

## Video Article

# Isolation of Human Atrial Myocytes for Simultaneous Measurements of $\text{Ca}^{2+}$ Transients and Membrane Currents

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Keywords: Cellular Biology, Issue 77, Medicine, Molecular Biology, Physiology, Anatomy, Cardiology, Pharmacology, human atrial myocytes, cell isolation, collagenase, calcium transient, calcium current, patch-clamp, ion currents, isolation, cell culture, myocytes, cardiomyocytes, electrophysiology, patch clamp

Date Published: 7/3/2013

Citation: Voigt, N., Zhou, X.B., Dobrev, D. Isolation of Human Atrial Myocytes for Simultaneous Measurements of  $\text{Ca}^{2+}$  Transients and Membrane Currents. *J. Vis. Exp.* (77), e50235, doi:10.3791/50235 (2013).

## Abstract

The study of electrophysiological properties of cardiac ion channels with the patch-clamp technique and the exploration of cardiac cellular  $\text{Ca}^{2+}$  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).<sup>1</sup> Here we describe a method for isolation of human atrial myocytes which are suitable for both patch-clamp studies and simultaneous measurements of intracellular  $\text{Ca}^{2+}$  concentrations. First, right atrial appendages obtained from patients undergoing open heart surgery are chopped into small tissue chunks ("chunk method") and washed in  $\text{Ca}^{2+}$ -free solution. Then the tissue chunks are digested in collagenase and protease containing solutions with 20  $\mu\text{M}$   $\text{Ca}^{2+}$ . Thereafter, the isolated myocytes are harvested by filtration and centrifugation of the tissue suspension. Finally, the  $\text{Ca}^{2+}$  concentration in the cell storage solution is adjusted stepwise to 0.2 mM. We briefly discuss the meaning of  $\text{Ca}^{2+}$  and  $\text{Ca}^{2+}$  buffering during the isolation process and also provide representative recordings of action potentials and membrane currents, both together with simultaneous  $\text{Ca}^{2+}$  transient measurements, performed in these isolated myocytes.

## Video Link

The video component of this article can be found at <https://www.jove.com/video/50235/>

## Introduction

Studying electrophysiological properties of cardiac ion channels with the patch-clamp technique and the exploration of cellular  $\text{Ca}^{2+}$  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<sup>2</sup> 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 Dissociation guide" provided by Worthington Biochemical Corp., USA ([www.tissuedissociation.com](http://www.tissuedissociation.com)).

Human atrial myocyte isolation protocols are generally derived from the method described by Bustamante, *et al.*<sup>3</sup> 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  $\text{Ca}^{2+}$  measurements.<sup>4-11</sup>

## Protocol

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 informed consent.

## 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 sample is transferred

immediately into a 50 ml Falcon tube with sterile  $\text{Ca}^{2+}$ -free transport solution containing 2,3-butanedione monoxime (**Table I**; BDM, contractile inhibitor, preventing myocyte contracture). With transport times between 30 and 45 min, 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.

## 1. Prearrangements

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.
2. Weigh the Collagenase I and Protease XXIV into three glass beakers and store it at room temperature. The enzymes will be diluted just before use.
3. Store 20 ml of  $\text{Ca}^{2+}$  free solution in a Petri dish at 4 °C.
4. Store 250 ml of  $\text{Ca}^{2+}$  free solution in a water bath at 37 °C.

## 2. Cleaning of the Tissue

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. 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.
3. Transfer the tissue sample into the Petri dish containing 20 ml  $\text{Ca}^{2+}$  free solution at 4 °C (see step 1.3) and chop the tissue sample into small chunks of approximately 1 mm<sup>3</sup> in size.
4. The following steps should be executed at 37 °C and under continuous gassing with 100% O<sub>2</sub>. A pipette tip can be placed onto the oxygen tube to avoid strong bubbling and generate continuous air flow.
5. Transfer the tissue chunks together with the  $\text{Ca}^{2+}$ -free solution into the jacketed beaker and stir carefully for about 3 min with a magnetic stirring bar.
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.
7. Refill the beaker with  $\text{Ca}^{2+}$  free solution and repeat the washing step 2.4 and 2.5 two times.

## 3. First Enzymatic Digestion

1. Re-suspend the tissue chunks with 20 ml Enzyme solution E1 (**Table I**) containing collagenase and protease and stir carefully for 10 min.
2. Add 40 µl of 10 mM  $\text{CaCl}_2$ -solution to obtain a final concentration of 20 µM  $\text{Ca}^{2+}$ .
3. After 35 min 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

1. Resuspend the tissue chunks again with 20 ml enzyme solution E2 containing collagenase I only (**Table I**). Add 40 µl of 10 mM  $\text{CaCl}_2$ -solution immediately to obtain a final concentration of 20 µM  $\text{Ca}^{2+}$ .
2. During the second digestion use scissors to further chop tissue clots occasionally.
3. After 5 min take a sample using a Pasteur pipette to check the dissociation of cells. Repeat this every 2-3 min until rod-shaped, striated cardiomyocytes appear.
4. Stop stirring and allow the tissue chunks to settle down for about 20-30 sec.
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.
6. Re-suspend the tissue chunks in the beaker with 20 ml storage solution (**Table I**)<sup>10</sup> 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.
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 $\text{Ca}^{2+}$ Concentration

1. Centrifuge both Falcon tubes A and B (see step 4.5 and 4.7) at 95 x g for 10 min.
2. Remove the supernatant from both tubes carefully by aspiration. Make sure not to disturb the pellet. Discard the supernatant.
3. Re-suspend both pellets in 1.5 ml storage solution (**Table I**) each (room temperature).
4. Add two times 7.5 µl of 10 mM  $\text{CaCl}_2$  solution to each Falcon and stir carefully. Incubate for 10 min after each step.
5. Add 15 µl of 10 mM  $\text{CaCl}_2$  solution to obtain a final  $\text{Ca}^{2+}$  concentration of 0.2 mM.

## 6. Loading of Myocytes with the Fluorescent $\text{Ca}^{2+}$ -indicator Fluo-3 AM (Figure 1)

1. Transfer 1.5 ml of cell suspension (tube A and/or tube B) into a 2 ml microcentrifuge tube (Eppendorf tube).
2. The following steps should be executed under consideration of the light sensitivity of the fluorescent  $\text{Ca}^{2+}$  Indicator Fluo-3.

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.
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.
5. Incubate the cell suspension for 10 min in an optically opaque box.
6. Briefly centrifuge at about 6,000 rpm.
7. Discard the supernatant and re-suspend the pellet in 1.5 ml bath solution (**Table IV**).
8. Leave the cell suspension for about 30 min for de-esterification before beginning with experiments.

## 7. Simultaneous Patch-clamp and Epifluorescent Ca<sup>2+</sup> 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.<sup>11-14</sup> For the sake of completeness we provide a brief summary of a protocol to measure action potentials or L-type Ca<sup>2+</sup> currents, both together with simultaneous Ca<sup>2+</sup>-transient recordings.

During experiments myocytes are superfused at 37 °C with bath solution (**Table IV**) using a rapid perfusion system (Octaflow IITM, ALA Scientific Instruments, NY). For voltage-clamp experiments, K<sup>+</sup> currents are blocked by adding 4-aminopyridine (5 mmol/L) and BaCl<sub>2</sub> (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<sup>2+</sup>]<sub>i</sub> assuming

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

where  $k_d$ =dissociation constant of Fluo-3 (864 nM),  $F$ =Fluo-3 fluorescence;  $F_{max}$ =Ca<sup>2+</sup>-saturated fluorescence obtained at the end of each experiment.<sup>12</sup> Both electrical signals and epifluorescent Ca<sup>2+</sup> signals are recorded simultaneously. Action potentials are stimulated at 0.5 Hz in current-clamp mode using 1 msec current pulses of 1.2x threshold strength. L-type Ca<sup>2+</sup>-currents are measured in voltage-clamp mode using a holding potential of -80 mV and a 100-msec ramp-pulse to -40 mV to inactivate the fast Na<sup>+</sup>-current, followed by a 100-msec 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<0.05; 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<sup>2+</sup> transients are given in **Figure 3**. In about 90% of the investigated cells, the action-potential-triggered Ca<sup>2+</sup> 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).<sup>15</sup> **Figure 4** shows representative simultaneous recordings of voltage-gated L-type Ca<sup>2+</sup> currents and cytosolic Ca<sup>2+</sup> transients. Application of the non-selective β-adrenoceptor agonist isoprenaline (1 µM) increases amplitudes of both I<sub>Ca,L</sub> and cytosolic Ca<sup>2+</sup> transients, suggesting intact β-adrenergic signal transduction cascade.

## Protocol for loading human atrial myocytes with Fluo-3 AM

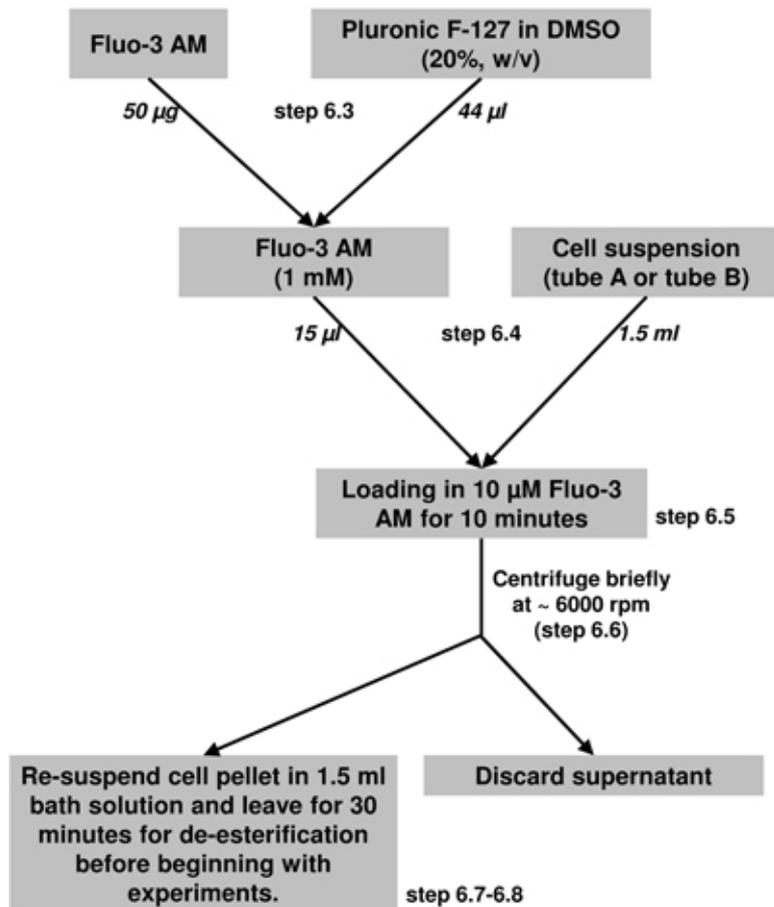
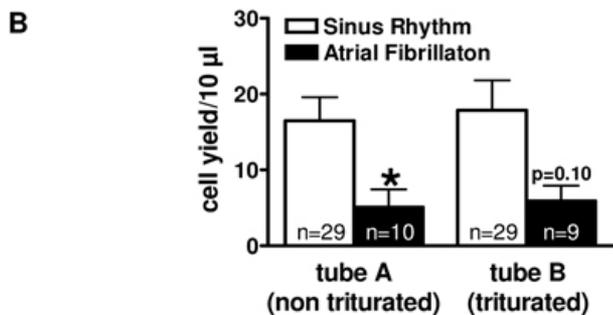
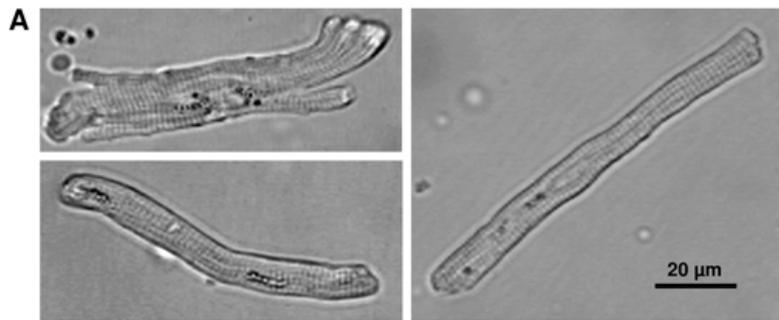
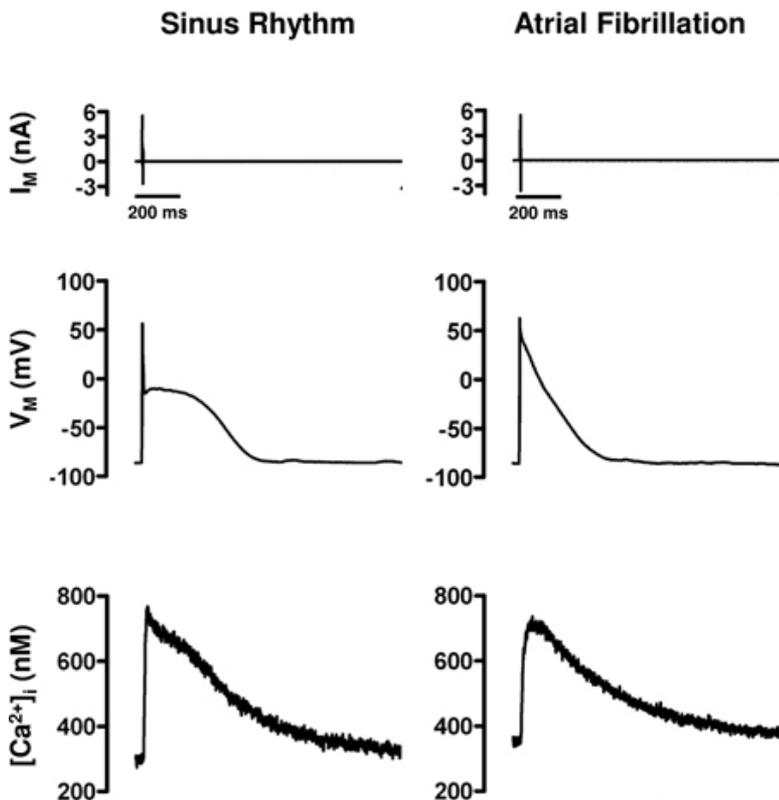


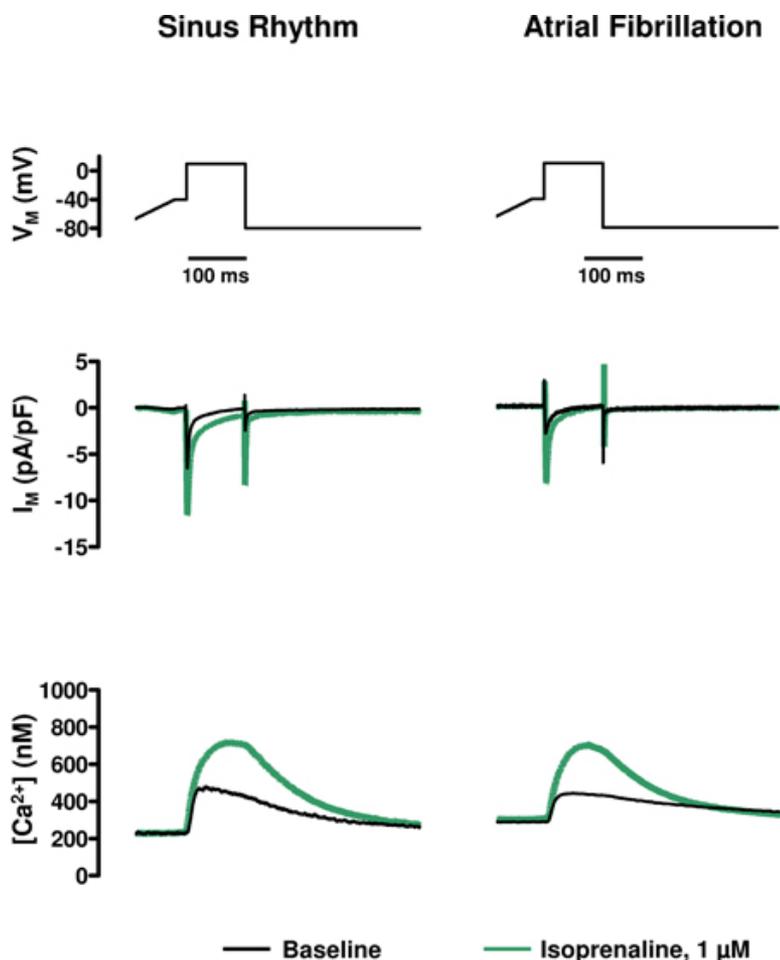
Figure 1. Flow chart of the myocytes Fluo-3 AM loading protocol (see step 6.1-6.5). m/v, mass/volume.



**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)<sup>15</sup>



**Figure 4.** Representative recordings of the isoprenaline (1  $\mu\text{M}$ ) effect on L-type  $\text{Ca}^{2+}$  current-triggered  $\text{Ca}^{2+}$ -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  $\text{Ca}^{2+}$  current (middle) and triggered CaT (bottom). (Replotted with permission from Voigt, *et al.* 2012)<sup>15</sup>

	Company	Catalogue number	Transport solution	$\text{Ca}^{2+}$ -free solution	Enzyme solution E1 and E2	Storage solution
Albumin	Sigma-Aldrich	A3059	–	–	–	1%
BDM	Sigma-Aldrich	31550	30	–	–	–
DL- $\beta$ -Hydroxybutyric 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
$\text{KH}_2\text{PO}_4$	Sigma-Aldrich	P5655	1.2	1.2	1.2	10
$\text{MgSO}_4$	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 I. Solutions.**

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

Table II. Specific equipment.

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

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

	Company	Catalogue number	Bath solution
4-aminopyridine*	Sigma-Aldrich	A78403	5
BaCl <sub>2</sub> *	Sigma-Aldrich	342920	0.1
CaCl <sub>2</sub> × 2H <sub>2</sub> O	Sigma-Aldrich	C5080	2
Glucose	Sigma-Aldrich	G8270	10
HEPES	Sigma-Aldrich	H9136	10
KCl	Merck	1049360250	4
MgCl × 6H <sub>2</sub> O	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 experiments

Table IV. Bath solution for patch-clamp.

	Company	Catalogue number	Pipette Solution
DL-aspartat K <sup>-</sup> -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 <sub>2</sub> ATP	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

Table V. Pipette solution for patch-clamp\*.

## 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<sup>2+</sup> we adapted a previously described method<sup>4-11</sup> by omitting EGTA from the storage solution.

Already in 1970 it was observed that although myocytes dissociate in the presence of Ca<sup>2+</sup> during digestion, all of them were in contracture and non-viable.<sup>16,17</sup> Therefore, cell isolation is performed in Ca<sup>2+</sup>-free solution. However, the re-introduction of physiological concentrations of Ca<sup>2+</sup> resulted in rapid Ca<sup>2+</sup> influx and cell death. This has been described as the Ca<sup>2+</sup> paradox phenomenon which was originally observed in perfused hearts by Zimmerman and Hulsman.<sup>18</sup> Modifications of the isolation media including reduction of the pH to 7.0,<sup>19</sup> addition of taurin<sup>20</sup> or of small amounts of Ca<sup>2+</sup> (see step 3.2 and 4.1),<sup>21</sup> as well as storage of isolated myocytes in EGTA containing storage-solution<sup>22</sup> have been suggested to prevent the Ca<sup>2+</sup> paradox.<sup>17</sup> However, it is well known that Ca<sup>2+</sup> buffering through EGTA reduces the amplitude of L-type Ca<sup>2+</sup> current-induced Ca<sup>2+</sup> transient amplitudes and results in a biphasic decay of the Ca<sup>2+</sup> transients.<sup>23</sup> Therefore, we omitted EGTA throughout the whole isolation process in order to obtain Ca<sup>2+</sup> transients with typical properties and monophasic decays. To protect the cells from the Ca<sup>2+</sup> paradox we increased the final Ca<sup>2+</sup> 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 different types.<sup>24</sup> Worthington collagenase Types I and II have been successfully used for isolation of human atrial myocytes.<sup>4-10,15,25-30</sup> 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/clis/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 hr for patch-clamp studies, Ca<sup>2+</sup> transient measurements and a combination of both.<sup>15</sup> 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).

## Disclosures

Worthington Biochemical Corp. supported this publication by covering production costs of video file. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

## Acknowledgements

In addition, we thank the cardiac surgeons at Heidelberg University for the provision of human atrial tissue and Claudia Liebetrau, Katrin Kupser and Ramona Nagel for their excellent technical support. Special thanks also to Andy W. Trafford for his helpful suggestions and advice during the establishment of the Ca<sup>2+</sup> transient measurements. The authors wish to express their deepest gratitude to the members of the Department of Pharmacology and Toxicology (head: Ursula Ravens) of the Dresden University of Technology for the opportunity they offered us to learn basic techniques and skills in cellular electrophysiology and cardiomyocyte isolation.

The authors' research is supported by the German Research Foundation (Do769/1-1-3), the German Federal Ministry of Education and Research through the Atrial Fibrillation Competence Network (01Gi0204) and the German Centre for Cardiovascular Research, the European Union through the European Network for Translational Research in Atrial Fibrillation (EUTRAF, FP7-HEALTH-2010, large-scale integrating project, Proposal No. 261057) and the European-North American Atrial Fibrillation Research Alliance (ENAFRA) grant of Fondation Leducq (07CVD03).

The schematic overview shown in the video file was produced using Servier medical art.

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