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Isolation of human atrial myocytes for simultaneous measurements of Ca2+ transients and membrane currents

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Cover Letter

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USA

Review article: Isolation of human atrial myocytes for simultaneous measurements of Ca^{2+} transients and membrane currents

Dear Ms. Meehan,

Enclosed please find the revised version of our manuscript.

We changed our work according to the reviewers' suggestions, which helped us to improve the manuscript. In addition we prepared a point by point answer to the reviewers' comments.

However, we regret that we are currently unable to cover the costs for the publication. Concerning this issue, we wish to cooperate with *Worthington Biochemical Corporation*. Their decision is still pending.

Please note that we, Drs. Xiao-Bo Zhou and Niels Voigt, share first co-authorship and Dr. Niels Voigt will be the corresponding author.

We would be very happy if you consider this method suitable for publication in JoVE.

Yours sincerely,

Drs. Niels Voigt and Xiao-Bo Zhou

Isolation of human atrial myocytes for simultaneous measurements of Ca^{2+} transients and membrane currents

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Short Abstract

We describe the isolation of human atrial myocytes which can be used for intracellular Ca^{2+} measurements in combination with electrophysiological patch-clamp studies.

Long Abstract

The study of electrophysiological properties of cardiac ion channels with the patch-clamp technique and the exploration of cardiac cellular Ca^{2+} handling abnormalities requires isolated cardiomyocytes. In addition, the possibility to investigate myocytes from patients using these techniques is an invaluable requirement to elucidate the molecular basis of cardiac diseases such as atrial fibrillation (AF).[1] Here we describe a method for isolation of human atrial myocytes which are suitable for both patch-clamp studies and simultaneous measurements of intracellular Ca^{2+} concentrations. First, right atrial appendages obtained from patients undergoing open heart surgery are chopped into small tissue chunks ("chunk method") and washed in Ca^{2+}-free solution. Then the tissue chunks are digested in collagenase and protease containing solutions with 20 µM Ca^{2+}. Thereafter, the isolated myocytes are harvested by filtration and centrifugation of the tissue suspension. Finally, the Ca^{2+} concentration in the cell storage solution is adjusted stepwise to 0.2 mM. We briefly discuss the meaning of Ca^{2+} and Ca^{2+} buffering during the isolation process and also provide representative recordings of action potentials and membrane currents, both together with simultaneous Ca^{2+} transient measurements, performed in these isolated myocytes.

Introduction

The study of electrophysiological properties of cardiac ion channels with the patch-clamp technique and the exploration of cellular Ca^{2+} handling abnormalities require isolated cardiomyocytes. These are usually obtained following the in vitro exposure of cardiac tissue samples to digestive enzymes (collagenase, hyaluronidase, peptidase etc.). Since the first report of isolation of viable cardiac myocytes in 1955[2] a large quantity of protocols has been developed in order to harvest single atrial and ventricular cardiomyocytes from different species including mouse, rat, rabbit, dog, guinea pig and human. In this review we focus on isolation of human atrial myocytes. Regarding procedures for isolation of myocytes from other species we refer to the "Worthington Tissue Dissociaton guide" provided by Worthington Biochemical Corp., USA (www.tissuedissociation.com).

Human atrial myocyte isolation protocols are generally derived from the method described by Bustamante et al.[3] Here we provide a step-by-step description of a technique, which is adapted from a previously published method, in order to obtain atrial myocytes suitable not only for patch-clamp experiments but also for simultaneous intracellular Ca^{2+} measurements.[4-11]

Protocol text:

Experimental protocols need to be approved by the local ethics committee and all patients need to give written informed consent. Our research was approved by the ethics committee of the Medical Faculty Mannheim, University of Heidelberg (#2011-216N-MA) and was performed in compliance with all institutional, national and international guidelines for human welfare. All patients gave written in formed consent.

25

0) Obtaining human atrial tissue

During routine cannulation procedures in patients undergoing open-heart surgery for cardiopulmonary bypass grafting, the tip of the right atrial appendage is usually removed and can be used for isolation of atrial cardiomyocytes. After excision the tissue
30 sample is transferred immediately into a 50 ml Falcon^{TM} tube with sterile Ca^{2+}-free transport solution containing 2,3-butanedione monoxime (**Table I**; BDM, contractile inhibitor, preventing myocyte contracture). With transport times between 30 and 45 minutes, we did not recognize a clear advantage of cooling or oxygenation with respect to both number and quality of isolated atrial cardiomyocytes. In general
35 transport to the lab should be as quick as possible. However, if longer transportation time cannot be avoided, transport at 4°C in oxygenated solution might be advantageous.

1) Prearrangements

40 1.1) Switch on the thermocirculator, which controls the temperature of the jacketed beaker (**Table II**). Make sure the temperature within the beaker equals 37°C. Cover the beaker with a glass lid to maintain a constant temperature within the beaker throughout the whole isolation process.

1.2) Weigh the Collagenase I and Protease XXIV into three glass beakers and store it
45 at room temperature. The enzymes will be diluted just before use.

1.3) Store 20 ml of Ca^{2+} free solution in a petri dish at 4°C.

1.4) Store 250 ml of Ca^{2+} free solution in a water bath at 37°C.

2) Cleaning of the tissue

2.1) Transfer the tissue sample together with the transport solution into a petri dish (~10 cm diameter) and remove fatty tissue roughly using scissors.

2.2) Weigh the tissue sample. Between 200 and 600 mg are used for cell isolation. The remaining tissue can be frozen in liquid nitrogen for later biochemical analysis.

2.3) Transfer the tissue sample into the petri dish containing 20 ml Ca^{2+} free solution at 4°C (see step 1.3) and chop the tissue sample into small chunks of approximately 1 mm^3 in size.

2.4) The following steps should be executed at 37°C and under continuous gassing with 100% O_2. A pipette tip can be placed onto the oxygen tube to avoid strong bubbling and generate continuous air flow.

2.5) Transfer the tissue chunks together with the Ca^{2+}-free solution into the jacketed beaker and stir carefully for about 3 minutes with a magnetic stirring bar.

2.6) Stop stirring and allow the tissue chunks to settle down for a few seconds. Carefully strain the supernatant through a nylon mesh (200 µm, **Table II**). Return the restrained tissue chunks from the mesh into the beaker using forceps.

2.7) Refill the beaker with Ca^{2+} free solution and repeat the washing step 2.4 and 2.5 two times.

3) First enzymatic digestion

3.1) Re-suspend the tissue chunks with 20 ml Enzyme solution E1 (**Table I**) containing collagenase and protease and stir carefully for 10 minutes.

3.2) Add 40 µl of 10 mM $CaCl_2$-solution to obtain a final concentration of 20 µM Ca^{2+}.

3.3) After 35 minutes strain the supernatant carefully through a nylon mesh (200 µm, **Table II**) in a way that most of the tissue chunks remain in the beaker. Return restrained tissue chunks from the mesh into the beaker.

4) Second enzymatic digestion

4.1) Resuspend the tissue chunks again with 20 ml enzyme solution E2 containing collagenase I only (**Table I**). Add 40 µl of 10 mM $CaCl_2$-solution immediately to obtain a final concentration of 20 µM Ca^{2+}.

4.2) During the second digestion use scissors to further chop tissue clots occasionally.

4.3) After 5 minutes take a sample using a Pasteur pipette to check the dissociation of cells. Repeat this every 2-3 minutes until rod-shaped, striated cardiomyocytes appear.

4.4) Stop stirring and allow the tissue chunks to settle down for about 20-30 seconds.

4.5) Strain the supernatant carefully through a nylon mesh (200 µm, **Table II**) into a 50 ml Falcon™ tube (Tube A). Return the restrained tissue chunks from the mesh into the beaker.

4.6) Re-suspend the tissue chunks in the beaker with 20 ml storage solution (**Table I**)[10] and further dissociate the cells by gentle mechanical trituration using a 20 ml serological pipette with dispenser. Try to avoid bubble formation since bubbles are detrimental to cell survival and quality.

4.7) Strain the supernatant carefully through a nylon mesh (200 µm, **Table II**) into a 50 ml Falcon™ Tube (Tube B).

5) Final preparation and adjustment of final Ca^{2+} concentration

5.1) Centrifuge both Falcon™ tubes A and B (see step 4.5 and 4.7) at 95 g for 10 minutes.

5.2) Remove the supernatant from both tubes carefully by aspiration. Make sure not to disturb the pellet. Discard the supernatant.

5.3) Re-suspend both pellets in 1.5 ml storage solution (**Table I**) each (room temperature).

5.4) Add two times 7.5 µl of 10 mM $CaCl_2$ solution to each Falcon™ and stir carefully for 10 minutes after each step.

5.5) Add 15 µl of 10 mM $CaCl_2$ solution to obtain a final Ca^{2+} concentration of 0.2 mM.

6) Loading of myocytes with the fluorescent Ca^{2+}-indicator Fluo-3 AM (Figure 1)

6.1) Transfer 1.5 ml of cell suspension (tube A and/or tube B) into a 2 ml microcentrifuge tube (Eppendorf™ tube).

6.2) The following steps should be executed under consideration of the light sensitivity of the fluorescent Ca^{2+} Indicator Fluo-3.

6.3) Dissolve 50 µg of the membrane permeable acetoxymethyl ester derivative of Fluo-3 (Fluo-3 AM, **Table III**) in 44 µl of the Pluronic F-127 (**Table III**) stock solution (20% w/v in anhydrous DMSO) to get a 1 mM Fluo-3 AM stock solution, which can be stored at -20 °C for at most 1 week.

6.4) Add 15 µl of the Fluo-3 AM stock solution to the microcentrifuge tube containing 1.5 ml of cell suspension (see step 6.1) and agitate carefully.

6.5) Incubate the cell suspension for 10 minutes in an optically opaque box.

6.6) Briefly centrifuge at about 6000 rpm.

6.7) Discard the supernatant and re-suspend the pellet in 1.5 ml bath solution (**Table IV**).

6.8) Leave the cell suspension for about 30 minutes for de-esterification before beginning with experiments.

7) Simultaneous patch-clamp and epifluorescent Ca^{2+} measurements

Since patch-clamp measurements are not the major topic of this review, we refer the interested reader to other publications providing a more in depth description of this technique.[11-14] For the sake of completeness we provide a brief summary of a protocol to measure action potentials or L-type Ca^{2+} currents, both together with simultaneous Ca^{2+}-transient recordings.

During experiments myocytes are superfused at 37°C with bath solution (**Table IV**) using a rapid perfusion system (Octaflow II^{TM}, ALA Scientific Instruments, NY). For voltage-clamp experiments, K^+ currents are blocked by adding 4-aminopyridine (5 mmol/L) and $BaCl_2$ (0.1 mmol/L) to the bath solution. Borosilicate glass microelectrodes are used and should have tip resistances of 2-5 MΩ when filled with pipette solution (**Table V**). In addition to the Fluo-3 AM loading of the myocytes (see step 6), Fluo-3 is also included in the pipette solution (**Table V**). Fluorescence is excited at 488 nm and emitted light (<520 nm) converted to $[Ca^{2+}]_i$ assuming

$$[Ca^{2+}]_i = k_d\left(\frac{F}{F_{\max} - F}\right)$$

where k_d=dissociation constant of Fluo-3 (864-nmol/L), F=Fluo-3 fluorescence; F_{max}=Ca^{2+}-saturated fluorescence obtained at the end of each experiment.[12]

Both electrical signals and epifluorescent Ca^{2+} signals are recorded simultaneously. Action potentials are stimulated at 0.5 Hz in current-clamp mode using 1 ms current pulses of 1.2x threshold strength. L-type Ca^{2+}-currents are measured in voltage-clamp mode using a holding potential of -80 mV and a 100-ms ramp-pulse to -40 mV to inactivate the fast Na^+-current, followed by a 100-ms test-pulse to +10 mV at 0.5 Hz.

Representative Results

Figure 2A shows three representative examples from isolated human right atrial myocytes. To quantify the cell yield we pipette 10 µl of cell suspension (step 5.5) on a CellFinder microscope slide (http://www.antenna.nl/microlab/index-uk.html). Averaged cell yields in **Figure 2B** clearly indicate that there is a tendency to lower cell yields in chronic AF (cAF) patient samples (tube A: 16.5±3.1 cells/10 µl (n=29) vs. 5.1±2.3 cells/10 µl (n=10) in SR and cAF, respectively, p<0.046; tube B: 17.9±3.9 cells/10 µl

(n=29) vs. 5.9±2.0 cells/10 µl (n=9) in SR and cAF, respectively, p=0.107).

Representative examples of action–potential measurements and simultaneous recordings of cytosolic Ca^{2+} transients are given in **Figure 3**. In about 90% of the investigated cells, the action-potential-triggered Ca^{2+} release causes clear and regular cell contractions. As reported previously, the resting membrane potential, which is an accepted indicator for cell integrity, averaged about -73.9±2.7 mV (n = 23/10 myocytes/patients) and -77.7±1.8 mV (n = 19/8 myocytes/patients) in SR and cAF respectively (p>0.05).[15] **Figure 4** shows representative simultaneous recordings of voltage-gated L-type Ca^{2+} currents and cytosolic Ca^{2+} transients. Application of the non-selective β-adrenoceptor agonist isoprenaline (1 µM) increases amplitudes of both $I_{Ca,L}$ and cytosolic Ca^{2+} transients, suggesting intact β-adrenergic signal transduction cascade.

Figure legends

Figure 1: Flow chart of the myocytes Fluo-3 AM loading protocol (see step 6.1-6.5). m/v, mass/volume.

Figure 2: A, Isolated human right atrial myocytes after one hour in storage solution. **B,** Mean±SEM of the cell yield counted in 10 µl of cells in storage solution (see step 5.5). n refers to the number of preparations within each group. *p<0.05.

Figure 3: Representative recordings of action-potential-triggered Ca^{2+}-transients (CaT) in an atrial myocyte from a sinus rhythm and a chronic atrial fibrillation patient. Top: Injected membrane current (I_M) used for stimulation (0.5 Hz). Below: Simultaneous recording of membrane potential (V_M), and triggered CaT (bottom). (Replotted with

permission from Voigt et al. 2012)[15]

Figure 4: Representative recordings of the isoprenaline (1 µM) effect on L-type Ca^{2+} current-triggered Ca^{2+}-transients (CaT) in an atrial myocyte from a sinus rhythm and a chronic atrial fibrillation patient. Top: Voltage-clamp protocol (0.5 Hz). Below: Simultaneous recording of total net membrane current (I_M), predominantly reflecting L-type Ca^{2+} current (middle) and triggered CaT (bottom). (Replotted with permission from Voigt et al. 2012)[15]

Discussion

Here we describe a method for isolation of human atrial myocytes from right atrial appendages obtained from patients undergoing open heart surgery. In order to use these myocytes for measurements of cytosolic Ca^{2+} we adapted a previously described method[4-11] by omitting EGTA from the storage solution.

Already in 1970 it was observed that although myocytes dissociate in the presence of Ca^{2+} during digestion, all of them were in contracture and non-viable.[16,17] Therefore, cell isolation is performed in Ca^{2+}-free solution. However, the re-introduction of physiological concentrations of Ca^{2+} resulted in rapid Ca^{2+} influx and cell death. This has been described as the Ca^{2+} paradox phenomenon which was originally observed in perfused hearts by Zimmerman and Hulsman.[18] Modifications of the isolation media including reduction of the pH to 7.0,[19] addition of taurin[20] or of small amounts of Ca^{2+} (see step 3.2 and 4.1),[21] as well as storage of isolated myocytes in EGTA containing storage-solution[22] have been suggested to prevent the Ca^{2+} paradox.[17] However, it is well known that Ca^{2+} buffering through EGTA reduces the amplitude of L-type Ca^{2+} current-induced Ca^{2+} transient amplitudes and results in a biphasic decay of the Ca^{2+}

transients.[23] Therefore, we omitted EGTA throughout the whole isolation process in order to obtain Ca^{2+} transients with typical properties and monophasic decays. To protect the cells from the Ca^{2+} paradox we increased the final Ca^{2+} concentration of the storage solution in a stepwise manner until 0.2 mM.

The choice of collagenase is probably the most critical step for successful myocyte isolation. Conventional collagenases are crude preparations obtained from Clostridium histolyticum and contain collagenase in addition to a number of other proteinases, polysaccharidases and lipases. Based on their general composition collagenases are subdivided into 4 types (Type I to Type IV).[24] Types I, II and IV have been successfully used for isolation of human atrial myocytes.[4-10,15,25-30] In our presently described protocol we recommend the use of collagenase Type I, although we were also able to obtain acceptable amounts of viable cells using collagenase Type II. However, even within a single collagenase type there is a significant batch-to-batch variation regarding the enzyme activities. These variations require careful batch selection and testing of various batches to optimize isolation procedure. The online available batch-selection tool from Worthington Biochemical Corp. (http://www.worthington-biochem.com/cls/match.php) may be used to find available batches with a composition that has been shown to be suitable for the isolation of human atrial myocytes. Currently we use collagenase type I with 250 U/mg collagenase activity, 345 U/mg caseinase activity, 2.16 U/mg clostripain activity and 0.48 U/mg tryptic activity (lot# 49H11338).

The cells obtained using the procedure described in this manuscript may be used within 8 hours for patch-clamp studies, Ca^{2+} transient measurements and a combination of both.[15] In addition, these cells allow measurements of cellular contraction in response to electric field stimulation or electric stimulation using the patch-clamp pipette

(unpublished observations).

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Disclosures

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Figure 1
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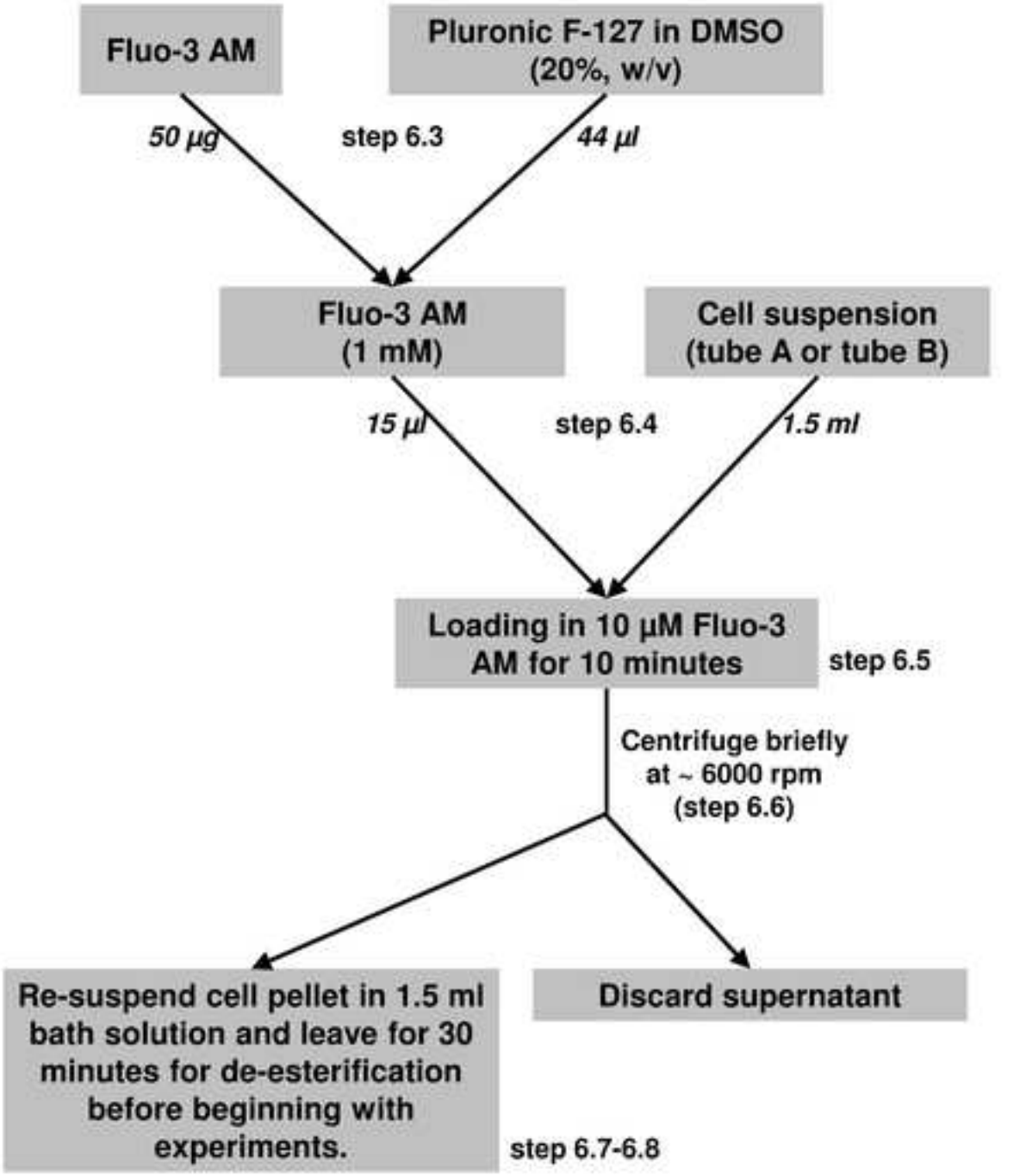
Figure 1

Protocol for loading human atrial myocytes with Fluo-3 AM



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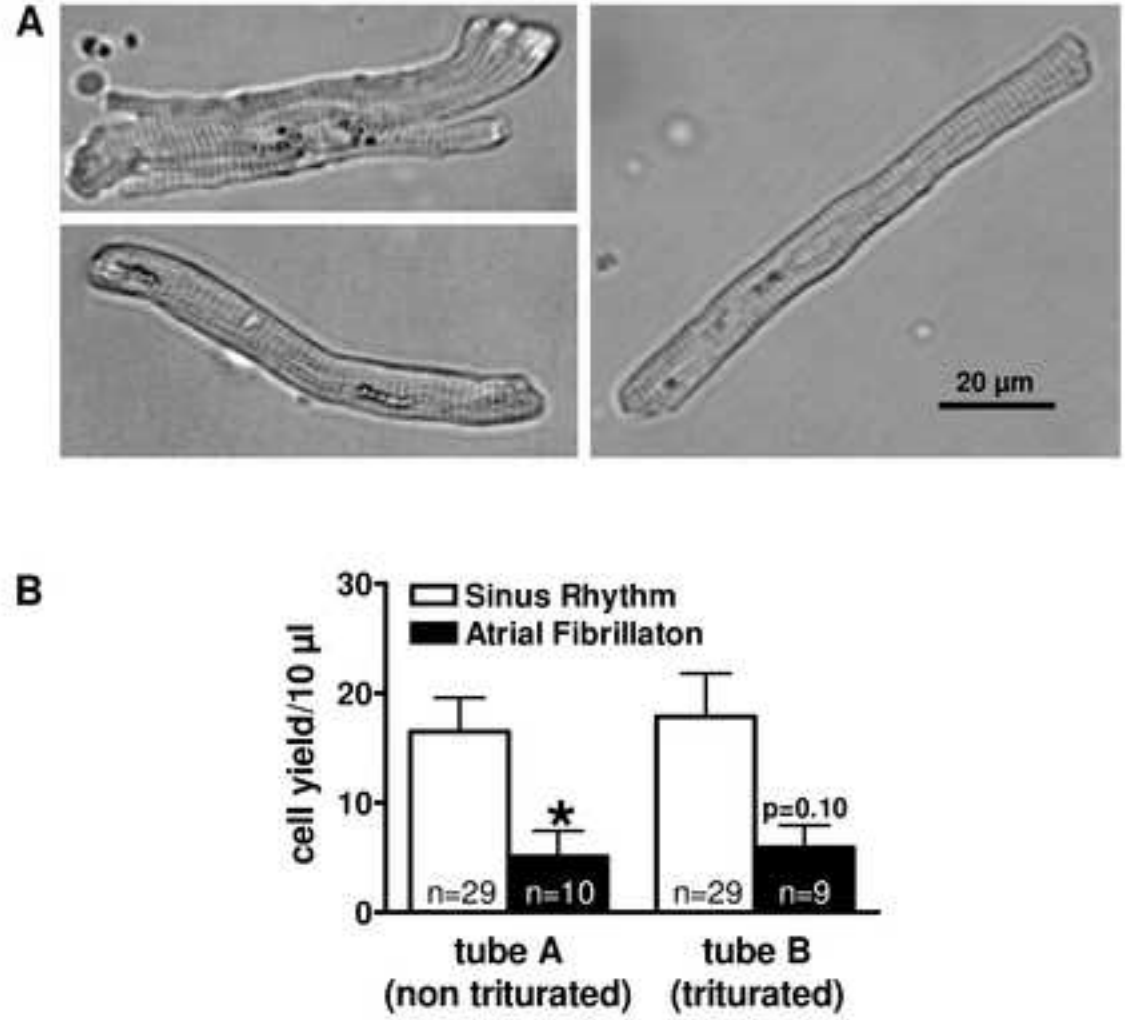

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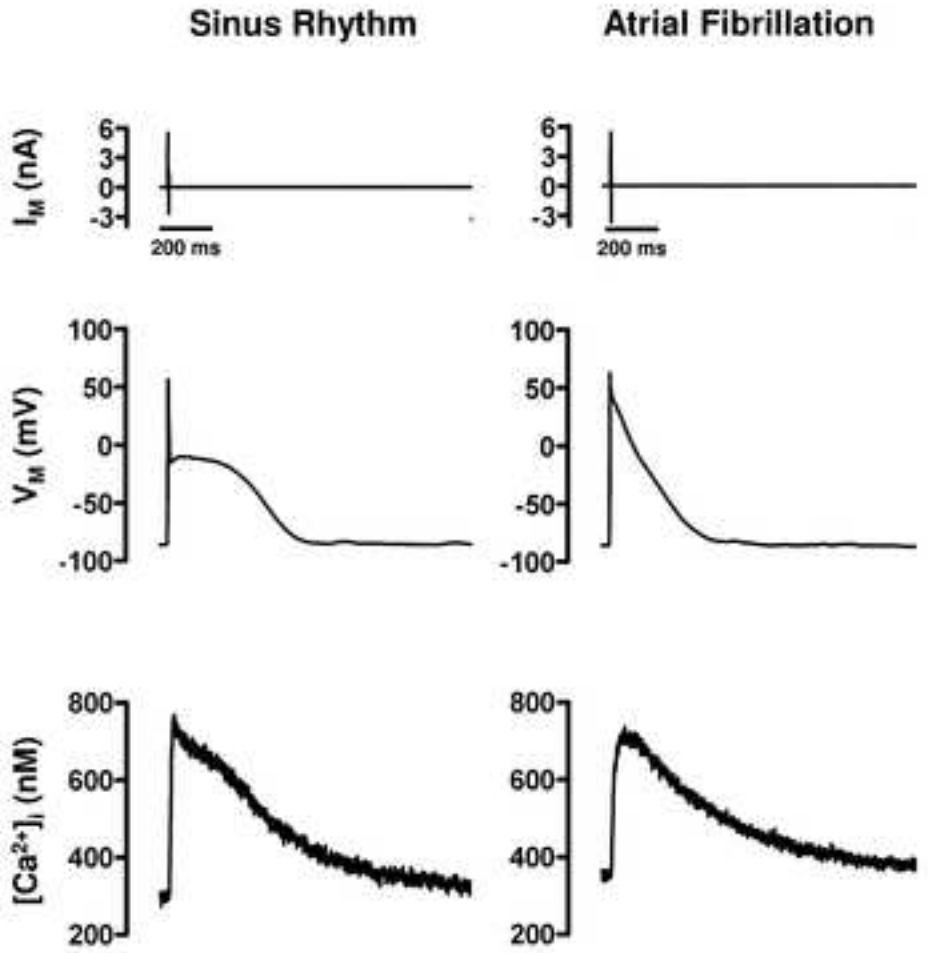

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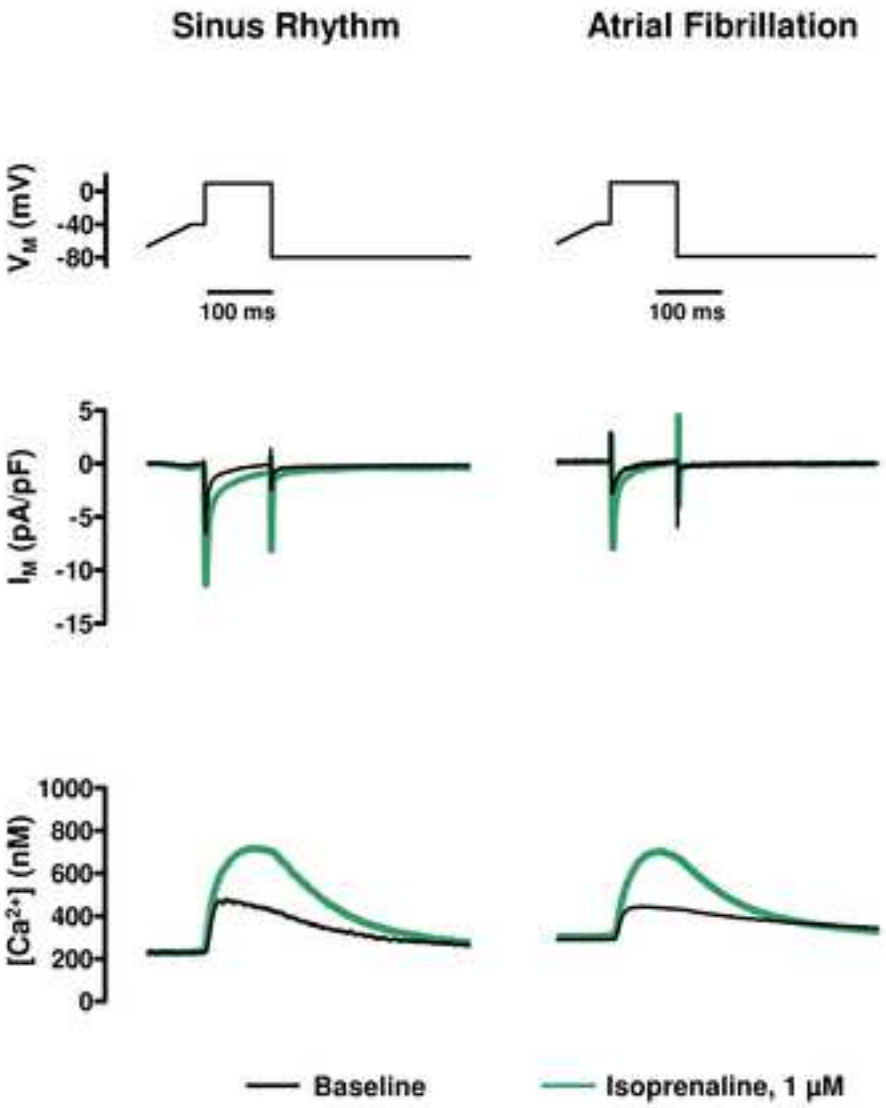
Figure 4



Animated/Video Figure

Click here to download Animated/Video Figure: Contracting myocyte.avi

*Table of Reagents/ Materials Used
Click here to download Table of Reagents/ Materials Used: Table III_R1_30082012b.xls

Table I: Solutions

	Company	Catalogue number	Transport solution	Ca^{2+}-free solution	Enzyme solution E1 and E2	Storage solution
Albumin	Sigma-Aldrich	A3059	–	–	–	1%
BDM	Sigma-Aldrich	31550	30	–	–	–
DL-β-Hydroxy-butyric acid	Sigma-Aldrich	H6501	–	–	–	10
Glucose	Sigma-Aldrich	G8270	20	20	20	10
L-Glutamic acid	Sigma-Aldrich	G1251	–	–	–	70
KCl	Merck	1049360250	10	10	10	20
KH_2PO_4	Sigma-Aldrich	P5655	1.2	1.2	1.2	10
$MgSO_4$	Sigma-Aldrich	M9397	5	5	5	–
MOPS	Sigma-Aldrich	M1254	5	5	5	–
NaCl	Sigma-Aldrich	S3014	100	100	100	–
Taurin	Sigma-Aldrich	86330	50	50	50	10
Collagenase I	Worthington	4196	–	–	286 U/ml	–
Protease XXIV	Sigma-Aldrich	P8038	–	–	5 U/ml*	–
pH			7.00	7.00	7.00	7.40
adjusted with			1 M NaOH	1 M NaOH	1 M NaOH	1 M KOH

Concentrations in mM unless otherwise stated. BDM, 2,3-Butanedione monoxime. *Protease XXIV is included in Enzyme solution E1 only.

Table II: Specific equipment

	Company	Catalogue number
Nylon mesh (200 µm)	VWR-Germany	510-9527
Jacketed reaction beaker	VWR	KT317000-0050

Table III: Substances for loading of myocytes with Fluo-3 AM

	Company	Catalogue number
Dimethyl-sulphoxide	Sigma-Aldrich	D2650
Fluo-3 AM (special packaging)	Invitrogen	F-1242
Pluronic F-127	Invitrogen	P6867

Table IV: Bath solution for patch-clamp

	Company	Catalogue number	Bath solution
4-aminopyridine*	Sigma-Aldrich	A78403	5
$BaCl_2$*	Sigma-Aldrich	342920	0.1
$CaCl_2 \cdot 2H_2O$	Sigma-Aldrich	C5080	2
Glucose	Sigma-Aldrich	G8270	10
HEPES	Sigma-Aldrich	H9136	10
KCl	Merck	1049360250	4
$MgCl \cdot 6H_2O$	Sigma-Aldrich	M0250	1
NaCl	Sigma-Aldrich	S3014	140
Probenecid	Sigma-Aldrich	P8761	2
pH			7.35
adjusted with			1 M HCl

*4-aminopyridine and BaCl were included for voltage-clamp

Table V: Pipette solution for patch-clamp*

	Company	Catalogue number	Bath solution
DL-aspartat K^+-salt	Sigma-Aldrich	A2025	92
EGTA	Sigma-Aldrich	E4378	0.02
GTP-Tris	Sigma-Aldrich	G9002	0.1
HEPES	Sigma-Aldrich	H9136	10
KCl	Merck	1049360250	48
MgATP	Sigma-Aldrich	A9187	1
Na_2ATP	Sigma-Aldrich	A2383	4
Fluo-3**	Invitrogen	F3715	0.1
pH			7.20
adjusted with			1 M KOH

*On experimental days pipette solution is stored on ice until use.

**Fluo-3 is added from a 1 mM stock solution on experimental days

*Rebuttal Comments
Click here to download Rebuttal Comments: Answer to Reviewers comments_R1_final.doc

Reviewer #1:

This manuscript provides documentation of a technique that can be successfully used to isolate atrial myocytes from right atrial appendage specimens obtained from consenting surgical patients undergoing cardiac bypass graft with cannulation via the right atrial appendage. The authors have published several high profile (Circulation, Circ. Research, Cardiovascular Research, etc.) studies (of calcium cycling, potassium currents, etc.) based on the use of this myocyte isolation technique. Their expertise is nicely conveyed to the readers of this technical manuscript. The background literature is well cited.

We thank the reviewer for his/her positive evaluation of our manuscript and the constructive suggestion. A point-by-point response is detailed below.

Major Concerns:
One of the primary challenges associated with myocyte isolation is the determination of appropriate lots of collagenase and identification of appropriate incubation time, temperature, etc. Please comment briefly on the general approach that the readers can use to troubleshoot and perform such optimization.

According to the reviewer's suggestion we added the following section to the discussion:

Page 12, line 202: "The choice of collagenase is probably the most critical step for successful myocyte isolation. Conventional collagenases are crude preparations obtained from Clostridium histolyticum and contain collagenase in addition to a number of other proteinases, polysaccharidases and lipases. Based on their general composition collagenases are subdivided into 4 types (Type I to Type IV).[1] Types I, II and IV have been successfully used for isolation of human atrial myocytes.[2-15] In our presently described protocol we recommend the use of collagenase Type I, although we were also able to obtain acceptable amounts of viable cells using collagenase Type II. However, even within a single collagenase type there is a significant batch-to-batch variation regarding the enzyme activities. These variations require careful batch selection and testing of various batches to optimize isolation procedure. The online available batch-selection tool from Worthington Biochemical Corp. (http://www.worthington-biochem.com/cls/match.php) may be used to find available batches with a composition that has been shown to be suitable for the isolation of human atrial myocytes. Currently we use collagenase type I with 250 U/mg collagenase activity, 345 U/mg caseinase activity, 2.16 U/mg clostripain activity and 0.48 U/mg tryptic activity (lot# 49H11338)."

It is stated (p. 9/31) that the myocytes retain the ability to contract, but this phenotype is not documented. Given that a relatively large concentration of BDM (30 mM for up to 30 min) is used during transport, please comment on the extent of contraction observed during an action potential (% cell length), under resting conditions, and during the presence of adrenergic stimulation. What fraction of cells respond to field stimulation? What is the typical range of resting potentials?

We agree with this reviewer that the cytoprotective properties of 2,3-butandione monoxime have been attributed to its negative inotropic effects. However, it has been shown, that the effects of BDM are reversible upon washout.[16-18] In accordance

preparation of atrial trabeculae, which were obtained using BDM containing solution, were successfully used for measurements of contractility[16,19,20]. Our data thus far showed that stimulation of patch-clamped human atrial myocytes with the patch-clamp pipette induces clear and rhythmic cell shortening in more than 90% of investigated cells. In the revised paper we included a representative video-clip showing cellular contraction of a human atrial myocyte upon stimulation with the patch-clamp pipette.

In addition we cited our recent publication to answer the reviewer's concerns regarding resting membrane potential and response to adrenergic stimulation. We added representative AP recordings (novel figure 2) and representative measurements of $I_{Ca,L}$ current and Ca^{2+} transients in response to isoprenaline application (modified novel figure 4).

We added the following text to the results part:

*Page 10, line 147: "Representative examples of action–potential measurements and simultaneous recordings of cytosolic Ca^{2+} transients are given in **Figure 3**. In about 90% of the investigated cells, the action-potential-triggered Ca^{2+} release causes clear and regular cell contractions. As reported previously, the resting membrane potential, which is an accepted indicator for cell integrity, averaged about -73.9±2.7 mV (n = 23/10 myocytes/patients) and -77.7±1.8 mV (n = 19/8 myocytes/patients) in SR and cAF respectively (p>0.05).[13] **Figure 4** shows representative simultaneous recordings of voltage-gated L-type Ca^{2+} currents and cytosolic Ca^{2+} transients. Application of the non-selective β-adrenoceptor agonist isoprenaline (1 µM) increases amplitudes of both $I_{Ca,L}$ and cytosolic Ca^{2+} transients, suggesting intact β-adrenergic signal transduction cascade."*

Tissues are typically obtained from patients with significant cardiac disease. Please comment briefly on your experience with the impact of persistent AF, HF and ischemic disease (CAD) on the yield of viable atrial myocytes.

According to this reviewers suggestion we included an analysis of cell yield depending on the rhythm status of the patient (see panel B of novel Figure 1). These data suggest, that specimen from AF patients tend to have lower cell yields. According to our experience other clinical parameters such as HF or CAD do apparently not influence cell yield.

We included the following text in the revised version:

*Page 9, line 142: "Averaged cell yields in **Figure 2B** clearly indicate that there is a tendency to lower cell yields in cAF patient samples (tube A: 16.5±3.1 cells/10 µl (n=29) vs. 5.1±2.3 cells/10 µl (n=10) in SR and cAF, respectively, p<0.046; tube B: 17.9±3.9 cells/10 µl (n=29) vs. 5.9±2.0 cells/10 µl (n=9) in SR and cAF, respectively, p=0.107)."*

Have you utilized the same technique on tissues from left atrial specimens? What is the impact of chamber on yield?

We agree with this reviewer, that quantification of Ca^{2+} handling properties in the left atrium is of great importance. Ectopic activity, which may contribute to the maintenance of AF and which may result from Ca^{2+} handling abnormalities, is more

frequently located in the left atrium, especially at the pulmonary veins. However, during the last 2 years we have not been able to obtain left atrial samples from sinus rhythm or from AF patients. Therefore we cannot comment on whether this method is suitable for isolation of left atrial myocytes.

Minor Concerns:
There is a typo in table 1, last column. The pH is listed as 7-April (presumably 7.4?) It is notable that the pH during other steps is maintained at a low value. Please comment briefly on the rationale for this - presumably to decrease calcium influx?

Sorry for the floppy oversight.

It has been established that heart tissues exposed to Ca^{2+}-free media for minutes are severely damaged with return to Ca^{2+}-containing solution.[21] Although the exact reason for this Ca^{2+} paradox is unclear, a reduction of the pH to 6.8 and the related increase in extracellular H^+ concentration may favor the extrusion of intracellular Na^+ by Na^+/H^+ exchanger and thereby reduce Na^+-load, which is assumed to play a crucial role in the pathogenesis of the Ca^{2+} paradox.[22]

In response to the reviewers comment we rephrased the text as follows:

Page 11, line 190: "Modifications of the isolation media including reduction of the pH to 7.0,[22] addition of taurin[23] or of small amounts of Ca^{2+} (see step 3.2 and 4.1),[24] as well as storage of isolated myocytes in EGTA containing storage-solution[25] have been suggested to prevent the Ca^{2+} paradox.[21]"

Reviewer #2:

Human atrial myocytes isolation is a very interesting technique for electrophysiology. This protocol allows to obtaine good quality cells. Description of the protocol is well detailed. However, I have some minor comments.

We thank the reviewer for his/her helpful comments and constructive critiques of our original manuscript that led us to improve substantially our revised manuscript. Detailed responses to individual points are provided below.

Major Concerns:
None.

Minor Concerns:
1) With respect to the solutions temperature, authors writtes that they didn't recognize any advantage on cooling the transport solution but the point 2.3 of the protocol is do it at 4ºC. Is it not better to maintain the tissue at 4ºC all the time until the step 2.5?

We agree with this reviewer that there might be a possible advantage of cooling the tissue during transport, especially because cooling has been shown to prevent the Ca^{2+} paradox.[21,24,26-29] However, because of practical reasons we did not transport the tissue samples on ice and did not experience any disadvantage, probably because of the presence of BDM, which has been shown to prevent the Ca^{2+} paradox as well.

However, since BDM was not present during the chopping of the tissue, this was performed at 4°C to reduce the cutting injury of the tissue.[16]

We modified the revised text as follows:

Page 4, line 32: "With transport times between 30 and 45 minutes, we did not recognize a clear advantage of cooling or oxygenation with respect to both number and quality of isolated atrial cardiomyocytes. In general transport to the lab should be as quick as possible. However, if longer transportation time cannot be avoided, transport at 4°C in oxygenated solution might be advantageous."

2) Time limit of transport should be mention.

Surprisingly, the tissue seems to tolerate quite long transport time with respect to cell yield and quality of cells. Thus we were able to isolated myocytes with good quality and quantity after storage of the tissue for more than one hour at room temperature. Therefore we cannot provide a specific limit of transport time. However, in general we managed to bring the tissue to the lab within a time frame of 30 and 45 minutes.

Considering this reviewer's concerns we rephrased the manuscript as follows:

Page 4, line 32: "With transport times between 30 and 45 minutes, we did not recognize a clear advantage of cooling or oxygenation with respect to both number and quality of isolated atrial cardiomyocytes. In general transport to the lab should be as quick as possible. However, if longer transportation time cannot be avoided, transport at 4°C in oxygenated solution might be advantageous."

3) Why don't you gas with O2 all the solutions, including transport solution?

We did not experience a significant advantage of gassing the transport solution during tissue transport to the lab and gassing of transport solution would be a disproportionally high effort. Having said this, it is unclear whether longer transportation time might be better tolerated with gassing of the transport solution. Therefore we rephrased the manuscript as follows:

Page 4, line 32: "With transport times between 30 and 45 minutes, we did not recognize a clear advantage of cooling or oxygenation with respect to both number and quality of isolated atrial cardiomyocytes. In general transport to the lab should be as quick as possible. However, if longer transportation time cannot be avoided, transport at 4°C in oxygenated solution might be advantageous."

4) It would be good to indicate, perhaps in the 4.6 protocol point that bubbles are detrimental to cell survival and quality.

The following text has been added to the revised version:

Page 6, line 57: "A pipette tip can be placed onto the oxygen tube to avoid strong bubbling and generate continuous air flow."

Page 8, line 86: "Try to avoid bubble formation since bubbles are detrimental to cell survival and quality."

5) In your patch-clamp experiments, what is the resting potential of your cells? More information about that could be given in the Representative Results.

We agree that the resting membrane potential is an important marker of cellular

integrity. We cited our recent publication to answer the reviewer's concerns regarding the resting membrane potential. In addition we added representative AP recordings (novel figure 3).

We added the following text to the results part:

*Page 10, line 147: "Representative examples of action–potential measurements and simultaneous recordings of cytosolic Ca^{2+} transients are given in **Figure 3**. In about 90% of the investigated cells, the action-potential-triggered Ca^{2+} release causes clear and regular cell contractions. As reported previously, the resting membrane potential, which is an accepted indicator for cell integrity, averaged about -73.9±2.7 mV (n = 23/10 myocytes/patients) and -77.7±1.8 mV (n = 19/8 myocytes/patients) in SR and cAF respectively (p>0.05).[13]"*

6) There are some topographic erors in Table I. 3 times 1-Feb in KH2PO4 and 1 time 7-Apr in pH.

We apologize for the sloppy oversights and corrected the typing errors in the revised version.

Reviewer #3:

The authors have developed an improved method for isolating human atrial myocytes from patient tissue samples. With their protocol they are able to produce myocytes that are sufficiently healthy for patch-clamp and Ca2+ imaging experiments. Although the paper is short, I think that the method they describe will be very useful for those working with human cardiac cell physiology. My major concern is, however, the originality of the method they describe (see comment #5 below).

We thank the reviewer for his/her helpful comments and constructive critiques on our original article. All suggestions have been addressed in the revised version of our manuscript, as detailed below.

Major Concerns:
#1. The authors do not report how long the cells retain their viability. For many applications cell are incubated in culture conditions from couple of hours up to couple of days. Does this protocol produce cells that are useful also for longer term experiments?

It is definitely of great interest to keep human atrial myocytes for a couple of days in culture. In fact we are currently trying to establish a method to keep these cells at physiological temperatures for more than 24 hours. However, the utility of this isolation method is limited under this aspect, since the cell yield is relatively low compared to previously described methods (compare: 40 cell/10 µl).[30]

We considered this point in the following paragraph:

Page 13, line 218: "The cells obtained using the procedure described in this manuscript may be used within 8 hours for patch-clamp studies, Ca^{2+} transient measurements and a combination of both.[13]"

#2. A good indicator of the integrity of the cells is the resting membrane potential. What is the range of resting potential of these cells?

We agree that the resting membrane potential is an important marker of cellular integrity. We cited our recent publication to answer the reviewer's concerns regarding the resting membrane potential. In addition we added representative AP recordings (novel figure 2).

We added the following text to the results part:

*Page 10, line 147: "Representative examples of action–potential measurements and simultaneous recordings of cytosolic Ca^{2+} transients are given in **Figure 3**. In about 90% of the investigated cells, the action-potential-triggered Ca^{2+} release causes clear and regular cell contractions. As reported previously, the resting membrane potential, which is an accepted indicator for cell integrity, averaged about -73.9±2.7 mV (n = 23/10 myocytes/patients) and -77.7±1.8 mV (n = 19/8 myocytes/patients) in SR and cAF respectively (p>0.05).[13]"*

#3. The method section does not include the solutions, conditions and methods used for patch-clamp and calcium-imaging experiments.

As suggested by this reviewer we included an additional section together with the novel figure 1 and novel tables III-V providing a step-by-step description of loading the myocytes with the Ca^{2+} fluorescent indicator Fluo-3 AM and a general description of the patch-clamp experiments, including tables with bath and pipette solutions. However, since the description of patch-clamp studies is not the main purpose of this manuscript, we refer the interested reader to literature with a more in depth discussion of this method.

Page 8, line 100:

*"6) **Loading of myocytes with the fluorescent Ca^{2+}-indicator Fluo-3 AM (Figure 1)***

6.1) Transfer 1.5 ml of cell suspension (tube A and/or tube B) into a 2 ml microcentrifuge tube (Eppendorf™ tube).

6.2) The following steps should be executed under consideration of the light sensitivity of the fluorescent Ca^{2+} Indicator Fluo-3.

*6.3) Dissolve 50 µg of the membrane permeable acetoxymethyl ester derivative of Fluo-3 (Fluo-3 AM, **Table III**) in 44 µl of the Pluronic F-127 (**Table III**) stock solution (20% w/v in anhydrous DMSO) to get a 1 mM Fluo-3 AM stock solution, which can be stored at -20 °C for at most 1 week.*

6.4) Add 15 µl of the Fluo-3 AM stock solution to the microcentrifuge tube containing 1.5 ml of cell suspension (see step 6.1) and agitate carefully.

6.5) Incubate the cell suspension for 10 minutes in an optically opaque box.

6.6) Briefly centrifuge at about 6000 rpm.

*6.7) Discard the supernatant and dissolve the pellet in 1.5 ml bath solution (**Table IV**).*

6.8) Leave the cell suspension for about 30 minutes for de-esterification before beginning with experiments.

Simultaneous patch-clamp and epifluorescent Ca^{2+} measurements

Since patch-clamp measurements are not the major topic of this review, we refer the interested reader to other publications providing a more in depth description of this technique.[30-33] For the sake of completeness we provide a brief summary of a protocol to measure action potentials or L-type Ca^{2+} currents, both together with simultaneous Ca^{2+}-transient recordings.

*During experiments myocytes are superfused at 37°C with bath solution (**Table IV**) using a rapid perfusion system (Octaflow II™, ALA Scientific Instruments, NY). For voltage-clamp experiments, K^+ currents are blocked by adding 4-aminopyridine (5 mmol/L) and $BaCl_2$ (0.1 mmol/L) to the bath solution. Borosilicate glass microelectrodes are used and should have tip resistances of 2-5 MΩ when filled with pipette solution (**Table V**). In addition to the Fluo-3 AM loading of the myocytes (see step 6), Fluo-3 is also included in the pipette solution (**Table V**). Fluorescence is excited at 488 nm and emitted light (<520 nm) converted to $[Ca^{2+}]_i$ assuming*

$$[Ca^{2+}]_i = k_d\left(\frac{F}{F_{\max} - F}\right)$$

where k_d=dissociation constant of Fluo-3 (864-nmol/L), F=Fluo-3 fluorescence; F_{max}=Ca^{2+}-saturated fluorescence obtained at the end of each experiment.[31]

Both electrical signals and epifluorescent Ca^{2+} signals are recorded simultaneously. Action potentials are stimulated at 0.5 Hz in current-clamp mode using 1 ms current pulses of 1.2x threshold strength. L-type Ca^{2+}-currents are measured in voltage-clamp mode using a holding potential of -80 mV and a 100-ms ramp-pulse to -40 mV to inactivate the fast Na^+-current, followed by a 100-ms test-pulse to +10 mV at 0.5 Hz."

#4. What does the IM means in the label of the y-axis in the fig. 2 (L-type calcium current), should it be I L-Ca or something like that?

I_M is the abbreviation for total net membrane current. Under the conditions we used I_M is predominantly determined by $I_{Ca,L}$. However, since other currents like I_{NCX} or capacitance artifacts also influence the recorded current, we found it more accurate to label the ordinate with "I_M".

However, to clarify that point we added the following explanation to the legend of the novel Figure 4:

Page 12, line 176: "Simultaneous recording of total net membrane current (I_M), predominantly reflecting L-type Ca^{2+} current (middle) and triggered CaT (bottom)."

#5. How does the isolation method described in this manuscript differ from the methods published previously by the same group (Voigt et al. 2010, Methods Enzymol.; Dobrev et al. 2000, Circulation; Voigt et al. 2012, Circulation)? Since the representative data in Fig.2 is replotted from Voigt et al. 2012, was the isolation method identical?

We agree with this reviewer, that these methods appear to be similar to the procedures described previously (Voigt et al. 2010, Methods Enzymol.; Dobrev et al. 2000, Circulation). However, in these protocols the EGTA containing storage medium

prevented the measurement of intracellular Ca^{2+} transients. Therefore we adapted these methods by excluding EGTA to measure and quantify Ca^{2+} transients in these cells (Voigt et al. 2012, Circulation). In addition these cells have resting membrane potentials of physiological size and remain contractile enabling the measurement of single cell shortening.

Against this background the described method is comparable to that previously published by our group (Voigt et al. 2012). However, in line with the journal's guidelines to publish a wide range of methods from "basic biology protocols to complex medical procedures", we feel that the detailed step by step description, the additional video, which will be produced according to our protocol and the provision of catalogue numbers for used substances, will enable other groups to establish this protocol in their lab more easily and will be therefore of great value to the readers.

Minor Concerns:
None.

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