiac myocytes. **A**, Representative voltage-dependent I_{Na} raw traces recorded

from a single ventricular cardiac myocyte. The voltage protocol is shown in the inset. **B,** Mean data for current-voltage relationship of I_{Na} current density (pA/pF; n=8 cells from 3 hearts). **C,** Representative raw traces showing voltage-dependent steady-state I_{Na} inactivation. The voltage protocol is shown in the inset. **D,** Mean data for I_{Na} inactivation curve (n=8 cells from 3 hearts).

Figure 6. Concomitant culture and study of cardiac myocytes and fibroblasts from the same mouse heart. **A**, Immunological staining of isolated cardiac myocytes (CM) and cardiac fibroblasts (CF) with cardiac troponin-T antibody (TNNT2, red), vimentin antibody (VIM, green), and DAPI, after 3 days culture. Specific staining demonstrates strong separation of myocyte and non-myocyte fractions. **B**, Transcriptional analysis of cultured CM, tail fibroblasts (TF) and CF after 3 days culture, and CF after one (p1) or two (p2) passages in culture. Expression of selected cardiac myocyte-related (CM), canonical fibroblast-related and cardiogenic-related genes was determined by QPCR, relative to *18S*, and presented in heat-map format. Data represent mean expression, n=2 independent experiments in biological triplicate. **C**, Co-culture of cardiac myocytes and fibroblasts from the same mouse heart. Cell fractions were isolated, recombined after 3 days separate culture, and maintained for 4 further days in the presence of 10% FBS. Cells were fixed and co-stained with antibodies against sarcomeric-alpha-actinin (ACTN2, green, CM) and vimentin (VIM, red, CF), and DAPI. **D**, Activation of isolated CFs following 24 h incubation with 10 ng/ml transforming growth factor beta (Tgfb) was detected by immunological staining for smooth muscle alpha-actin (ACTA2) production. **E**, The potential for activation of isolated CFs with Tgfb persisted for at least two passages. Data show mean ± s.d, n=2 independent experiments in biological triplicate, *Acta2* expression relative to *18S*. ***P<0.01*, ****P<0.001*, Student's t-test, compared to relevant unstimulated controls.

Figure 7. Isolated cardiac non-myocytes represent a heterogeneous population. **A**, Relative proportions of non-myocyte-fraction cells detected positive for putative identity markers: ACTA2 (smooth muscle), THY1 (cardiac fibroblast), CD146 and GSL-Isolectin-B4 (endothelial), CD45 (immunocyte), by flow cytometry. Data averaged from 3 independent experiments. **B**, Immunological staining of CD31 (PECAM-1) in sub-confluent non-myocyte fraction cultures marked clusters of endothelial-like cells (green). **C**-**D**, In post-confluent cultures, these cells were observed to form CD31-positive (green), actin-rich (stained using fluorophore-conjugated phalloidin; red) networks (**C**), that stained negative for the fibroblast marker vimentin (VIM; green; **D**). All scale bars are 100 μm.

Novelty and Significance

What Is Known?

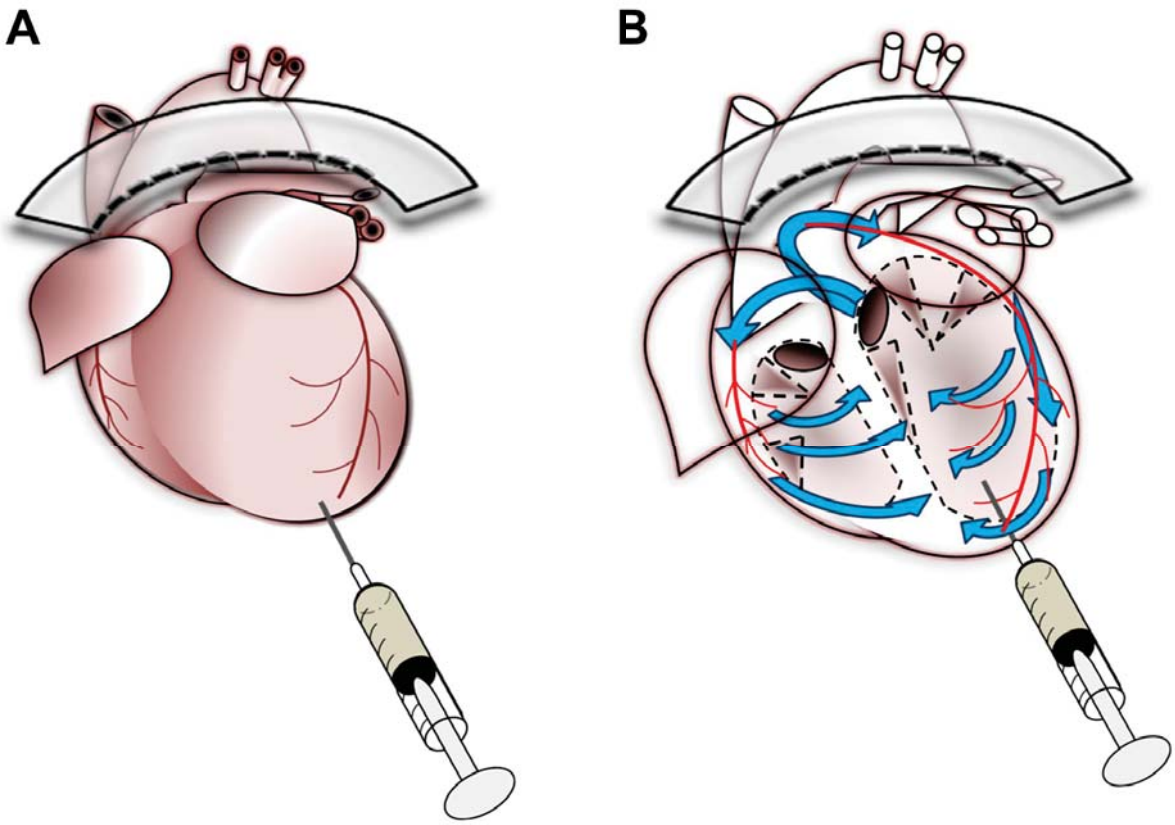
- Isolation of healthy, intact cardiac myocytes from the mouse heart is challenging and a barrier to progress in cardiac research.
- Current established protocols rely on the use of Langendorff apparatus, which requires considerable technical expertise.

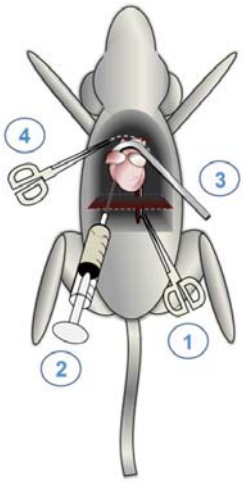
What New Information Does This Article Contribute?

- We describe a convenient, alternative approach, using direct needle perfusion of the LV ex vivo, allowing the robust isolation and culture of adult mouse cardiac myocytes using only common surgical and laboratory equipment.
- Myocytes are isolated with yields, viability and functionality comparable to those in published Langendorff-based methods.
- The technique also permits concurrent isolation, separation and co-culture of non-myocyte cardiac cell populations.

Progress in cardiac research is hampered by unique complexities associated with the isolation of viable myocytes from the adult mouse heart. Current protocols rely on reverse aortic perfusion using specialised Langendorff apparatus, which poses considerable logistical and technical barriers to researchers, and demands extensive training. We therefore sought to validate an alternative, simplified approach. Our protocol achieves yields of myocytes comparable to those in published Langendorff-based methods, by direct needle perfusion of the LV ex vivo, using only common surgical and laboratory equipment. Isolated myocytes are viable, functional, and amenable to a full range of investigative techniques. Furthermore, the methodology permits concurrent isolation, separation and co-culture of myocyte and non-myocyte cardiac populations, including fibroblasts and endothelial cells. We anticipate that this new approach will expand and accelerate innovative research in the field of cardiac biology.

FIGURE 1





Chest cavity of anaesthetised mouse is opened to below diaphragm (red) to fully expose heart.

(1) Descending aorta and inferior vena cava are cut.
(2) 7 ml EDTA buffer is injected into apex of right ventricle.
(3) Lahey forceps reach behind heart to clamp aorta.
(4) Heart is removed by cutting behind clamp.

Clamped heart is submerged in 60 mm dish of EDTA buffer. 10 ml EDTA buffer is injected into apex of left ventricle (LV).

Heart is transferred to dish of perfusion buffer. 3 ml perfusion buffer is injected into apex of LV.

Heart is transferred to dish of collagenase buffer. 30-40 ml collagenase buffer injected into apex of LV, until digestion is apparent.

Clamp is removed. Heart may be separated into respective chambers as desired. Tissue is then pulled gently into ~1mm^3 pieces using forceps, and dissociated by gentle pipetting.

Stop buffer is added. Cell suspension is passed through 100 µm strainer and myocytes gravity settle for 20 min.

Supernatant containing non-myocyte cells, debris and extracellular matrix is collected. Myocytes then undergo two further rounds of gravity settling to achieve a pure population that may be harvested, applied in acute studies, or plated for in vitro culture.

Similarly, supernatant fractions are combined and centrifuged to isolate non-myocyte populations.

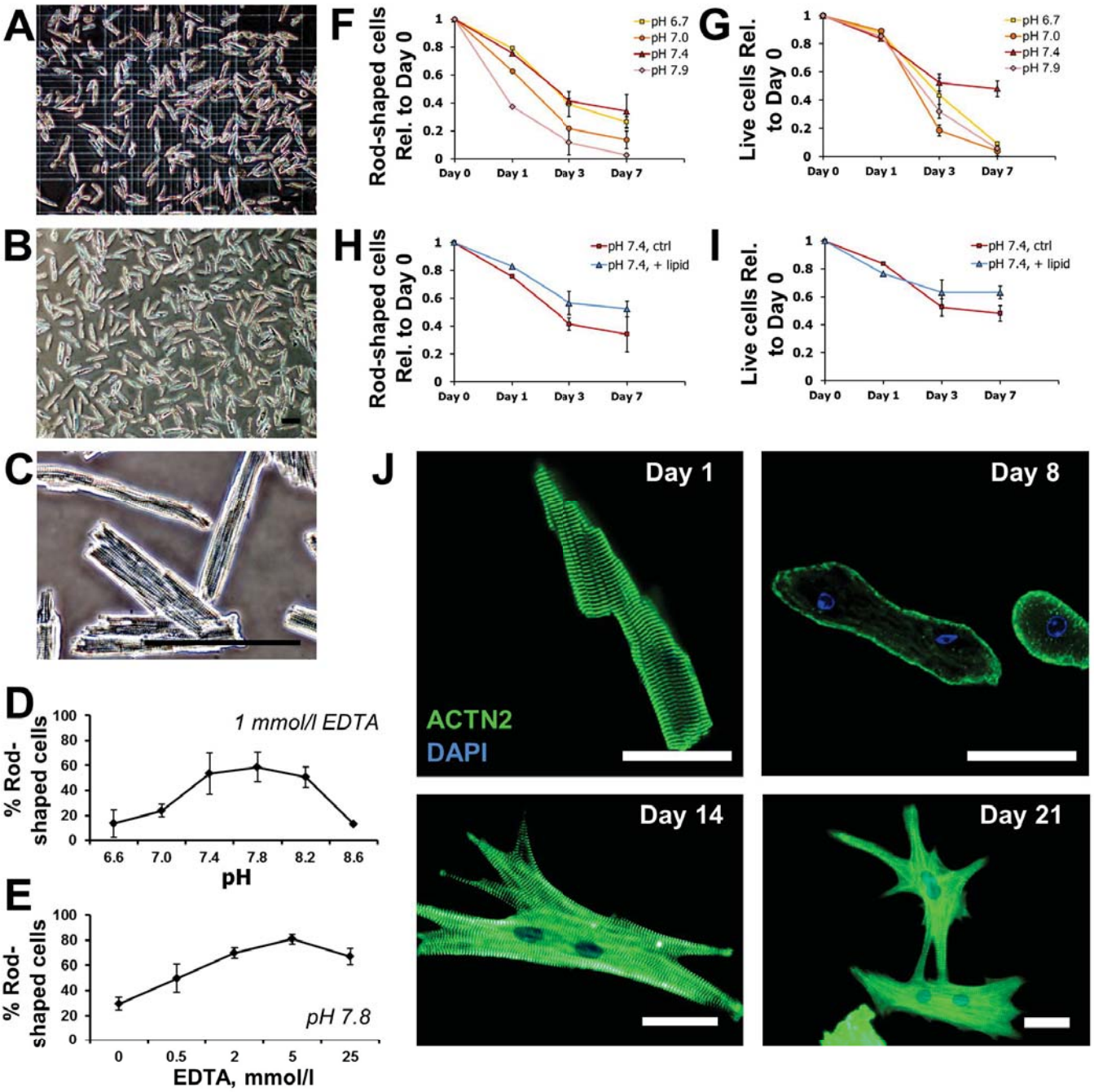
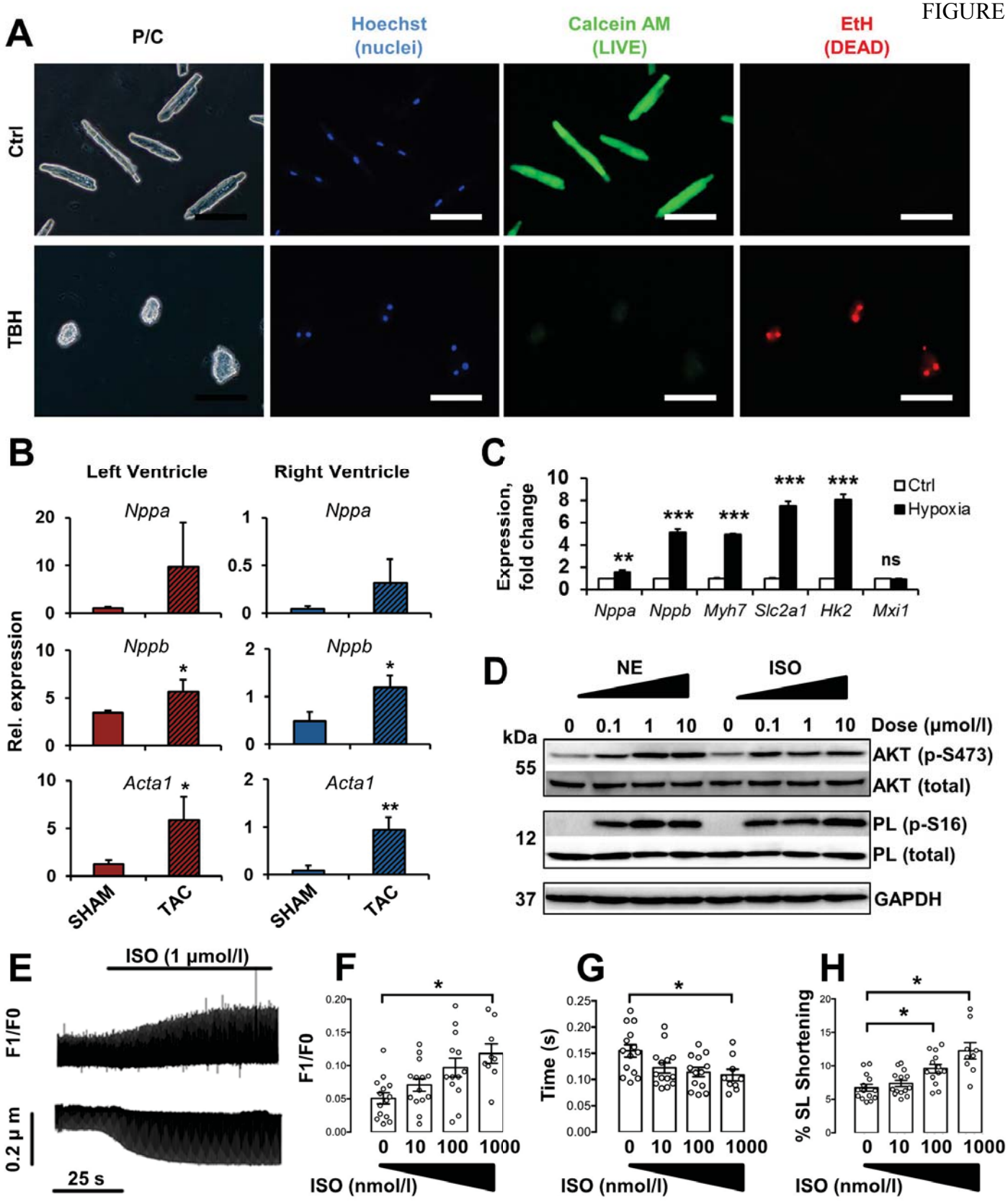


FIGURE 4



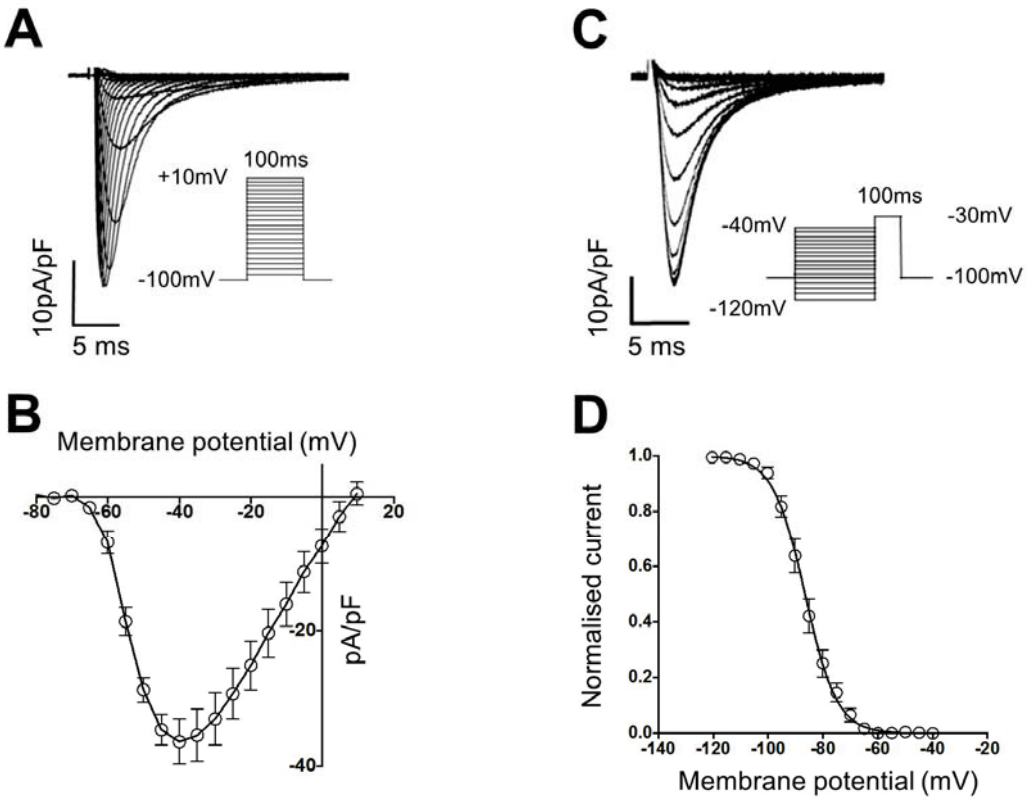
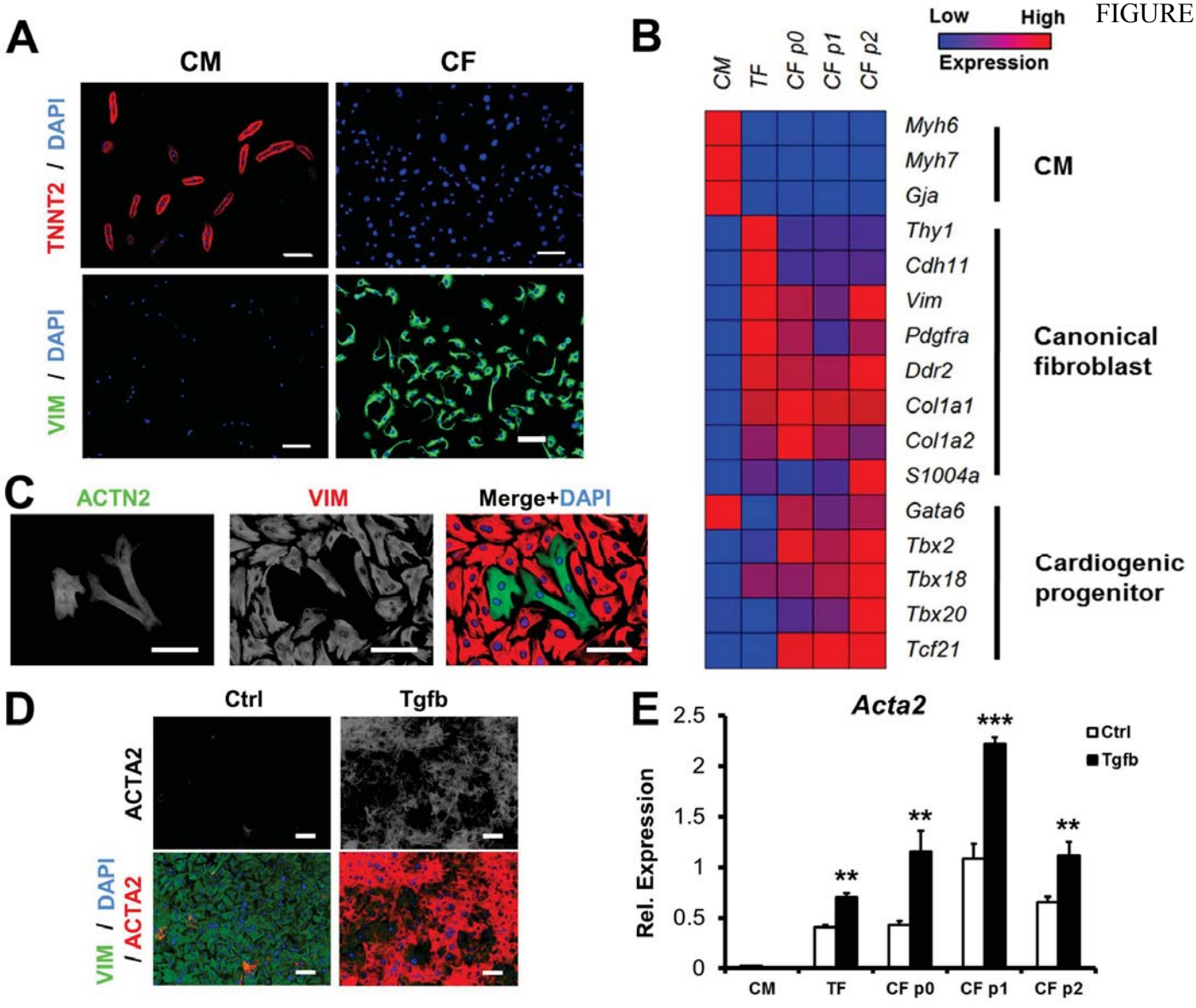
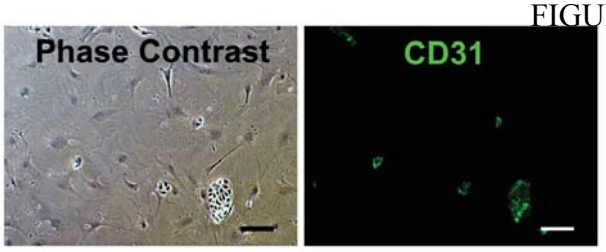


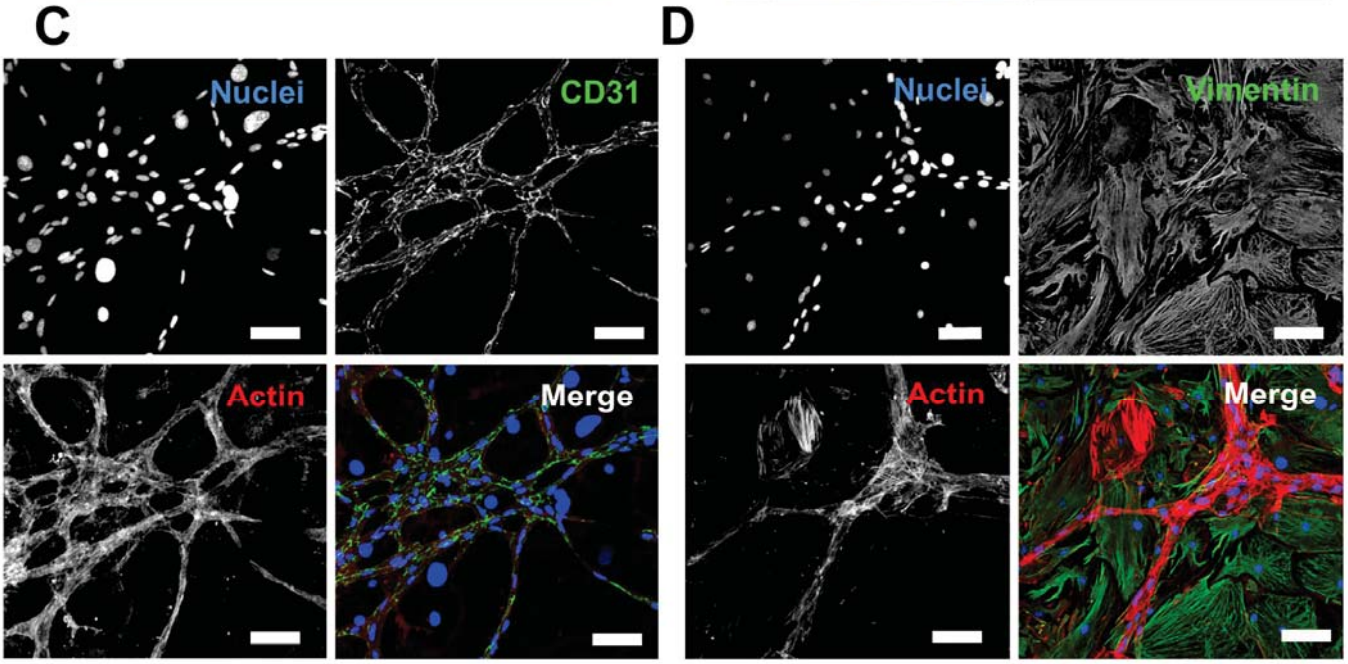
FIGURE 6



A

Cell Mark	% Positive count	Std. Dev.
ACTA2	*7.5*	*+/- 3.5%*
THY1	*20.7*	*+/- 3.8%*
[Isolectin B4]	*21.0*	*+/- 4.7%*
CD146	*21.4*	*+/- 1.9%*
CD45	*26.8*	*+/- 7.9%*





Circulation Research
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A Simplified, Langendorff-Free Method for Concomitant Isolation of Viable Cardiac Myocytes and Non-Myocytes from the Adult Mouse Heart

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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.

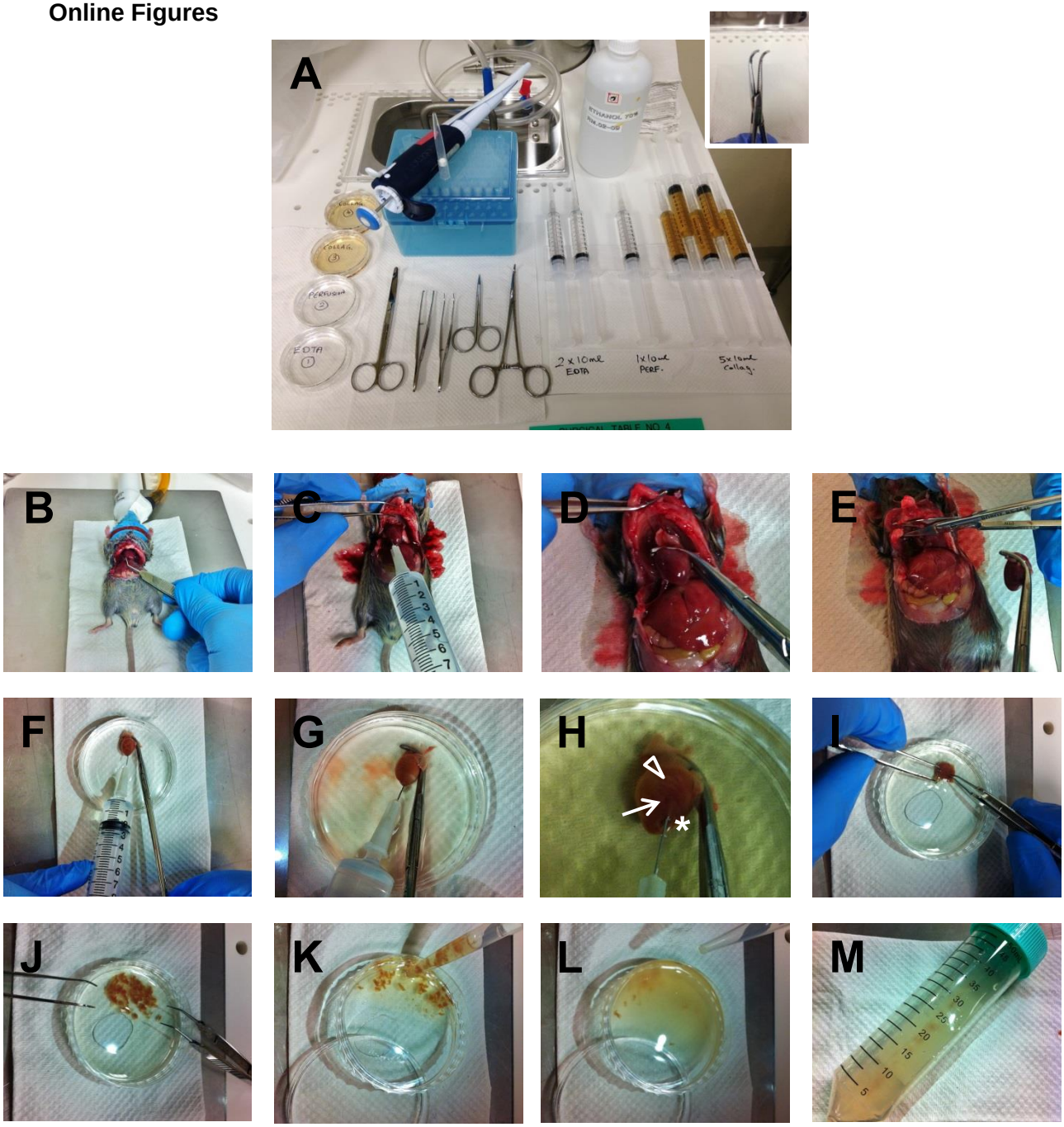
Online video III

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.

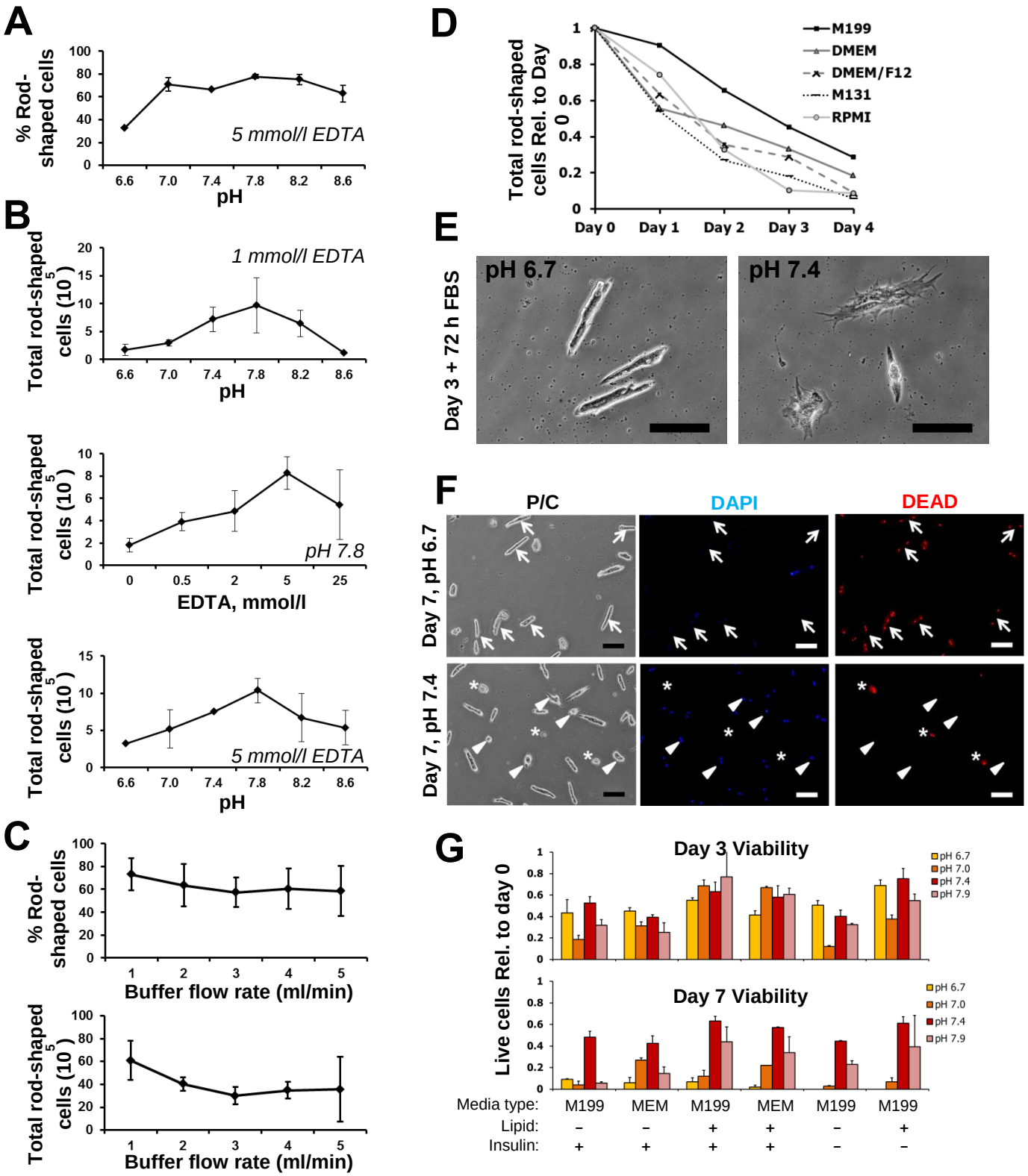
Online video IVA and IVB

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

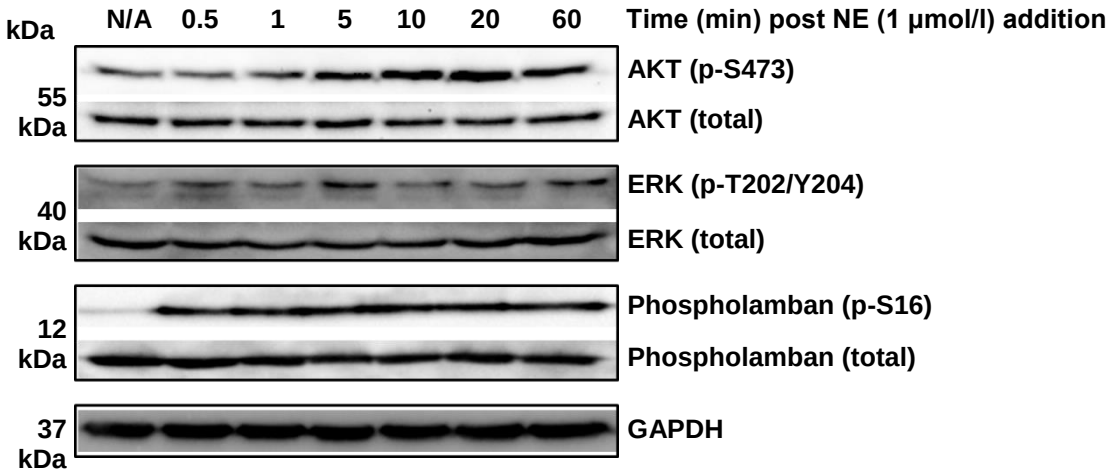


Online Figure I. Photographic images of the cardiac myocyte isolation procedure. A, The entire procedure requires only equipment and surgical instruments found readily in most animal surgical facilities. Inset image shows our preferred choice of full-curved-ended Reynolds haemostatic forceps to clamp 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.

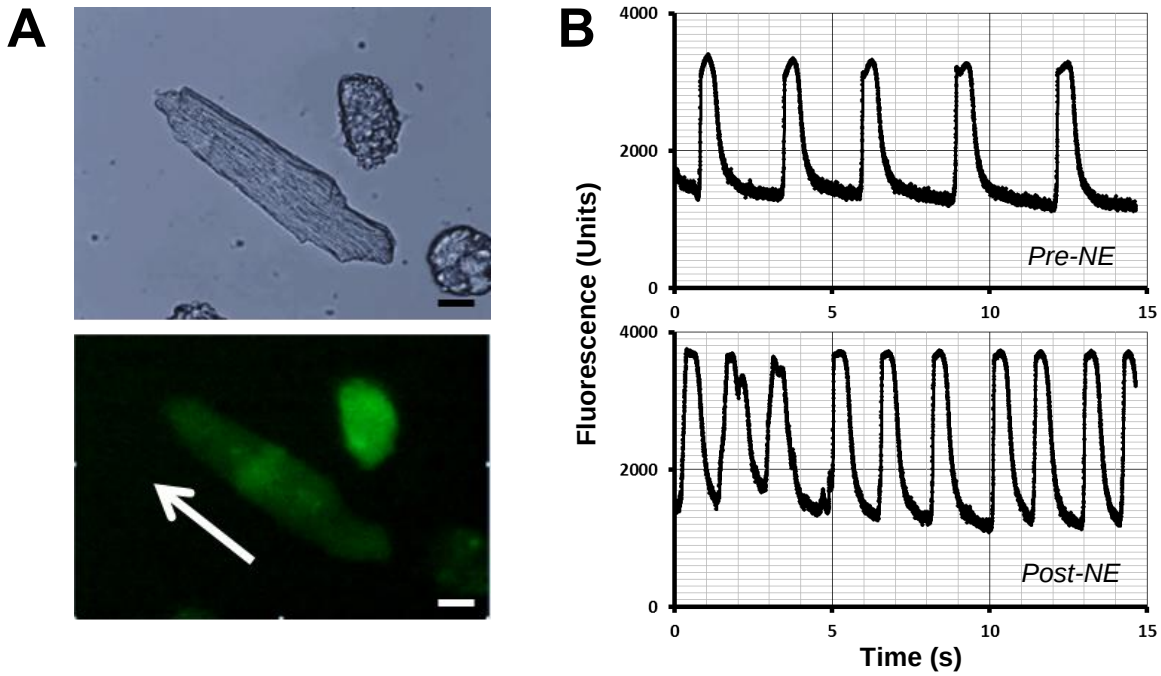


Online Figure II. Protocol optimisation and compatibility with automation. A, Confirmation that pH 7.8 remains optimal for isolation of rod-shaped myocytes, when using 5 mmol/l EDTA in EDTA buffer. **B**, Conditions producing the highest proportions of viable rod-shaped cardiac myocytes correspond to 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.*

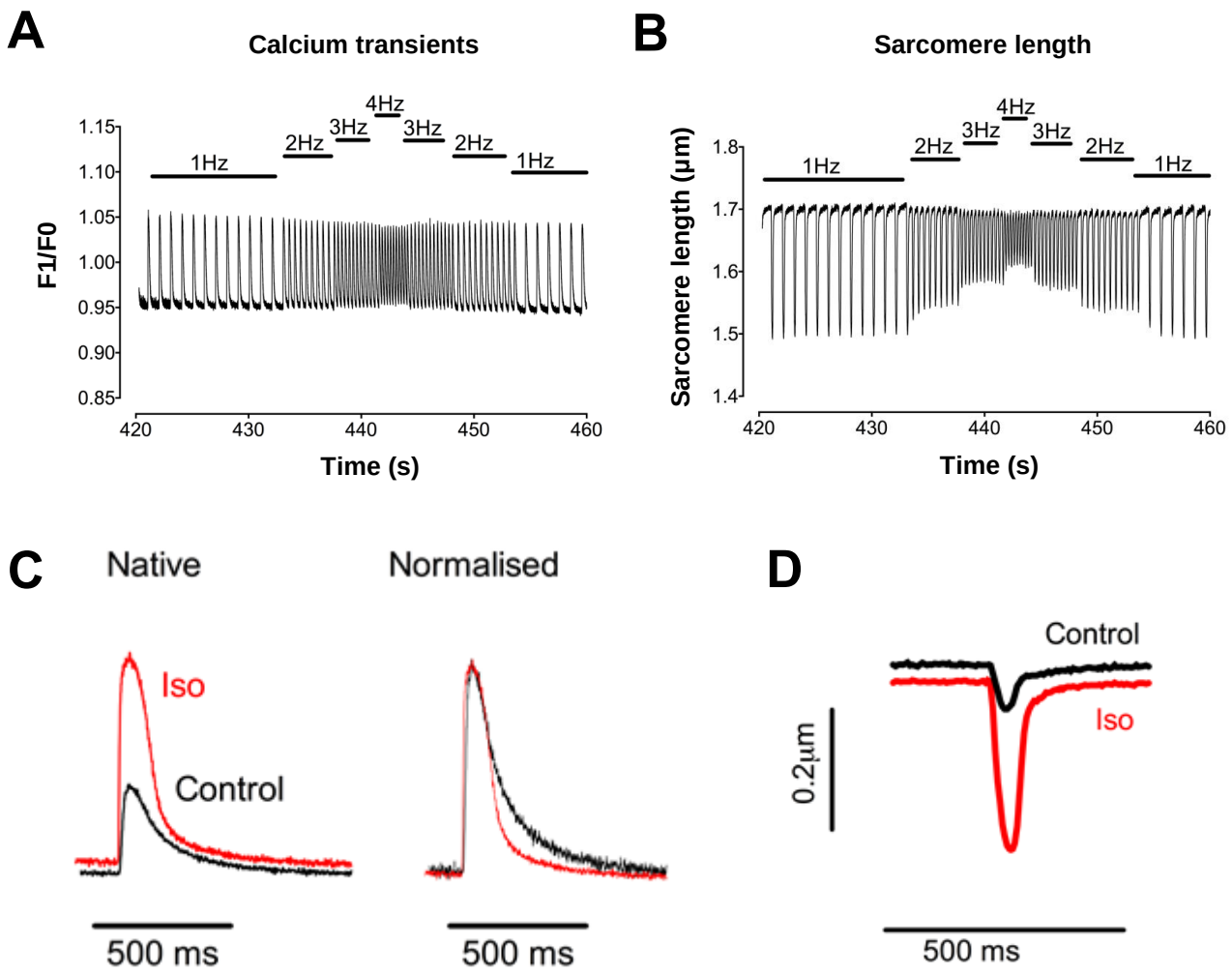
Online Figure II (Cont.) D, Initial testing of various basal media for maintenance of cultured myocytes. Culture media prepared using M199, DMEM, DMEM/F12, M131, or RPMI, all without addition of lipid, were tested for ability to maintain rod shaped myocytes in culture over a period of 4 days. Cell counts 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.



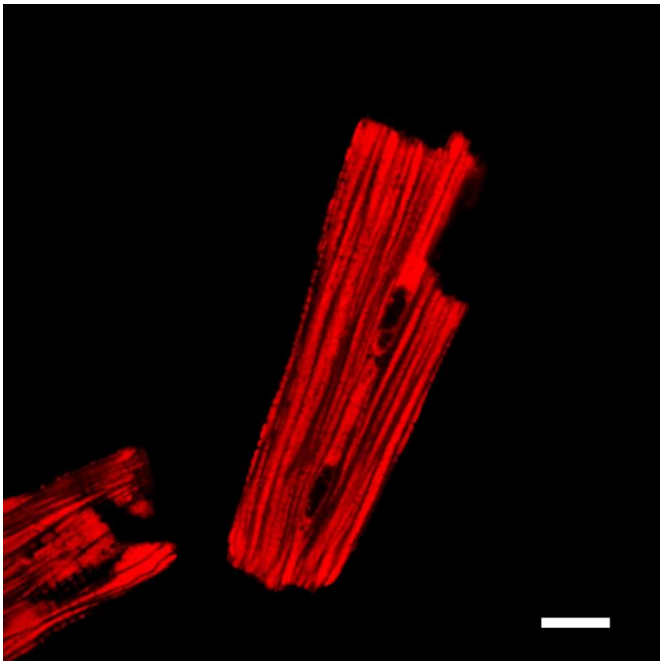
Online Figure III. Isolated myocyte preparations are suitable for biochemical signalling experiments. Myocytes were incubated in the presence of 1 µmol/l norepinephrine (NE), 4 hours after plating, for varying times as indicated. Lysates were subsequently analysed by Western blotting with specific antibodies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



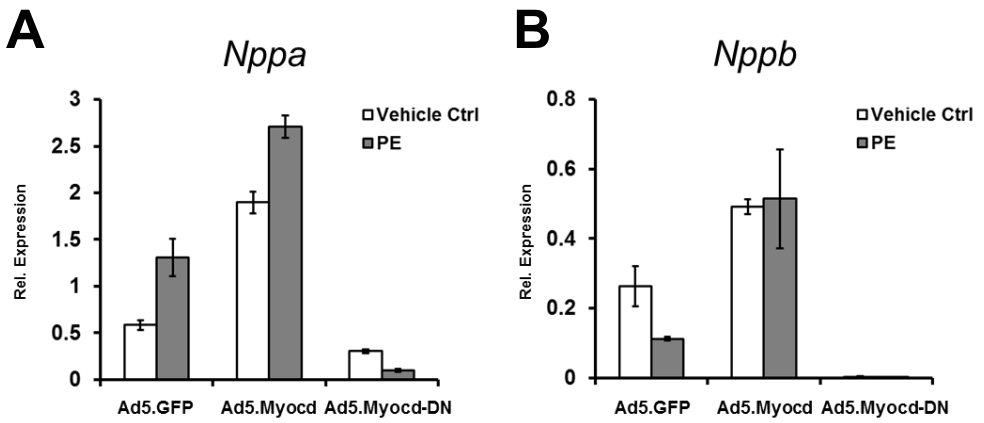
Online Figure IV. Visualisation of spontaneous calcium transients in isolated, plated myocytes. Myocytes were loaded with Fluo-4 AM and imaged. White arrow indicates direction of calcium wave propagation. Scale bars are 10 μm. **E**, Representative quantification of calcium transients in a single cell,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



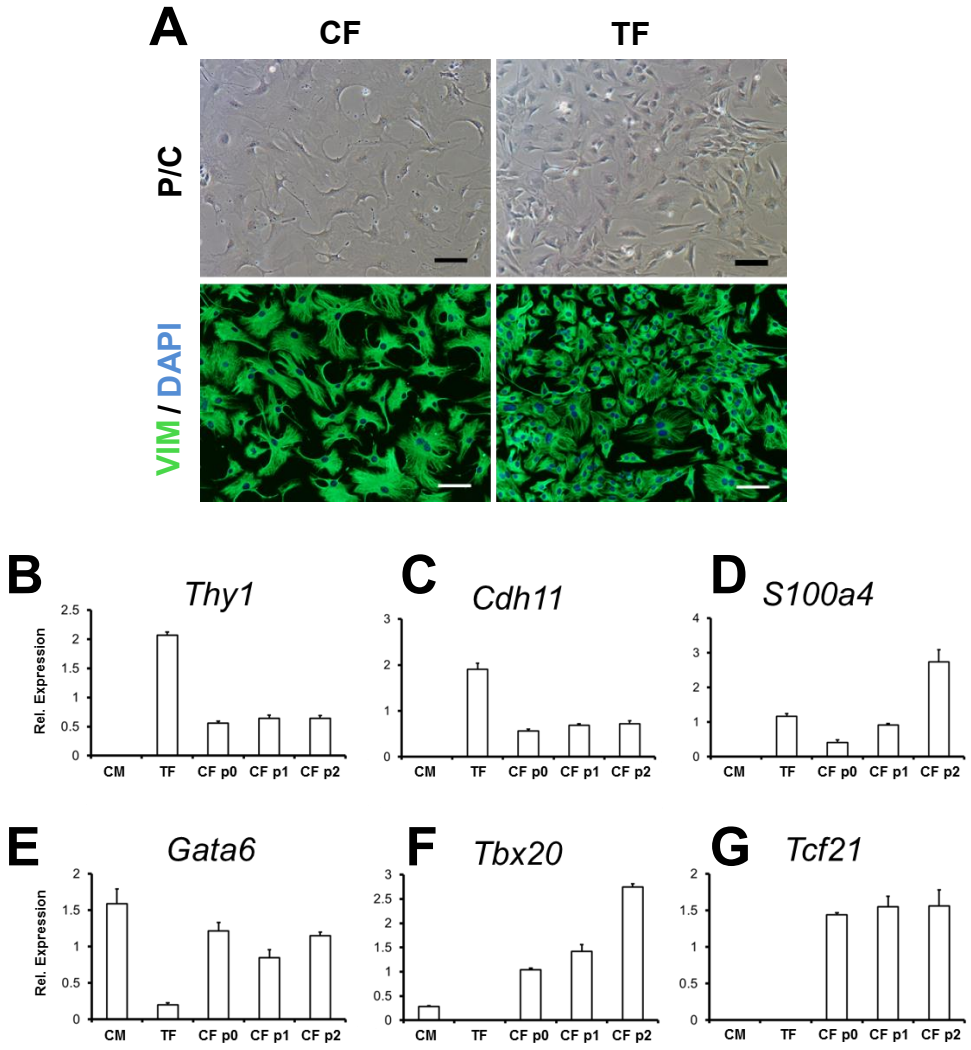
Online Figure V. Isolated cardiomyocytes respond to changes in stimulation frequency and isoproterenol (ISO). Myocytes were loaded with fura2-AM and paced at 2 Hz in the presence or absence of ISO. Calcium transients and sarcomere length shortening were measured in single myocytes using the integrated 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).



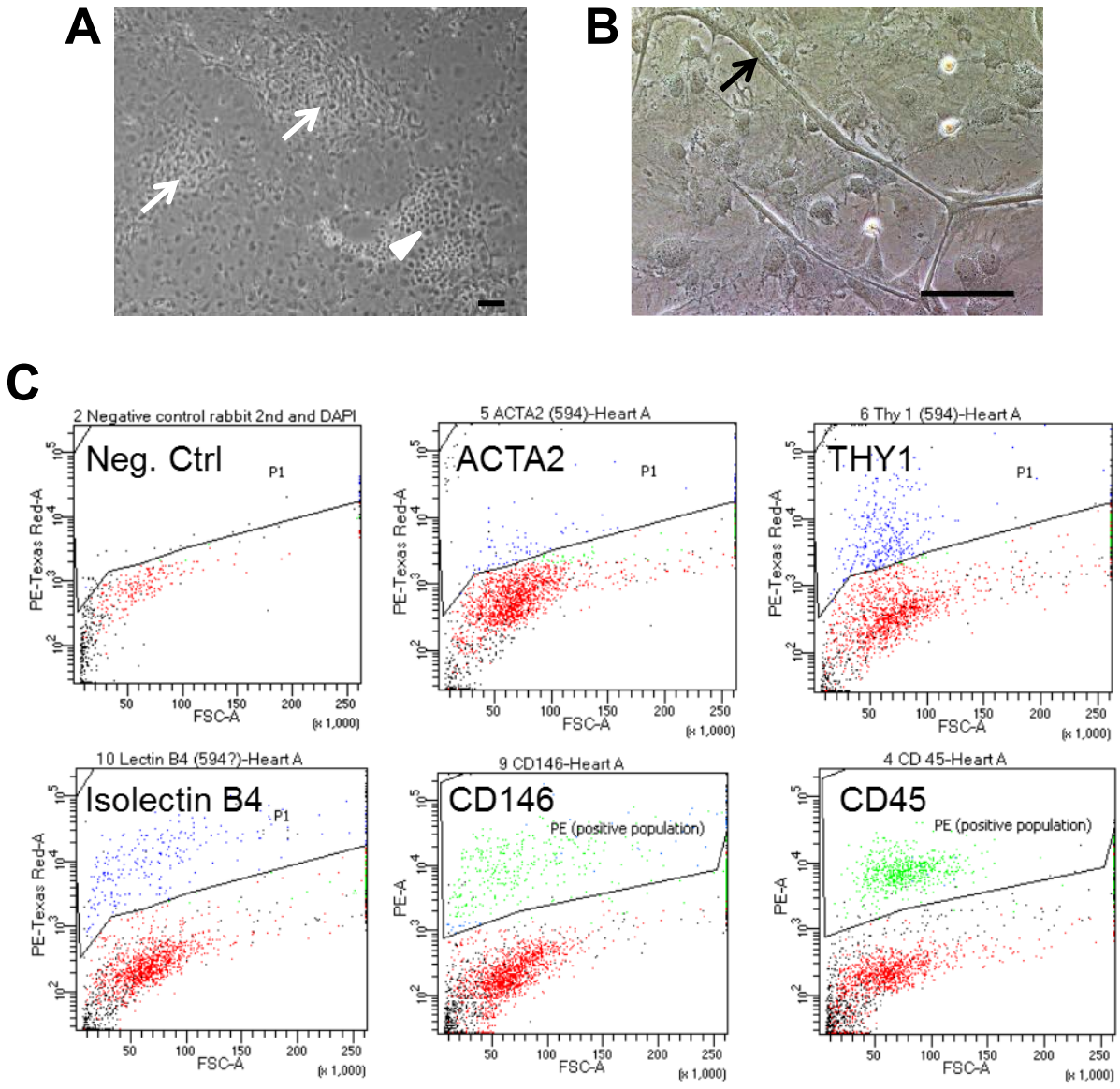
Online Figure VI. Visualisation of active mitochondria in cultured myocytes. Cells were loaded with MitoTracker Red, fixed and imaged using confocal microscopy. Scale bar is 10 μm.



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.