Video Article

Isolation of Human Ventricular Cardiomyocytes from Vibratome-Cut Myocardial Slices

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Abstract

The isolation of ventricular cardiac myocytes from animal and human hearts is a fundamental method in cardiac research. Animal cardiomyocytes are commonly isolated by coronary perfusion with digestive enzymes. However, isolating human cardiomyocytes is challenging because human myocardial specimens usually do not allow for coronary perfusion, and alternative isolation protocols result in poor yields of viable cells. In addition, human myocardial specimens are rare and only regularly available at institutions with on-site cardiac surgery. This hampers the translation of findings from animal to human cardiomyocytes. Described here is a reliable protocol that enables efficient isolation of ventricular myocytes from human and animal myocardium. To increase the surface-to-volume ratio while minimizing cell damage, myocardial tissue slices 300 µm thick are generated from myocardial specimens with a vibratome. Tissue slices are then digested with protease and collagenase. Rat myocardium was used to establish the protocol and quantify yields of viable, calcium-tolerant myocytes by flow-cytometric cell counting. Comparison with the commonly used tissue-chunk method showed significantly higher yields of rod-shaped cardiomyocytes (41.5 ± 11.9 vs. 7.89 ± 3.6%, $p < 0.05$). The protocol was translated to failing and non-failing human myocardium, where yields were similar as in rat myocardium and, again, markedly higher than with the tissue-chunk method (45.0 ± 15.0 vs. 6.87 ± 5.23 cells/mg, $p < 0.05$). Notably, with the protocol presented it is possible to isolate reasonable numbers of viable human cardiomyocytes (9–200 cells/mg) from minimal amounts of tissue (<50 mg). Thus, the method is applicable to healthy and failing myocardium from both human and animal hearts. Furthermore, it is possible to isolate excitable and contractile myocytes from human tissue specimens stored for up to 36 h in cold cardioplegic solution, rendering the method particularly useful for laboratories at institutions without on-site cardiac surgery.

Introduction

A seminal technique that has paved the way to important insights into cardiomyocyte physiology is the isolation of living ventricular cardiomyocytes from intact hearts[1]. Isolated cardiomyocytes can be used to study normal cellular structure and function, or the consequences of in vivo experiments; for example, to assess changes in cellular electrophysiology or excitation-contraction coupling in animal models of cardiac disease. Additionally, isolated cardiomyocytes can be used for cell culture, pharmacological interventions, gene transfer, tissue engineering, and many other applications. Therefore, efficient methods for cardiomyocyte isolation are of fundamental value to basic and translational cardiac research.

Cardiomyocytes from small mammals, such as rodents, and from larger mammals, such as pigs or dogs, are commonly isolated by coronary perfusion of the heart with solutions containing crude collagenases and/or proteases. This has been described as the “gold standard” method for cardiomyocyte isolation, resulting in yields of up to 70% of viable cells[2]. The approach has also been used with human hearts, resulting in acceptable cardiomyocyte yields[3,4,5]. However, because coronary perfusion is only feasible if the intact heart or a large myocardial wedge containing a coronary artery branch is available, most human cardiac specimens are not suited for this approach due to their small size and a lack of appropriate vasculature. Therefore, the isolation of human cardiomyocytes is challenging.

Human myocardial specimens mostly consist of tissue chunks of variable size (approximately 0.5 x 0.5 x 0.5 cm to 2 x 2 x 2 cm), obtained through endomyocardial biopsies[6], septal myectomies[7], VAD implantations[8], or from explanted hearts[9]. The most common procedures for cardiomyocyte isolation start with mincing the tissue using scissors or a scalpel. Cell-to-cell contacts are then disrupted by immersion in calcium-free or low-calcium buffers. This is followed by multiple digestion steps with crude enzyme extracts or purified enzymes like proteases (e.g., trypsin), collagenase, hyaluronidase, or elastase, resulting in a disintegration of the extracellular matrix and liberation of cardiomyocytes. In a final, critical step, a physiological calcium concentration has to be carefully restored, or cellular damage can occur due to the calcium-paradox[10,11,12]. This isolation approach is convenient but usually inefficient. For instance, one study found that nearly 1 g of myocardial tissue was required to obtain a sufficient number of cardiomyocytes suitable for subsequent experiments[13]. A possible reason for low yields is the relatively harsh method of mincing the tissue. This may particularly damage cardiomyocytes located at the chunk edges; although the myocytes are most likely to be released by enzymatic digestion.

Another aspect that may influence isolation efficiency and quality of cells obtained from human specimens is the duration of tissue ischemia. Most protocols mention short transportation times to the laboratory as a prerequisite for best results. This restricts the study of human ventricular cardiomyocytes to laboratories with nearby cardiac surgery facilities. Together, these restrictions hamper the verification of important findings

from animal models in human cardiomyocytes. Improved isolation protocols that allow for high cardiomyocyte yields from small amounts of tissue, preferably without serious damage after extended transportation times, are therefore desirable.

Described here is an isolation protocol based on the enzymatic digestion of thin myocardial tissue slices generated with a vibratome[14,15]. We demonstrate that isolation from tissue slices is much more efficient than that from tissue chunks minced with scissors. The described method not only allows for high yields of viable human cardiomyocytes from small amounts of myocardial tissue but is also applicable to specimens stored or transported in cold cardioplegic solution for up to 36 h.

Female Wistar rats (150–200 g) were commercially obtained, anesthetized by injecting 100 mg/kg of thiopental-sodium intraperitoneally, and euthanized by cervical dislocation followed by thoracotomy and excision of the heart. Human cardiac tissue samples were collected from the left-ventricular apical core during implantation of mechanical assist devices, from septal myectomy, from tetralogy of Fallot corrective surgery, or from the free left-ventricular wall of explanted hearts. The following protocol describes the isolation from human ventricular tissue. The isolation of rat cardiomyocytes was performed accordingly, but with different enzymes (see **Table of Materials**). A schematic workflow of the protocol is illustrated in **Figure 1**.

Protocol

All experiments with rats were approved by the Animal Care and Use Committee Mittelfranken, Bavaria, Germany. Collection and use of human cardiac tissue samples was approved by the Institutional Review Boards of the University of Erlangen-Nürnberg and the Ruhr-University Bochum. Studies were conducted according to Declaration of Helsinki guidelines. Patients gave their written informed consent prior to tissue collection.

1. Preparation of buffers, solutions, and enzymes

1. Prepare buffers and solutions as listed in **Table 1**.
2. Warm up solutions 1, 2, 3 and the modified Tyrode's solution to 37 °C. Store the cutting solution at 4 °C until use.
   NOTE: For 1–2 myocardial slices a total of approximately 15 mL, 8 mL, and 5 mL of solutions 1, 2, and 3 are required for the isolation in one 35 mm tissue culture dish. Scale up accordingly for simultaneous myocyte isolations in multiple dishes. Cutting solution can be frozen and kept at -20 ˚C for several months.
3. Weigh 1 mg of proteinase XXIV (see **Table of Materials**) into a prechilled 15 mL centrifuge tube and store on ice until use. This is for processing one sample. Scale up the amount accordingly. Do not mix the proteinase and collagenase.
4. Weigh 8 mg of collagenase CLSI (see **Table of Materials**) into a prechilled 15 mL centrifuge tube and store on ice until use. This is for processing one sample. Scale up the amount accordingly. Do not mix the proteinase and collagenase.
   CAUTION: Wear a face mask or work under a fume hood to avoid inhalation of enzyme powder.
   NOTE: Enzyme activity may vary in different lots. Therefore, the optimal concentration may differ and should be determined with each newly purchased enzyme[16].

2. Storage and transport of myocardial tissue

1. Store and transport human cardiac samples in cooled, 4 ˚C cutting solution (**Table 1**).
2. Use the same solution for further tissue processing and vibratome slicing.
   NOTE: Biopsies and surgical heart samples should be transferred immediately to the cutting solution at 4 °C and can then be stored or transported at 4 °C for a maximum of 36 h before the application of this protocol.

3. Processing and slicing of the tissue

NOTE: The protocol for tissue slicing follows Fischer et al.[15].

1. Trimming of the tissue block
   CAUTION: Human cardiac tissue is potentially infectious ays use gloves. Carefully handle used blades and discard in safety containers.
   1. Place the specimen into a 100 mm tissue culture dish filled with 20 mL cold cutting solution and keep on a cooled, 4 °C plate.
   2. Remove excess fibrotic tissue and epicardial fat with a scalpel. In case of a transmural specimen, remove trabeculae and tissue layers near the endocardium.
      NOTE: Fibrotic tissue is stiff and appears white. Fat is typically soft and appears white to yellow. Trabeculae and endocardial tissue layers can be identified from their loose tissue composition and a nonaligned fiber orientation compared to myocardium of the epicardial layers
   3. For optimal vibratome processing, cut rectangular tissue blocks of approximately 8 mm x 8 mm x 8 mm with a scalpel from a larger tissue specimen. For smaller biopsies skip this step and move to agarose embedding.

2. Embedding cardiac tissue into low-melting-point agarose
   1. Boil 400 mg of low-melting point agarose in 10 mL of cutting solution in a glass beaker.
      CAUTION: Wear gloves and safety glasses to avoid burns. Handle hot glassware only with heat protection wear.
   2. Fill a 10 mL syringe with the hot, dissolved agarose gel. Seal the syringe and allow the agarose to equilibrate in a 37 °C water bath for at least 15 min.
   3. Use forceps to place the trimmed cardiac specimen or biopsy into a clean 35 mm tissue culture dish with the epicardium facing down and remove excess fluid with a sterile swab.
   4. Pour the equilibrated agarose (step 3.2.2) over the tissue by emptying the syringe. Secure the tissue against movement with forceps while pouring the agarose. Make sure that the tissue is completely immersed in agarose.

5. Immediately place the dish on ice and let the agarose solidify for 10 min.

3. Slicing the myocardium
    1. Mount an unused blade to the blade holder of the vibratome and calibrate the vibratome by adjusting the z-deflection of the blade if possible.
    NOTE: This protocol uses a vibratome with an infrared-assisted calibration device to align the blade in a horizontal position with minimal z-deflection (measured deflection <0.1 µm).
    2. Use a scalpel to excise an agarose-tissue block that fits the specimen holder of the vibratome. To ensure stability, make sure the tissue is still sufficiently immersed in agarose (agarose margins ≥ 8 mm).
    3. Fix the agarose block to the specimen holder with a thin layer of cyanoacrylate glue and gentle pressure.
    4. Place the specimen holder with the tissue into the vibratome bath. Fill the bath with cutting solution and keep at 4–6 °C throughout the processing with crushed ice, filled in the outer cooling tank of the vibratome.
    5. Generate 300 µm thick slices with an advancing speed of ≤0.1 mm/s, an oscillating frequency of 80 Hz, a lateral amplitude of 1.5 mm, and a blade angle of 15°. When handling the slices, hold the agarose instead of the tissue itself to avoid tissue damage. Store the slices in the cutting solution at 4 °C for a maximum of 2 h, if necessary.
    NOTE: Cardiomyocytes are oriented in parallel to the epicardium. Therefore, it is important to cut in parallel to the epicardium to avoid excessive myocyte damage. It is recommended to discard the first 1–3 slices, as only uniform slices of constant thickness should be used for the isolation. For small tissue biopsies, however, only discard the first slice.
    6. Check cardiomyocyte alignment under a standard light microscop

4. Tissue digestion

1. Place the heat plate on the lab shaker and warm it to 37 °C. Start the lab shaker at 65 rpm.
2. Dissolve the proteinase (prepared in step 1.3) in 2 mL of solution 1 (step 1.1 and 1.2) and incubate at 37 °C until use. Do not mix the proteinase and collagenase.
3. Dissolve the collagenase (prepared in step 1.4) in 2 mL of solution 1 (step 1.1 and 1.2) and incubate at 37 °C until use. Do not mix the proteinase and collagenase.
    CAUTION: Wear protective eyewear and gloves, because dissolved enzymes can cause skin and eye injuries.
4. Add calcium chloride ($CaCl_2$) to the collagenase containing solution (prepared in step 4.3) to a final concentration of 5 µM.
5. Use forceps to transfer a tissue slice from the vibratome bath to a clean 60 mm tissue culture dish filled with 5 mL of prechilled cutting solution (step 1.1 and 1.2) and keep on ice.
6. Carefully remove the agarose from the myocardial tissue with blade or forceps.
    NOTE: Avoid excess tension and shear stress on the myocardial slices, because they can damage the cardiomyocytes.
7. To perform the initial wash, place a clean 35 mm tissue culture dish on the heat plate and fill it with 2 mL of prewarmed (37 °C) solution 1. Transfer 1–2 myocardial slices to the prepared dish with forceps. Aspirate the solution with a 1 mL pipette and perform the wash steps 2x to remove remnants of cutting solution.
    NOTE: Do not aspirate the cardiac slices. The solutions and the slices should remain at a constant temperature of 35 °C on the agitated heat plate. Adjust the heat plate temperature if necessary.
8. Remove solution 1 from the dish and add 2 mL of the proteinase solution (step 4.2). Incubate for 12 min on the heat plate at 65 rpm.
9. Wash 2x with 2 mL of prewarmed solution 1 (37 °C).
10. Remove solution 1 from the dish and add 2 mL of collagenase solution (steps 4.3 and 4.4). Incubate at least 30 min on the heat plate at 65 rpm.
11. Check for free individual myocytes at 30, 35, 40 min, etc., by placing the dish under a light microscope. Work quickly to avoid significant cooling of the solution.
    NOTE: The required digestion time may vary depending on the tissue constitution and the degree of fibrosis. As soon as the tissue gets visibly soft and dissociates readily when gently pulled, the optimal digestion time has been reached. If enough tissue is available, several slices can be digested in parallel with varying digestion times.
12. When the tissue is digested and individual myocytes are visible (step 4.11), wash 2x with 2 mL of prewarmed solution 2 (37 °C) and fill again with 2 mL.

5. Tissue dissociation

1. Dissociate the digested tissue slices with forceps by carefully pulling the fibers apart.
2. Carefully pipette several times with a single-use Pasteur pipette (opening diameter >2 mm).
    NOTE: The use of forceps and pipetting can induce mechanical stress and cause cardiomyocyte damage. However, it is a crucial step for separation of the cells. Use fine forceps to minimize cell damage and carefully dissociate the slices to smaller pieces.
3. Check for the liberated rod-shaped cardiomyocytes under the light microscope.

6. Reintroduction of physiological calcium concentration

1. Slowly increase the calcium concentration from 5 µM to 1.5 mM while agitating at 35 °C on the heat plate. Use 10 mM and 100 mM $CaCl_2$ stock solutions. Recommended steps: 20, 40, 80, 100, 150, 200, 400, 800, 1,200, 1,500 µM. Allow the cells to adapt to the increased calcium levels in 5 min incubation intervals between each step.
2. Remove undigested tissue chunks carefully with forceps or filter the cell suspension through a nylon mesh with 180 µm pore size at the end of the calcium increase.

7. Removal of mechanical uncoupling agent

1. Stop agitation and slowly remove one third of the solution (~700 µL) from the top with a 1,000 µL pipette. Avoid aspiration of the cardiomyocytes.
   NOTE: If undigested tissue was removed, the cardiomyocytes will accumulate in the center of the dish, which facilitates aspiration of cell-free solution. If not, transfer the cell solution to a 15 mL centrifuge tube and allow the cells to sediment for 10 min at 35 °C, then aspirate 700 µL of the supernatant and discard, resuspend the cells, transfer them back to a 35 mm tissue culture dish and proceed with step 7.2.
2. Add 700 µL of solution 3 to the cells, resume agitation on the heat plate and incubate for 10 min.
3. Repeat steps 7.1 and 7.2.
4. Transfer the solution to a 15 mL centrifuge tube and allow the cardiomyocytes to sediment for a minimum of 10 min and a maximum of 30 min at room temperature or spin at 50 x *g* for 1 min. Remove the supernatant completely and resuspend in modified Tyrode's solution or the desired experimentation buffer.
   NOTE: Cardiomyocytes can be stored in modified Tyrode's solution (**Table 1**) for several hours at 37 °C and 5% CO_2 before use.
5. Verify the cell quality with a standard light microscope at 40x and 200x magnification.
   NOTE: Around 30–50% of the cardiomyocytes should be rod-shaped, smooth without membrane blebs, and display clear cross-striations. Only 5–10% of the viable cells should show spontaneous contractions.

Representative Results

To verify isolation efficiency, the protocol was used with rat myocardium and the resulting number of viable myocytes was compared with the numbers obtained by isolation via coronary perfusion and by isolation from small tissue chunks (chunk isolation, **Figure 2**). Chunk isolation and isolation from tissue slices were performed from the same hearts. For the isolation via coronary perfusion, however, the whole heart was used. Coronary perfusion yielded predominantly rod-shaped and cross-striated cardiomyocytes. With isolations from myocardial slices a lower proportion of rod-shaped cells were observed, but the total number was still high. In contrast, after isolation from tissue chunks, only few rod-shaped cells were recovered (**Figure 2A**). Flow cytometry was used to compare the results quantitatively. Due to their relatively large size and cross-striation, cardiomyocytes typically show large values for both forward and side scatter. These properties were used to count rod-shaped myocytes with a flow-cytometric cell analyzer, applying a simple gating scheme that discriminated rod-shaped from hypercontracted and rounded cardiomyocytes. (**Supplemental Figure 1**). Representative dot plots are depicted in **Figure 2B**. Statistical analysis showed distinctively higher counts in the cardiomyocyte gate CM1 for isolation from slices than from tissue chunks (41.5 ± 11.9 vs. 7.89 ± 3.6%, respectively; n =3 isolations; $p < 0.05$, paired two-tailed t-test). As expected from visual inspection, coronary perfusion yielded the highest counts, with 71.0 ± 9.4% (n = 3 isolations; $p < 0.05$, unpaired two-tailed t-test) (**Figure 2C**). Thus, the isolation of cardiomyocytes from tissue slices does not reach the yield obtained by coronary perfusion, but results in a considerably larger number of rod-shaped myocytes than isolation from tissue chunks, providing enough cells for subsequent experiments. In addition to cell count, structural parameters of rat cardiomyocytes were quantified. Cell length, cell width, and sarcomere length were not different in cells isolated by perfusion or from tissue slices (length = 110.0 ± 5.4 µm vs. 99.4 ± 3.2 µm, $p > 0.05$; width = 33.9 ± 1.9 µm vs. 30.6 ± 1.2 µm, $p > 0.05$, for n = 19 and n = 21 cells, respectively; sarcomere length = 1.62 ± 0.04 µm vs. 1.68 ± 0.04 µm, $p = 0.28$, n = 24 and n = 23 cells respectively) (**Supplemental Figure 2A–C**). Using Fluo-4 loaded myocytes subjected to electrical field stimulation[17] functional parameters were also assessed (**Supplemental Figure 2D–F**). Mean activation time (27.5 ± 1.5 vs. 23.6 ± 2.5 ms, n = 100 vs. n = 31 cells; perfusion vs. slice, $p > 0.05$) (**Supplemental Figure 2D**) and relative shortening (12.2 ± 1.1 vs. 11.3 ± 2.5%, n = 42 vs. n= 7 cells, perfusion vs. slice, $p > 0.05$) (**Supplemental Figure 2F**) of cardiomyocytes that responded to stimulation did not differ significantly between the two groups. The calcium transient amplitude (maximal normalized Ca^{2+}, F/F_0) (**Supplemental Figure 2E**) was slightly augmented in cardiomyocytes isolated from slices when compared to cardiomyocytes isolated by perfusion (4.9 ± 0.2 vs. 5.7 ± 0.3, n = 104 vs. n = 25 cells, perfusion vs. slice, $p < 0.05$). Although the fraction of rod-shaped cardiomyocytes responding to electrical stimulation was approximately 15% higher in preparations obtained by perfusion, the majority of cells was also excitable in preparations from tissue slices (74.3 ± 4.2% vs. 62.1 ± 2.9%, perfusion vs. slice, $p < 0.05$, ten fields of view, 452 vs. 276 counted cells, respectively) (**Supplemental Figure 2G**). To assess cell quality after cultivation, the percentage of myocytes contracting in response to electrical field stimulation after 48 h in cell culture was determined[17]. The fraction of responding cells was reduced by approximately half in both groups (41.9 ± 3.6% vs. 31.5 ± 2.3%, perfusion vs. slice, $p < 0.05$, 10 fields of view, n = 371 vs. n = 329 counted cells, respectively) (**Supplemental Figure 2H**).

Next, the protocol was applied to human myocardium. The number of rod-shaped and viable isolated human ventricular cardiomyocytes obtained from tissue slices was compared pairwise with the number obtained from tissue chunks of the same myocardial specimens (**Figure 3**). We found that the isolation from myocardial slices yielded a large proportion of rod-shaped and only a small proportion of rounded cardiomyocytes (**Figure 3A**). We counted rod-shaped myocytes from wide-field images and normalized their number by the tissue wet weight used for the isolation. The quantification showed that isolation from myocardial slices resulted in a strikingly higher number of rod-shaped myocytes than isolation from tissue chunks (45.0 ± 15.0 vs. 6.87 ± 5.23 cells/mg, n = 7 isolations, $p < 0.05$, paired two-tailed t-test) (**Figure 3B**). Furthermore, the viability was assessed with an MTT viability assay[14], normalizing the photometric formazan absorption to the total amount of protein in the cell lysate. The photometric quantification confirmed a substantially higher myocyte viability after isolation from myocardial slices (4.76 ± 0.47 vs. 1.09 ± 0.18 AU, n = 3 isolations, $p < 0.05$, paired two-tailed t-test) (**Figure 3C**).

In total, the method was applied to myocardial specimens from 30 human hearts with transportation times of up to 36 h. From 23 of the 30 specimens' viable cells were obtained for subsequent experiments (see also **Supplemental Table 1**). An example of an unsuccessful experiment (i.e., <100 cells per dish) is shown in **Supplemental Figure 3**. Here, most cells did not tolerate high extracellular calcium and hypercontracted. We assessed contractility in myocytes from 14 isolations, and in two cases the cells did not respond to electrical stimulation. Note that the technique not only worked with large specimens obtained from adult hearts, but also with small biopsies (as little as 40 mg) from child hearts (**Supplemental Figure 4**).

To demonstrate that human cells isolated with the described protocol can be subjected to structural analysis, they were stained with alpha-actinin, the EC coupling proteins L-type calcium channel (LTCC) and ryanodine receptor (RyR), as well as the cell membrane and nuclei, according to a staining method described recently in rat myocytes[17] (**Figure 4**). Alpha-actinin staining revealed a dense, regular z-line pattern

and a mean sarcomere length of 1.92 ± 0.06 µm in resting cardiomyocytes (n = 4, **Figure 4A**). LTCC and RyR showed clear clusters and were, as expected, colocalized near the cell membrane and t-tubules (**Figure 4B**).

The potentiometric dye FluoVolt[18] and the calcium sensitive dye Fluo-4[17] were used to demonstrate that human cardiomyocytes isolated with the described protocol were excitable and could be used for studies on cellular electrophysiology or excitation-contraction coupling (**Figure 5**). Action potential (AP) shape and duration were analyzed (**Figure 5A**,**B**). The values of 50% and 90% AP duration (APD_{50}: 400.6 ± 41.1 ms, APD_{90}: 748.9 ± 56.6 ms, n = 10 cells) were in the range reported by others for ventricular cardiomyocytes from failing human hearts[4,6,7,9]. The calcium transients recorded by confocal line scanning of Fluo4-loaded cells (**Figure 5C**,**D**) showed a clear upstroke after stimulation and an acceptable signal-to-noise ratio. The calcium transient amplitude (maximum F/F_0) was 2.9 ± 0.5 (n = 31 cells). Cardiomyocyte shortening by contraction can be seen in the example from the deflection of the lower cell border, which had a mean of 4.6 ± 0.8% (n = 31 cells).

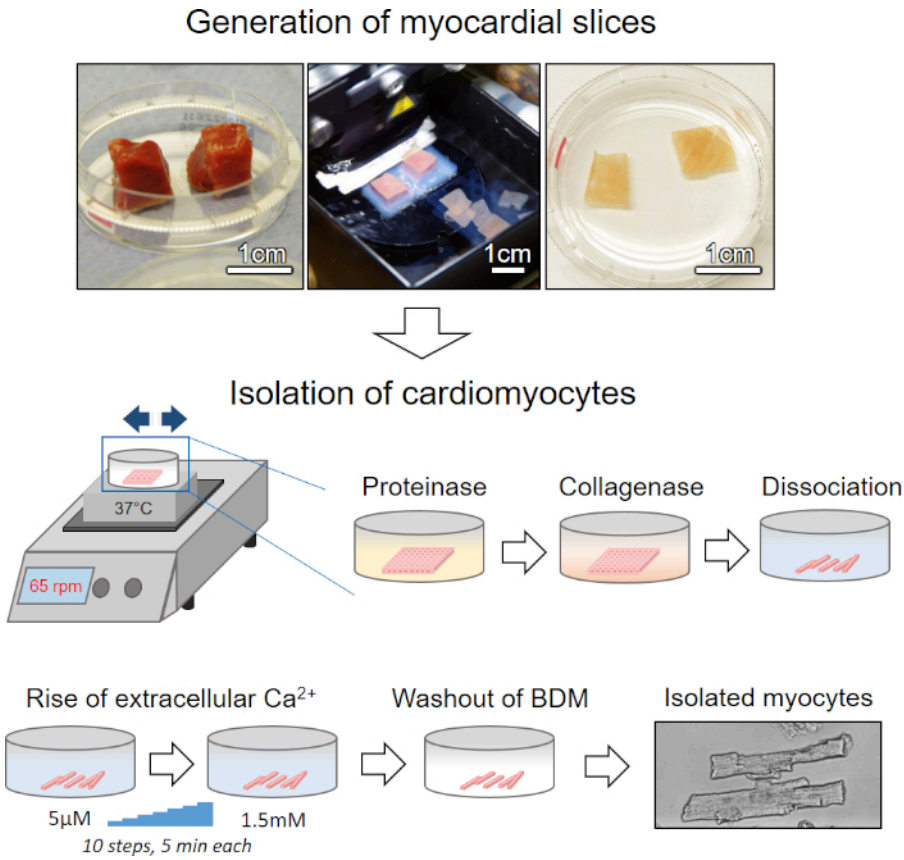

Figure 1: Workflow of the isolation protocol from human cardiac tissue slices. Tissue blocks or biopsies from human myocardium (top left) are embedded in low-melting-point agarose and 300 µm thick slices are generated with the oscillating blade of a vibratome (top middle). The slices are transferred to a culture dish and removed from the surrounding agarose (top right). Tissue digestion is carried out at ~35 °C and 65 rpm on a heated and agitated plate (middle, left) and includes: (step 4.8) Incubation in nominally calcium-free solution supplemented with proteinase, (step 4.10) Digestion of the extracellular matrix with collagenase, (step 5) Dissociation and liberation of individual cardiomyocytes with forceps and pipetting. (step 6) The extracellular calcium concentration is raised slowly with adaptation intervals of 5 min from 5 µM to 1.5 mM. (step 7) Stepwise removal of the mechanical uncoupler 2,3-butanedione monoxime (BDM). Rod-shaped and cross-striated human cardiomyocytes are retrieved. Please click here to view a larger version of this figure.

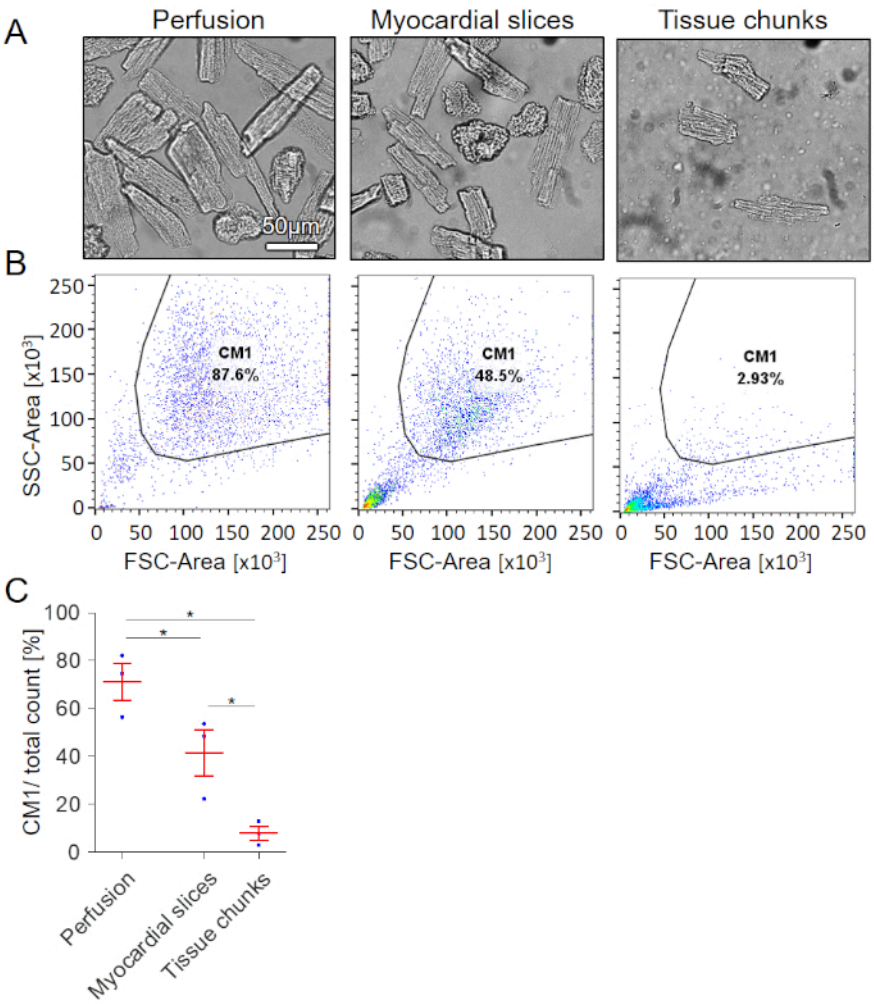

Figure 2: Cardiomyocyte yields of myocardial slice, perfusion, and chunk isolation. (**A**) Representative light micrographs of cardiomyocytes isolated either via coronary perfusion (Perfusion), from vibratome-cut tissue (Myocardial slices), or from minced tissue (Tissue chunks). (**B**) Respective representative flow cytometry dot plots from cardiomyocyte isolations described in **A**. Side scatter area (SSC-Area) and forward scatter area (FSC-Area) were used as indicators of cellular granularity and size, respectively. The gating scheme of the gate CM1 for cardiomyocytes is indicated by the black line and the respective fractions of total counts in percent are displayed. (**C**) Mean ± standard error of the fractions of cardiomyocytes (CM1) assessed by flow-cytometric analysis as described in **B** from n = 3 cell isolations per group. The isolation from myocardial slices and tissue chunks was performed from the same hearts (paired two-tailed t-test). Isolation by perfusion was performed from whole hearts of different animals and compared by the unpaired two-tailed t-test. *p < 0.0 Please click here to view a larger version of this figure.

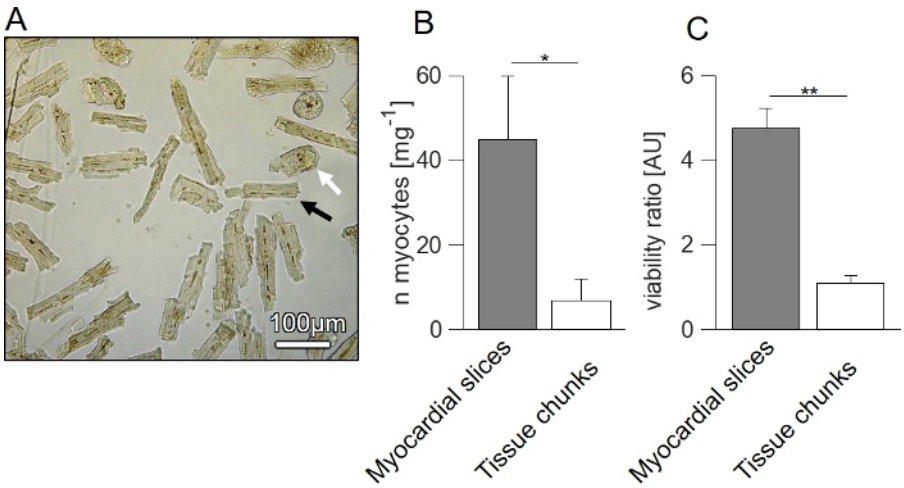

Figure 3: Comparison of yield and viability of human cardiomyocytes after isolation from myocardial slices and minced tissue chunks. (**A**) Representative image of calcium-tolerant human ventricular cardiomyocytes after isolation from tissue slices. Rod-shaped and striated cardiomyocytes are predominant (black arrow), with only few rounded and hypercontracted cells (white arrow). (**B**) Quantification of calcium-tolerant, rod-shaped myocytes isolated in parallel, either from myocardial slices or tissue chunks counted from wide field micrographs and normalized to the wet weight of the input material (in milligrams) from n = 7 paired isolations. (**C**) Quantification of the cardiomyocyte viability by photometric measurement of formazan dye absorbance after MTT assay. Absorbance was normalized to the total protein amount, assessed by BCA assay from n = 3 paired isolations. *p < 0.05, **p < 0.01 (paired two-tailed t-test). Please click here to view a larger version of this figure.

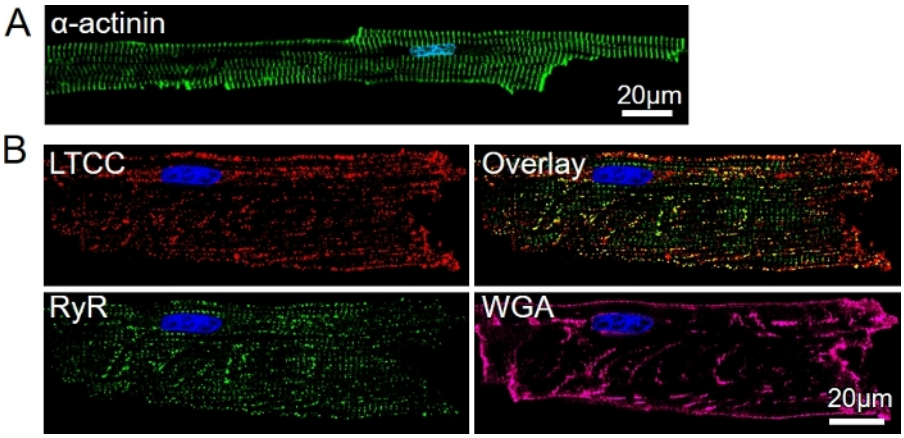

Figure 4: Microstructural characterization of human cardiomyocytes after isolation. (**A**) Representative confocal microscopic image after immunostaining of alpha-actinin (green) and nuclear stain with 4′,6-diamidin-2-phenylindol (DAPI, blue). The sarcomere length was 1.92 ± 0.06 µm (n = 4 myocytes), determined by analyzing the Fourier spectrum. (**B**) Representative confocal microscopic images of a fixed human ventricular cardiomyocyte coimmunostained for L-type calcium channel (LTCC) and cardiac ryanodine receptor (RyR). The overlay of RyR and LTCC (overlay) and the staining of the extracellular matrix with wheat germ agglutinin (WGA, magenta) is also shown. Nuclei were stained with DAPI (blue). Please click here to view a larger version of this figure.

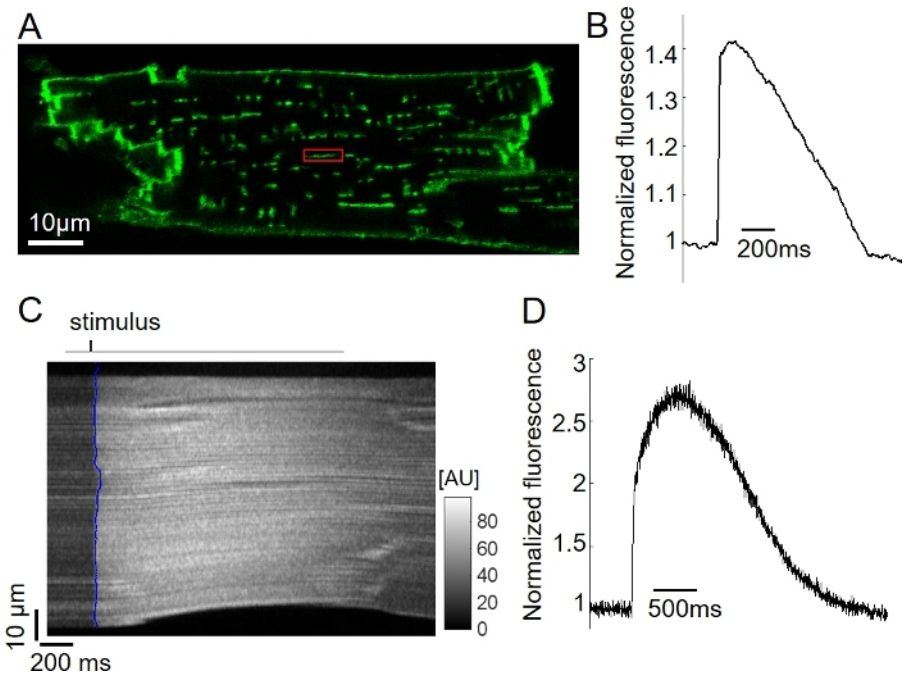

Figure 5: Action potential and intracellular calcium transient in human ventricular myocytes. (**A**) Two-dimensional confocal image of an isolated human cardiomyocyte loaded with the potentiometric dye FluoVolt (green, 488 nm excitation wavelength). The red box indicates an element of the t-tubular system that was measured with a fast line scan during electrical field stimulation at 0.5 Hz. (**B**) Averaged and baseline-normalized FluoVolt fluorescence (F/F_0) of five consecutive action potentials obtained by confocal imaging as described in **A**. (**C**) Line scan image of an isolated human cardiomyocyte loaded with the calcium sensitive dye Fluo-4 AM (488 nm excitation wavelength) along the longitudinal cell axis. The fluorescence intensity is shown in grey values [AU] and the blue dots indicate the maximal rise of the fluorescence intensity (dF/dt_{max}) of each pixel along the scanned line. The sampling rate was 529 Hz. (**D**) Calcium transient obtained by averaging the fluorescence of each pixel along the scanned line from the image in **C** and normalization to baseline values before stimulation (F/F_0). Please click here to view a larger version of this figure.

Name	Composition in mmol/L	Supplements	pH	Required volume in mL
Solution 1	20 KCl, 10 KH_2PO_4, 10 $MgCl_2$, 10 glucose, 70 glutamic acid, 20 taurin, 10 beta-hydroxybutyrate, 30 BDM	2 mg/ml bovine serum albumin	7.3 with KOH	300
Solution 2	20 KCl, 10 KH_2PO_4, 10 $MgCl_2$, 10 glucose, 70 glutamic acid, 20 taurin, 10 beta-hydroxybutyrate, 0.005 $CaCl_2$, 30 BDM	10 mg/ml bovine serum albumin	7.3 with KOH	300
Solution 3	20 KCl, 10 KH_2PO_4, 10 $MgCl_2$, 10 glucose, 70 glutamic acid, 20 taurin, 10 beta-hydroxybutyrate, 1.5 $CaCl_2$	10 mg/ml bovine serum albumin	7.3 with KOH	300
Modified Tyrode's solution	130 NaCl, 0.4 NaH_2PO_4, 5.8 $NaHCO_3$, 5.4 KCl, 0.5 $MgCl_2$, 1.5 $CaCl_2$, 25 HEPES, 22 glucose	2 mg/ml bovine serum albumin	7.3 with NaOH at 5 % CO_2	100
Cutting solution	138 NaCl, 0.33 NaH_2PO_4, 5.4 KCl, 2 $MgCl_2$, 0.5 $CaCl_2$, 10 HEPES, 10 glucose, 30 BDM		7.3 with NaOH	500

Table 1: Buffers and Solutions. Composition of the required buffers and solutions.

Supplemental Figure 1: Validation of the cardiomyocyte (CM1) gating scheme. Dot plots from flow cytometry, measuring forward- and side-scatter area (FSC-A, SSC-A) indicating size and granularity of the detected cells in a control sample (CTRL) from perfusion-isolated rat cardiomyocytes and after incubation of cardiomyocytes from the same preparation for 15 min at 37 °C in modified Tyrode's solution containing

100 mM calcium (calcium overdose). The high extracellular calcium causes hypercontraction and rounding of rod-shaped cardiomyocytes, resulting in a reduction of cells (87.6% vs. 6.00%) in the defined gate (CM1). Please click here to download this figure.

Supplemental Figure 2: Cardiomyocyte characteristics isolated by perfusion or from slices. Cell length (**A**) and cell width (**B**) were determined from rat cardiomyocytes fixed directly after isolation via perfusion or from myocardial slices by microscopy (n = 19 and n = 21 cells, respectively). The sarcomere length (**C**) was determined from microscopic images after immunostaining with alpha-actinin and Fourier analysis (n = 24 and n = 23 cells, respectively). The mean activation time (**D**), the maximum F/F_0 (**E**) and the relative shortening (**F**) were assessed from Fluo-4 loaded cardiomyocytes isolated by perfusion or from myocardial slices and responsive to electrical stimulation (n = 100/31 myocytes in D, n = 104/25 myocytes in E and n = 42/7 myocytes in F). The fraction of rod-shaped cardiomyocytes (**G**) that contracted upon electrical stimulation, assessed by counting the total number of rod-shaped cells and the contractile cells in ten fields of view at 200x magnification in a standard light microscope was assessed for perfusion and slice isolation (n = 452/276 cells, respectively). (**H**) Analysis as performed in G but after 48 h of cultivation (n = 371/329 cells, respectively). Please click here to download this figure.

Supplemental Figure 3: Human cardiomyocyte isolation with low yield. Representative light microscope image of myocytes from an isolation with predominantly hypercontracted (blue) or rounded cells (black) and only few rod-shaped and striated cells (white). Please click here to download this figure.

Supplemental Figure 4: Cardiomyocyte isolation from infant cardiac biopsies. (**A**) Image of an endomyocardial biopsy (wet weight approximately 40 mg) obtained during corrective surgery for tetralogy of Fallot in an infant. (**B**) Myocardial slice from the tissue shown in **A**, embedded in agarose. (**C**) Cardiomyocytes isolated from a myocardial slice as shown in **B**. Please click here to download this figure.

Supplemental Table 1: Human patient data. A list of the number of samples from human patients included in this study is given. Samples were categorized according to surgery types, age, disease etiology, and transportation time, with the respective total number of samples and the number of successful myocyte isolations. Please click here to download this table.

Discussion

Although the isolation of living cardiomyocytes was established more than 40 years ago and is still a prerequisite for many experimental approaches in cardiac research, it remains a difficult technique with unpredictable outcomes. Cardiomyocyte isolation via perfusion of the coronary arteries with enzyme solution is commonly used for hearts of small animals and yields large numbers of viable cells. However, this requires a relatively complex system and expertise. Furthermore, most human tissue samples are not suited for this method, due to their small size or the absence of coronary artery branches, which makes new methods for the isolation of human cardiomyocytes desirable. The protocol described here is based on the atraumatic generation of thin cardiac tissue slices, which are then subjected to enzymatic digestion. It can be applied to myocardial specimens from animal hearts and human myocardium such as apical cores from left-ventricular assist-device implantation, endomyocardial biopsies from septal myectomy, or explanted hearts (see **Supplemental Table 1**), and reliably produces sufficient amounts of viable, calcium-tolerant cardiomyocytes that can be used for subsequent experiments, like cardiomyocyte cultivation[17].

A major reason for higher yields of myocytes from vibratome-cut tissue slices than from minced tissue chunks might be a lower degree of myocyte damage during the slicing. When coronary perfusion is not possible, slicing or mincing the tissue specimen is necessary to increase the contact surface for digesting enzymes. Slice dimensions of approximately 8 mm x 8 mm x 0.3 mm allow for good access of enzymes due to a relatively large surface-to-volume ratio (~7 mm^{-1}). Using scissors, a similar surface-to-volume ratio (~6 mm^{-1}) can be achieved by mincing the specimens into small pieces of approximately 1 mm x 1 mm x 1 mm. Although myocyte damage in slices and minced chunks before enzymatic digestion was not quantified, slicing on a vibratome likely causes considerably less damage because it enables gentle cutting in the fiber direction. In fact, it was estimated that in vibratome-cut slices fewer than 5% of the myocytes are damaged[19]. The results show that human cardiomyocytes can be isolated very efficiently with the protocol provided, with a much higher proportion of viable cells compared to tissue chunks. This is in accordance with a protocol that used slices from atrial tissue[20]. Our method yields up to 200 calcium-tolerant ventricular myocytes per milligram of tissue and provides the possibility to store or transport cardiac tissue for up to 36 h prior to isolation. This renders the protocol applicable for laboratories without on-site cardiac surgery and thereby extends the possibilities for collaborations.

Critical steps in the protocol are the correct processing of myocardium to slices on a vibratome and their digestion as well as the final steps of calcium-reintroduction and washout of BDM. In contrast to other methods[19,21], the cardiac tissue is embedded in agarose with a low melting temperature to avoid motion during the slicing procedure[14,15]. This was necessary to stabilize the tissue block and to generate uniform slices. When embedding the tissue, the agarose should still be liquid, but the temperature should not exceed 37–38 °C to avoid cell damage by hyperthermia. Conversely, if the agarose is too cold (<35 ˚C), it does not bind well to the tissue block and may break during the slicing. To test for myocyte viability after the vibratome processing, a simple MTT assay that allows for quick evaluation of cell viability by light microscopy and also for quantification by photometrical analysis is suggested[14,22]. Cardiomyocyte damage can also occur during reintroduction of extracellular calcium after a period of incubation in calcium-free solution[23,24]. This calcium paradox phenomenon in isolated cells can be mitigated by a slow, stepwise increase of the calcium levels to allow the cardiomyocytes to adapt. This step should be carried out carefully during continuous agitation at 35 °C and with intervals of 5 min. Slight hypothermia (21 °C) increases the calcium tolerance of rat cardiomyocytes during reintroduction, which is in accordance with earlier reports[25]. However, human cardiomyocytes did not show major differences in calcium tolerance at 35 °C or 21 °C. The use of BDM during cutting, digestion, and reintroduction of calcium prevents myocyte contraction and cellular energy expenditure as well as hypercontraction and is therefore protective. However, for subsequent experiments assessing cardiac contractility or excitation-contraction coupling, BDM needs to be removed[26]. A gradual washout was applied to minimize spontaneous hypercontractions. BDM can be omitted, but this will markedly increase the amount of hypercontracted myocytes, as observed in previous experiments and studies[6].

The described protocol uses a solution that contains high potassium and only traces of sodium for digestion. This solution gave the highest yields of rod-shaped, cross-striated cardiomyocytes. For enzymatic digestion, however, it required a higher concentration of collagenase (4 mg/mL) compared to digestion in normal Tyrode's solution (1.5 mg/mL). High extracellular sodium may lead to intracellular sodium overload, exacerbating the negative effects of the calcium paradox, thus reducing cell yields[27]. Therefore, it is suggested to use solutions with high

potassium and low sodium. A high extracellular magnesium concentration is frequently employed in cardiomyocyte isolation protocols[3,28], and has been shown to reduce ischemia-reperfusion injury[29,30] and improve cardiac function after extended periods of cold ischemia[31]. Therefore, the solution applied here also contains a high concentration of magnesium (10 mM). However, it was shown that excitability, contraction force, and conduction velocity may be reduced by high magnesium concentrations[32]. Although these effects were shown to be reversible, effects on isolated cardiomyocytes cannot be excluded. Thus, using magnesium at physiological concentrations (1–2 mM), might result in a lower overall cell yield but improve excitability.

The optimal digestion time is quite variable, as the amount of extracellular matrix or fibrosis can vary considerably in human failing myocardium. Should the tissue still be firmly connected at the time of dissociation, with only few viable myocytes released, increasing the digestion time in steps of 5 min and regularly checking for free myocytes and the texture of the slices is recommended. If the tissue remains undigested after prolonged periods (>45 min), increasing the concentration of collagenase in steps of 1 mg/mL or testing a different enzyme batch is recommended[16]. The respective values presented in this work can serve as a primer for a robust isolation of cardiomyocytes, but the conditions for optimal yield and viability may be refined in each laboratory, taking into account the great variability of enzyme activities and tissue constitutions. An advantage of the method is that due to the small amounts of required tissue and the simple workflow in small batches, several tests can be carried out in parallel. Thus, an optimal protocol can be achieved quickly.

The generation of high-quality myocardial slices requires a high precision vibratome. The need for a high-precision tissue slicer or vibratome is a possible drawback of the method, because it is not easily available at every laboratory. The device used for this study is described in more detail elsewhere[14,15]. Different devices have been used successfully to generate myocardial slices by other groups[33,34,35]. Hence, if the settings of advancing speed, blade amplitude, and oscillation frequency can be met and a horizontal blade orientation with a z-deflection below 0.1 µm is available, successful slicing and isolation should be feasible with other vibratomes. In addition, the tissue slicing is elaborate and significantly prolongs the duration of the protocol (approximately 1–2 h). The use of BDM as a mechanical uncoupling agent has protective effects by reducing cellular energy exposure, ischemic injury, and hypercontraction[26,36,37]. Although the negative inotropic effect of BDM was shown to be quickly reversible after washout[38,39], it is not clear if BDM may have unknown consequences on cardiomyocyte function[40]. This study shows that cells can be isolated with high yields after tissue storage times of up to 36 hours and that human cardiomyocytes can be cultured for up to three days after isolation with this method[17]. We did not notice functional or structural differences between myocytes with short and long storage times. However, this was not assessed systematically. On the other hand, for whole rabbit hearts, it was shown that functional differences between fresh hearts and hearts exposed to cold ischemia in extracellular solution containing 30 mM/L BDM were negligible[31], and the same may be true in this case.

The presented protocol provides a solid basis for all kinds of experiments with isolated ventricular cardiomyocytes and might be especially valuable for research on human myocardial specimens. With some adaptations, the isolation of other cardiac cells, such as fibroblasts or endothelial cells also seems possible[41]. Because it requires only small amounts of tissue that can be shipped in cold storage solution from other laboratories, application of the protocol may reduce the number of sacrificed laboratory animals. In fact, the protocol has been successfully applied to rabbit and porcine cardiac tissue. Furthermore, as experiments with long-term cultivated myocardial tissue slices increase[21,33,42] and are constantly improved to provide optimal culture conditions[15,43,44], the method can be readily applied after slice cultivation. Single cell isolation from cultivated myocardium may therefore help to characterize changes in cardiomyocytes after pharmacological or physical interventions in vitro.

Disclosures

The authors have nothing to disclose.

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