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MacDougall, DA and Calaghan, S (2013) A novel approach to the Langendorff technique: preparation of isolated cardiomyocytes and myocardial samples from the same rat heart. Experimental Physiology, 98 (8). 1295 - 1300. ISSN 0958-0670

https://doi.org/10.1113/expphysiol.2013.072827

Title: A novel approach to the Langendorff technique: preparation of isolated cardiomyocytes and myocardial samples from the same rat heart

Running title: A novel approach to the Langendorff technique

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Keywords: Cardiomyocyte; myocardium; Langendorff.

Word Count: 2236

Subject Area: Heart/Cardiac Muscle

Abbreviations: ARVM, adult rat ventricular myocyte; β2-AR, β2-adrenoceptor; Cav-1, caveolin-1; Cav-3, caveolin-3; cTnI, cardiac troponin I; EC, endothelial cells; EM, electron microscopy; eNOS, endothelial nitric oxide synthase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; hsp90, heat-shock protein 90; SDGF, sucrose density gradient fractionation.

New Findings

1. What is the central question of this study?

The isolation of cardiomyocytes and the homogenisation of myocardium are commonly performed on separate rodent hearts. We asked whether it might be viable to derive cells as well as whole muscle (homogenised or cryopreserved) from one organ.

2. What is the main finding and its importance?

We provide examples of six diverse experiments that can be performed using cells and tissue samples generated by a modified Langendorff technique. The volume and power of resultant data is increased and the method may be especially beneficial in models of cardiovascular disease.

Abstract

Researchers in biomedical sciences must continually re-evaluate their investment in experiments using laboratory animals. Our group is interested in various signalling pathways underlying physiological and pathophysiological function of the mammalian heart. Two important resources for this type of work are isolated cardiomyocytes and homogenised or preserved sections of whole myocardium. In order to ethically improve our experimental approach, we devised an adaptation of the Langendorff whole heart retrograde perfusion technique that allows the isolation of adult rat ventricular cardiomyocytes and processing/storage of myocardium from the same heart. This could result in a 50% reduction in the number of animals required for certain experiments. We believe that a generalised adoption of this method would represent a meaningful reduction of animal use in our field of research and, furthermore, strengthens datasets by permitting correlation between myocyte function and various parameters of myocardial biochemistry/structure in individual hearts. This approach is of particular relevance for studies of cardiac pathology, given the cost and time involved in generating experimental disease models.

Introduction

The use of animals in biomedical research permits the discovery of novel therapeutic approaches to the alleviation of human disease. A controlled legal framework makes the undertaking of such experiments impossible without strong justification [1]. Any justified approach includes the implementation of measures to limit the number of animals used. One research arena which critically depends on fresh tissue is cardiac (patho)physiology. A range of model systems, from isolated ventricular cardiomyocytes to the whole heart, have allowed major breakthroughs in basic science to instruct and inform treatments in the clinic.

The isolation of cardiomyocytes is typically achieved through retrograde perfusion of hearts from small mammals (rat, mouse) with collagenase-containing solution in a Langendorff system [2][3]. Our laboratory routinely uses the isolated adult rat ventricular myocyte (ARVM) as a model for understanding fundamental signalling pathways in the heart, e.g. [4-6]. However, like many others, we also require intact myocardial samples for certain types of analysis. Myocardium is the preferred

source material for methods which require substantial quantities of protein from homogenates as well as assessment of intact muscle structure.

Separate animals are normally used for cell isolation and processing of myocardial material. We outline here a simple modification of the Langendorff technique in which ventricular cardiomyocytes can be isolated and large sections of myocardium dissected from the same heart. This approach significantly expands the range of data that can be gathered from one experimental animal. We provide examples of six experiments that can be undertaken.

Materials and methods

Ethical approval. Appropriate approval was obtained from the University of Leeds' Research Ethics Council.

Experimental animals. Two adult (250-300 g) male Wistar rats were used according to guidelines set out in the *Animals (Scientific Procedures) Act 1986*.

Modified Langendorff technique. Two researchers were required to efficiently perform this technique: one to isolate ARVM and the other to cryopreserve and homogenise myocardial tissue. The animal was humanely killed and the heart quickly excised and cannulated on a standard Langendorff retrograde perfusion assembly as described in [7]. The coronary circulation was perfused with HEPES-based isolation solution [IS (in mM): 130 NaCl, 5.4 KCl, 1.4 $MgCl_2$, 0.4 NaH_2PO_4, 10 creatine, 20 taurine, 10 glucose, 5 HEPES (pH 7.4)] containing 0.75 mM $CaCl_2$. Immediately after the vessels had cleared of blood, the heart was cut transversely into two (basal and apical) sections. The position of the scission, at the midline from base to apex, was judged carefully in order to retain most of the right coronary and left anterior descending arteries in the basal section (Figure 1). The flat, cut edge of the basal section was brought into close contact with the bottom of a small (5 cm diameter) Petri dish. Along with the perfusate collecting in the dish, this provides a degree of back-pressure that maintains adequate coronary artery perfusion. After the section, two procedures were performed in parallel.

(1) Perfusion of the mounted heart tissue was switched to IS containing 0.1 mM EGTA for 4 min, and then IS supplemented with digest enzymes: 1 mg/ml collagenase (type II; Worthington) plus 0.08 mg/ml protease (type XIV) for 7 min. The solution volume in the supporting dish was kept low to allow rapid exchange of solutions surrounding the heart. Once all EGTA had been flushed out of the vasculature, enzyme-containing IS was continually collected and returned to the supply reservoir for recirculation. After 7 min perfusion with enzyme, ventricular tissue was minced and agitated in enzyme-containing IS supplemented with 1% ($^{w}/_{v}$) BSA at 37°C under a continuous oxygen supply. Isolated cardiomyocytes were filtered from this suspension at 5 min intervals and resuspended in IS containing 0.75 mM Ca^{2+}.

(2) Around 450 mg of tissue – approximately half of the wet weight of an adult rat heart – was available for sample preparation by the second researcher. The apical portion of the heart was minced and placed into one of two different buffers for immunoblotting or sucrose density gradient fractionation (SDGF). For immunoblotting, ≈100 mg tissue was homogenised for 4 x 10 s in 2 ml cooled (≈15 °C) Laemmli buffer without β-mercaptoethanol at maximum power (Ultra-Turrax T8 hand-held homogeniser, Ika) [7]. After the final homogenisation step, tubes were centrifuged (2000 g, 10 min at 15 °C) to sediment cell debris and unprocessed material. The supernatant was immediately stored at -20 °C. For

SDGF ≈300 mg tissue was homogenised for 6 x 20 s in 2.5 ml of 500 mM Na_2CO_3 solution (pH 11.0; 4 °C) containing protease inhibitor cocktail (Roche). Samples were stored at -20 °C. The remaining portion of tissue (≈50 mg) was snap frozen in liquid N_2 for later biochemical analysis. Alternatively, this tissue could be frozen in isopentane, cooled to melting point over liquid N_2, for sectioning.

ARVM shortening and Ca^{2+} transient measurement. ARVM were loaded with fura-2 by incubation in IS containing 1.5 µM fura-2-AM for 10 min at room temperature. Cell shortening and intracellular Ca^{2+} (Ca^{2+}_i) transients were recorded using IonWizard6 software (IonOptix) as described previously [6].

Electron microscopy (EM). ARVM were fixed in EM buffer (100 mM Na cacodylate, pH 7.3) containing 2.5% ($^v/_v$) glutaraldehyde and stored at 4 °C until sample processing. EM was performed as described in [8]. Digital images were obtained using a Technai G^2 Spirit microscope (FEI Company).

Separation of ARVM cytosol, membranes, and myofilaments. Cells were disrupted and material separated into various fractions using the Subcellular Fractionation Kit from Pierce (Thermo Fisher Scientific).

Myocardial homogenate discontinuous SDGF. Samples were sonicated for 6 x 10 s at maximum power (Vibra Cell, Sonics) on ice before fractionation on a discontinuous sucrose density gradient as recently described [5]. Protein expression and cholesterol levels in fractionated samples were measured by immunoblotting and Amplex Red assay (Invitrogen) respectively.

Immunoblotting. 5% ($^v/_v$) β-mercaptoethanol was added to all samples prior to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred from polyacrylamide gels to polyvinylidene fluoride (PVDF) membranes, after which a standard immunoblotting procedure was employed [5].

Materials. All materials were from Sigma-Aldrich unless stated otherwise. Primary antibodies raised against Hsp90 (#4875), Akt (#9272), troponin I (#4002), and phospho-troponin I (Ser23/24) (#4004) were from Cell Signalling Technology. Purified mouse anti-eNOS (#610296), anti-phospho-eNOS (Ser1177) (#612392), anti-caveolin 3 (#610421), anti-caveolin 1 (#610406) and anti-adaptin β (#610382) were from BD Biosciences. Anti-β2-AR antibody (#sc-569) was from Santa Cruz Biotechnology. Anti-Desmin (#M0760) antibody was from DAKO.

Results

Populations of ARVM isolated using the modified Langendorff method showed comparable viability (percentage of rod-shaped quiescent cells) to those isolated in the intact heart Langendorff system. The viability of previous ARVM isolations from the intact heart system was found to be 63.5 ± 4.7 % (n=8; mean ± S.D.). In the small number of experiments performed using the modified method, the ARVM viability fell within this range. There was also no overt difference in the amount of undigested tissue remaining following Langendorff perfusion on the intact versus truncated heart. Together these data suggest that perfusion of the coronary vasculature of the basal section is adequate and that this method does not compromise the number of cells isolated (per wet weight of myocardium).

A wealth of material is generated using the method reported here; we focus on subsequent analytical techniques pertinent to our research aims. We have been able to isolate enough ARVM

from one basal section of heart to perform at least 3 different types of experiment. One of these is the simultaneous assessment of cell shortening and Ca^{2+}_i transients from fura-2-loaded individual cells. A typical recording is shown in Figure 2 (*a*); robust shortening (lower traces, *black*) and tightly controlled Ca^{2+}_i (upper traces, *grey*) are seen (which are further indications of cell viability). Only a relatively small number of cells are required for investigation of cell function in response to intervention (e.g. drug treatment). For another experiment, a small aliquot of cells was taken on the day of isolation, fixed in EM buffer and stored at 4 °C for subsequent processing and analysis. We are interested in caveolae, specialised invaginated membrane microdomains rich in cholesterol, sphingolipids and caveolin proteins. Figure 2 (b) shows a typical transmission EM image showing a section of surface sarcolemma with caveolae. Similar assessments could be made in sections of intact myocardium. As well as single cell analyses, ARVM can be pooled for further measurements. For example, we lysed ARVM populations and separated the lysate into cytosolic, membrane and insoluble phases using a commercially available kit (Figure 2 (c)). This type of procedure is useful for studying membrane localisation of proteins of interest. As can be seen by specific labelling using marker proteins, there was a clean separation of phases. Using populations of cells in this way allows multiple experimental manoeuvres to be performed on a single heart. For example, we are interested in the migration of the small GTPase Rac1 from cytosol to membrane following agonist challenge with endothelin 1, so will be able determine its subcellular localisation before and after treatment.

The generation of myocardial homogenates and preservation of ventricular muscle expands the range of information that can be gathered from one heart. Figure 2 (d) shows typical data from immunoblotting of homogenates to measure expression levels of total and phosphorylated cardiac troponin I (cTnI) and endothelial nitric oxide synthase (eNOS). Analysis can be extended to examine the distribution of proteins among the various membrane fractions obtained after SDGF. Figure 2 (e) illustrates representative data from SDGF with the buoyant caveolar fraction (6 in this instance) identified by the presence of caveolin-1 and -3 (Cav-1 and Cav-3) and non-caveolar fractions (heavy fractions 9-12; HF) indicated by the presence of β-adaptin. Samples generated in this way can additionally be used for other analyses, e.g. Figure 2 (f) shows the typical cholesterol concentration profile across myocardial membrane fractions and confirms its enrichment in the buoyant fraction.

An experimental manoeuvre in the whole animal which might be expected to alter ARVM contractile parameters through changes in underlying biochemical properties or even cellular ultrastructure can easily be assessed by the array of experiments outlined above. Of course, an ARVM population from a whole heart can be split to provide source material for the same experiments but the amount of sample generated for SDGF/immunoblotting is comparatively scant. This occurs because of significant loss of tissue during the digestion process. For example, in our experience, ARVM from multiple hearts have to be pooled to generate sufficient sample to layer on a single sucrose density gradient.

Discussion

There are limitations in the application of this technique to certain animal models of cardiovascular disease. Whilst it could be suitable for any model characterised by global cardiomyopathy (e.g. hypertrophy/heart failure *via* aortic constriction), it would not be appropriate to use this for infarct models which target discrete areas of the heart since isolated cardiomyocytes from one region may not have undergone the same pathological progression as myocardium from a different region.

Where the success of myocyte isolation is already compromised (e.g. diabetic cardiomyopathy or failure models), a conceivable drawback might be the potential for less viable populations of isolated ARVM than in the intact heart system. This, however, has not yet been tested. The inherent longitudinal bias in the technique could also affect comparison of cells and whole muscle (taken from apical and basal regions, respectively). For example, regional differences in L-type Ca^{2+} channel [9] and sodium-calcium exchanger [10] expression/activity have been reported. The interpretation of data derived from protein homogenates from whole ventricle must always incorporate the fact that cell types other than cardiomyocytes (cardiac fibroblasts, vascular smooth muscle cells, endothelial cells (EC)) are abundant in the source tissue. When examining proteins (like cTnI) which are expressed exclusively in cardiomyocytes, there should be no concern with 'contamination' from other cell sources. eNOS, for example, is found in EC as well as cardiomyocytes so quantification of protein level in ventricle homogenates reflects contributions from more than one cell type. However, in some situations, the express aim may be to quantify expression of ubiquitously expressed proteins in all tissues of the heart: the approach outlined here would accommodate such assessments. In principle the current technique could be applied to other rodent heart systems, but it remains untested in mouse heart, a source generally considered more difficult from which to obtain healthy myocytes.

We have outlined a new procedure that yields, from a single rat heart, dissociated cardiomyocytes, cardiac muscle homogenates and cryopreserved tissue that can be used in a number of different applications. The examples we provide are not limiting but illustrate that this approach can produce multiple different streams of data that feed into a common research aim. This is advantageous because: (i) correlations can be made between cardiomyocyte function and numerous parameters of myocardial biochemistry in individual hearts and (ii) statistical power is increased. Indeed, we consider that the reduction in animals required for a particular study that can be achieved by implementation of this modified Langendorff technique is of great relevance to studies of cardiac pathology, given the cost and time involved in generating experimental models of cardiac disease.

Our technique could be incorporated into to a wider analytical framework to maximise the experimental observations made per heart. Such a framework could include *in vivo* monitoring of heart function with telemetry probes in an unrestrained animal, followed by echocardiography under anaesthesia, and culminate in modified Langendorff technique-derived cells and myocardial samples.

It is hoped that other groups may test and, where suitable, adopt this method to reduce the use of experimental animals in basic cardiology research.

Figure legends

Figure 1. Scheme depicting the modified Langendorff set-up. Standard apparatus for retrograde perfusion of the whole heart according to Langendorff is shown. Three separate chambers (labelled 1, 2, and 3) connected in series to a peristaltic pump allow successive switching of Ca^{2+}-, EGTA-, and enzyme-containing solutions (see Materials and Methods). Standard myocyte dissociation is then performed on the basal section, whilst the apical section can be further dissected into right ventricle, septum and left ventricle. Tissue from the latter three sources is snap-frozen or homogenised. The upper rectangular inset shows a larger version of the stylised heart to indicate where the section is made (*grey dashed line*). Larger arterial vessels supplying the left ventricle are named in italics.

Figure 2. Examples of experiments performed on cell and muscle samples. The upper panel shows representative data from experiments using isolated ARVM: A, Cell shortening and intracellular Ca^{2+} transients in an individual fura-2 loaded ARVM; B, transmission EM of a sarcolemmal region from a single ARVM, *m* indicates a mitochondrion, and *z* the sarcomeric z-line (18500x magnification); C, separation of cell populations into cytosolic (*C*), membrane (*M*), and insoluble (*I*) fractions demonstrated by the marker proteins heat-shock protein 90 (Hsp90), β2-AR and desmin, respectively; 10 µg protein was loaded per lane. The lower panel shows representative data from experiments carried out on myocardial homogenates: D, levels of total and phosphorylated cTnI and eNOS using immunoblotting; E, protein distribution in different membrane compartments separated by SDGF (Cav-1, caveolin 1; Cav-3, caveolin 3; β-adap, β adaptin; HF, heavy fractions 9-12); F, cholesterol distribution in different membrane fractions measured using Amplex Red cholesterol assay.

Disclosures

None.

Acknowledgements

This work was funded by the British Heart Foundation. We would like to thank Professor Ed White for useful discussion of this technique.

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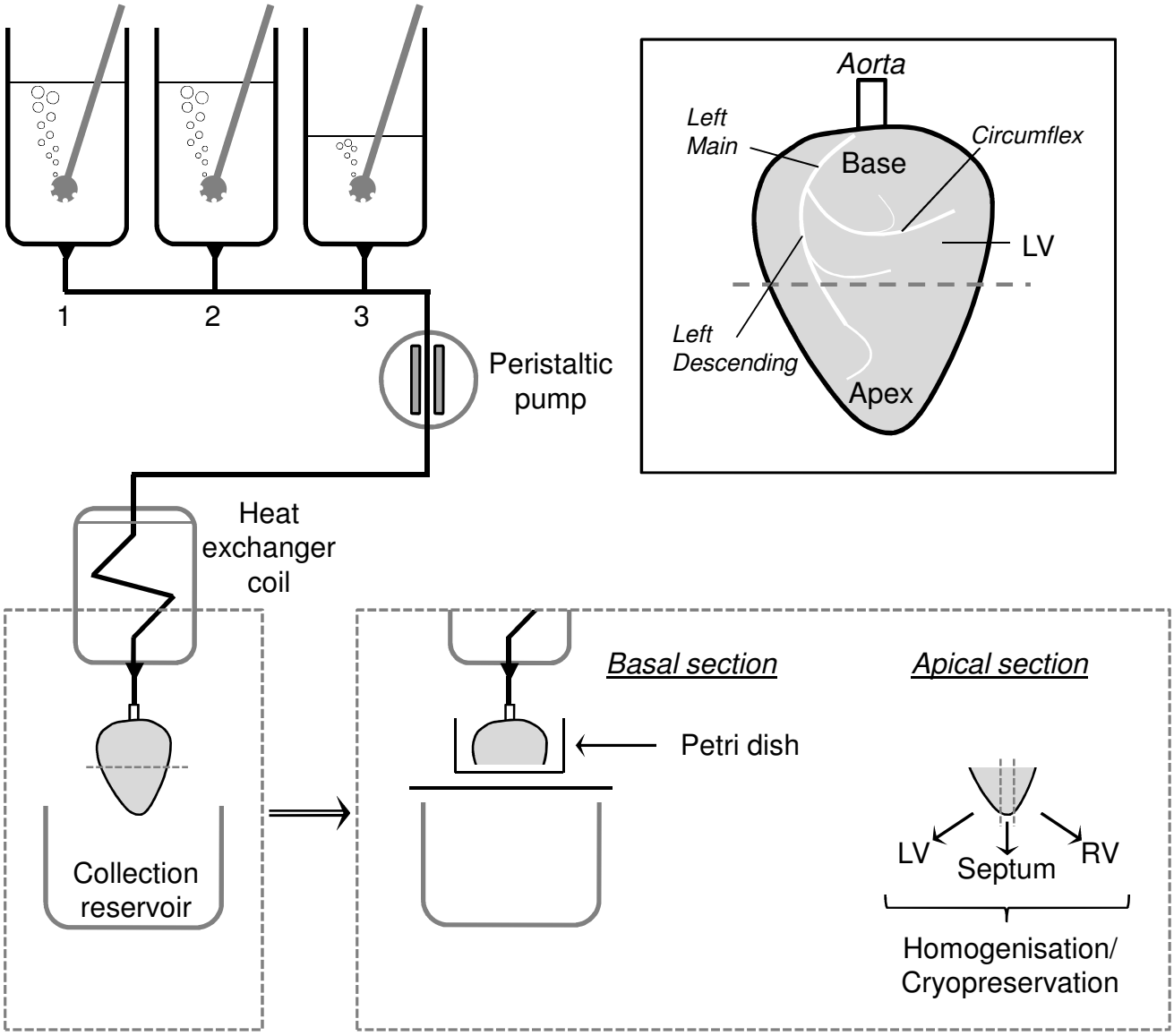
1
2
3
Peristaltic pump
Heat exchanger coil
Collection reservoir
Aorta
Left Main
Circumflex
Base
LV
Left Descending
Apex
Basal section
Apical section
Petri dish
LV
Septum
RV
Homogenisation/ Cryopreservation

Figure 1

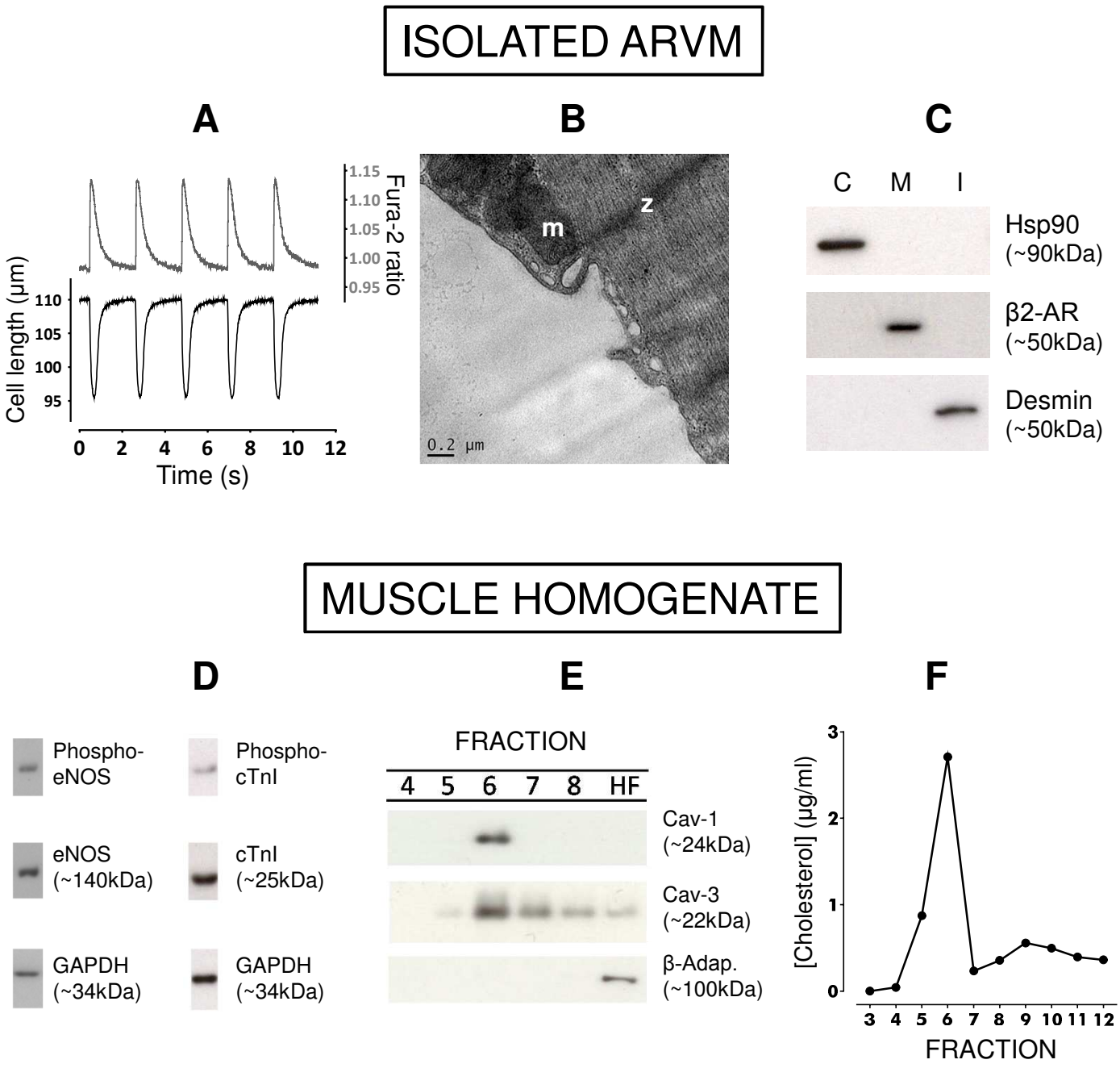
ISOLATED ARVM
A
B
C
Cell length (µm)
110
105
100
95
0 2 4 6 8 10 12
Time (s)
1.15
1.10
1.05
1.00
0.95
Fura-2 ratio
m
z
0.2 µm
C M I
Hsp90
(~90kDa)
β2-AR
(~50kDa)
Desmin
(~50kDa)
MUSCLE HOMOGENATE
D
E
F
Phospho-
eNOS
eNOS
(~140kDa)
GAPDH
(~34kDa)
Phospho-
cTnI
cTnI
(~25kDa)
GAPDH
(~34kDa)
FRACTION
4 5 6 7 8 HF
Cav-1
(~24kDa)
Cav-3
(~22kDa)
β-Adap.
(~100kDa)
[Cholesterol] (µg/ml)
3
2
1
0
3 4 5 6 7 8 9 10 11 12
FRACTION

Figure 2