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Cover Letter

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Editorial Office

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**Submission of Revised Manuscript**

Dear Editors:

We have submitted a thoroughly revised version of our manuscript entitled *Isolation of human ventricular cardiomyocytes from vibratome-cut myocardial slices*.

According to the reviewers' comments, we added additional data to demonstrate the functional quality of isolated cardiomyocytes. Furthermore, we added more details to the protocol as requested. We also reviewed our manuscript to address the editorial comments.

We hope that you will find the revised version of our manuscript acceptable for publication in JoVE.

Sincerely,

Dominik Fiegle, Tilmann Volk and Thomas Seidel
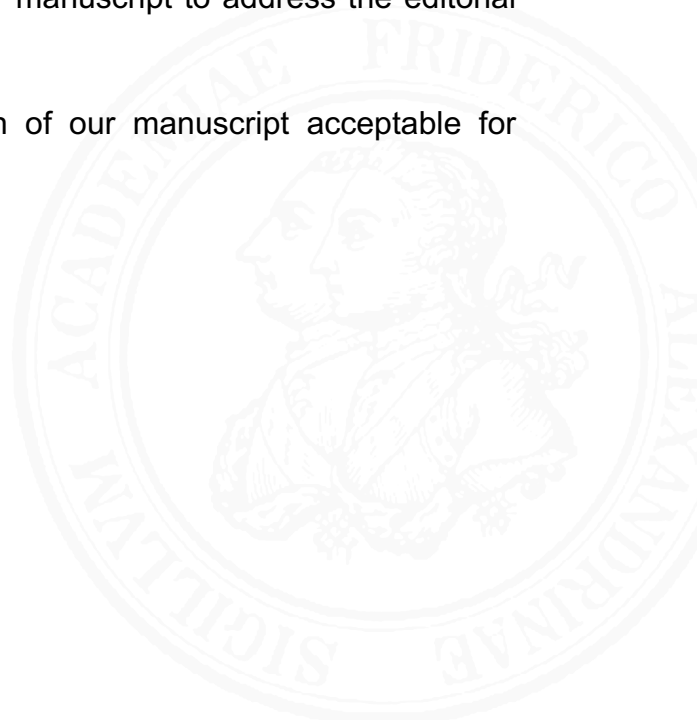
1 **TITLE:**
2 **Isolation of Human Ventricular Cardiomyocytes from Vibratome-Cut Myocardial Slices**
3

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17 **KEYWORDS:**
18 human, heart failure, myocardial slices, cardiomyocyte isolation, vibratome, contractile
19 myocytes, calcium imaging, cardiac excitation-contraction coupling
20

21 **SUMMARY:**
22 Presented is a protocol for the isolation of human and animal ventricular cardiomyocytes from
23 vibratome-cut myocardial slices. High yields of calcium-tolerant cells (up to 200 cells/mg) can be
24 obtained from small amounts of tissue (<50 mg). The protocol is applicable to myocardium
25 exposed to cold ischemia for up to 36 h.
26

27 **ABSTRACT:**
28 The isolation of ventricular cardiac myocytes from animal and human hearts is a fundamental
29 method in cardiac research. Animal cardiomyocytes are commonly isolated by coronary
30 perfusion with digestive enzymes. However, isolating human cardiomyocytes is challenging
31 because human myocardial specimens usually do not allow for coronary perfusion, and
32 alternative isolation protocols result in poor yields of viable cells. In addition, human myocardial
33 specimens are rare and only regularly available at institutions with on-site cardiac surgery. This
34 hampers the translation of findings from animal to human cardiomyocytes. Described here is a
35 reliable protocol that enables efficient isolation of ventricular myocytes from human and animal
36 myocardium. To increase the surface-to-volume ratio while minimizing cell damage, myocardial
37 tissue slices 300 µm thick are generated from myocardial specimens with a vibratome. Tissue
38 slices are then digested with protease and collagenase. Rat myocardium was used to establish
39 the protocol and quantify yields of viable, calcium-tolerant myocytes by flow-cytometric cell
40 counting. Comparison with the commonly used tissue-chunk method showed significantly higher
41 yields of rod-shaped cardiomyocytes (41.5 ± 11.9 vs. $7.89 \pm 3.6\%$, $p < 0.05$). The protocol was
42 translated to failing and non-failing human myocardium, where yields were similar as in rat
43 myocardium and, again, markedly higher than with the tissue-chunk method (45.0 ± 15.0 vs.
44 6.87 ± 5.23 cells/mg, $p < 0.05$). Notably, with the protocol presented it is possible to isolate

45   reasonable numbers of viable human cardiomyocytes (9–200 cells/mg) from minimal amounts of
46   tissue (<50 mg). Thus, the method is applicable to healthy and failing myocardium from both
47   human and animal hearts. Furthermore, it is possible to isolate excitable and contractile
48   myocytes from human tissue specimens stored for up to 36 h in cold cardioplegic solution,
49   rendering the method particularly useful for laboratories at institutions without on-site cardiac
50   surgery.
51
52   **INTRODUCTION:**
53   A seminal technique that has paved the way to important insights into cardiomyocyte physiology
54   is the isolation of living ventricular cardiomyocytes from intact hearts[1]. Isolated cardiomyocytes
55   can be used to study normal cellular structure and function, or the consequences of in vivo
56   experiments; for example, to assess changes in cellular electrophysiology or excitation-
57   contraction coupling in animal models of cardiac disease. Additionally, isolated cardiomyocytes
58   can be used for cell culture, pharmacological interventions, gene transfer, tissue engineering, and
59   many other applications. Therefore, efficient methods for cardiomyocyte isolation are of
60   fundamental value to basic and translational cardiac research.
61
62   Cardiomyocytes from small mammals, such as rodents, and from larger mammals, such as pigs
63   or dogs, are commonly isolated by coronary perfusion of the heart with solutions containing
64   crude collagenases and/or proteases. This has been described as the "gold standard" method for
65   cardiomyocyte isolation, resulting in yields of up to 70% of viable cells[2]. The approach has also
66   been used with human hearts, resulting in acceptable cardiomyocyte yields[3–5]. However, because
67   coronary perfusion is only feasible if the intact heart or a large myocardial wedge containing a
68   coronary artery branch is available, most human cardiac specimens are not suited for this
69   approach due to their small size and a lack of appropriate vasculature. Therefore, the isolation of
70   human cardiomyocytes is challenging.
71
72   Human myocardial specimens mostly consist of tissue chunks of variable size (approximately 0.5
73   x 0.5 x 0.5 cm to 2 x 2 x 2 cm), obtained through endomyocardial biopsies[6], septal myectomies[7],
74   VAD implantations[8], or from explanted hearts[9]. The most common procedures for cardiomyocyte
75   isolation start with mincing the tissue using scissors or a scalpel. Cell-to-cell contacts are then
76   disrupted by immersion in calcium-free or low-calcium buffers. This is followed by multiple
77   digestion steps with crude enzyme extracts or purified enzymes like proteases (e.g., trypsin),
78   collagenase, hyaluronidase, or elastase, resulting in a disintegration of the extracellular matrix
79   and liberation of cardiomyocytes. In a final, critical step, a physiological calcium concentration
80   has to be carefully restored, or cellular damage can occur due to the calcium-paradox[10–12]. This
81   isolation approach is convenient but usually inefficient. For instance, one study found that nearly
82   1 g of myocardial tissue was required to obtain a sufficient number of cardiomyocytes suitable
83   for subsequent experiments[13]. A possible reason for low yields is the relatively harsh method of
84   mincing the tissue. This may particularly damage cardiomyocytes located at the chunk edges;
85   although the myocytes are most likely to be released by enzymatic digestion.
86
87   Another aspect that may influence isolation efficiency and quality of cells obtained from human
88   specimens is the duration of tissue ischemia. Most protocols mention short transportation times

89   to the laboratory as a prerequisite for best results. This restricts the study of human ventricular
90   cardiomyocytes to laboratories with nearby cardiac surgery facilities. Together, these restrictions
91   hamper the verification of important findings from animal models in human cardiomyocytes.
92   Improved isolation protocols that allow for high cardiomyocyte yields from small amounts of
93   tissue, preferably without serious damage after extended transportation times, are therefore
94   desirable.
95
96   Described here is an isolation protocol based on the enzymatic digestion of thin myocardial tissue
97   slices generated with a vibratome[14,15]. We demonstrate that isolation from tissue slices is much
98   more efficient than that from tissue chunks minced with scissors. The described method not only
99   allows for high yields of viable human cardiomyocytes from small amounts of myocardial tissue
100 but is also applicable to specimens stored or transported in cold cardioplegic solution for up to
101 36 h.
102
103 Female Wistar rats (150–200 g) were commercially obtained, anesthetized by injecting
104 100 mg/kg of thiopental-sodium intraperitoneally, and euthanized by cervical dislocation
105 followed by thoracotomy and excision of the heart. Human cardiac tissue samples were collected
106 from the left-ventricular apical core during implantation of mechanical assist devices, from septal
107 myectomy, from tetralogy of Fallot corrective surgery, or from the free left-ventricular wall of
108 explanted hearts. The following protocol describes the isolation from human ventricular tissue.
109 The isolation of rat cardiomyocytes was performed accordingly, but with different enzymes (see
110 **Table of Materials**). A schematic workflow of the protocol is illustrated in **Figure 1**.
111
112 **PROTOCOL:**
113
114 All experiments with rats were approved by the Animal Care and Use Committee Mittelfranken,
115 Bavaria, Germany. Collection and use of human cardiac tissue samples was approved by the
116 Institutional Review Boards of the University of Erlangen-Nürnberg and the Ruhr-University
117 Bochum. Studies were conducted according to Declaration of Helsinki guidelines. Patients gave
118 their written informed consent prior to tissue collection.
119
120 **1. Preparation of buffers, solutions, and enzymes**
121
122 1.1.   Prepare buffers and solutions as listed in **Table 1**.
123
124 1.2.   Warm up solutions 1, 2, 3 and the modified Tyrode's solution to 37 °C. Store the cutting
125 solution at 4 °C until use.
126
127 NOTE: For 1–2 myocardial slices a total of approximately 15 mL, 8 mL, and 5 mL of solutions 1, 2,
128 and 3 are required for the isolation in one 35 mm tissue culture dish. Scale up accordingly for
129 simultaneous myocyte isolations in multiple dishes. Cutting solution can be frozen and kept at -
130 20 ˚C for several months.
131
132 1.3.   Weigh 1 mg of proteinase XXIV (see **Table of Materials**) into a prechilled 15 mL centrifuge

133 tube and store on ice until use. This is for processing one sample. Scale up the amount
134 accordingly. Do not mix the proteinase and collagenase.
135
136 1.4. Weigh 8 mg of collagenase CLSI (see **Table of Materials**) into a prechilled 15 mL centrifuge
137 tube and store on ice until use. This is for processing one sample. Scale up the amount
138 accordingly. Do not mix the proteinase and collagenase.
139
140 CAUTION: Wear a face mask or work under a fume hood to avoid inhalation of enzyme powder.
141
142 NOTE: Enzyme activity may vary in different lots. Therefore, the optimal concentration may differ
143 and should be determined with each newly purchased enzyme[16].
144
145 **2. Storage and transport of myocardial tissue**
146
147 2.1. Store and transport human cardiac samples in cooled, 4 ˚C cutting solution (**Table 1**).
148
149 2.2. Use the same solution for further tissue processing and vibratome slicing.
150
151 NOTE: Biopsies and surgical heart samples should be transferred immediately to the cutting
152 solution at 4 °C and can then be stored or transported at 4 °C for a maximum of 36 h before the
153 application of this protocol.
154
155 **3. Processing and slicing of the tissue**
156
157 NOTE: The protocol for tissue slicing follows Fischer et al.[15].
158
159 3.1. Trimming of the tissue block
160
161 CAUTION: Human cardiac tissue is potentially infectious. Always use gloves. Carefully handle used
162 blades and discard in safety containers.
163
164 3.1.1. Place the specimen into a 100 mm tissue culture dish filled with 20 mL cold cutting solution
165 and keep on a cooled, 4 °C plate.
166
167 3.1.2. Remove excess fibrotic tissue and epicardial fat with a scalpel. In case of a transmural
168 specimen, remove trabeculae and tissue layers near the endocardium.
169
170 NOTE: Fibrotic tissue is stiff and appears white. Fat is typically soft and appears white to yellow.
171 Trabeculae and endocardial tissue layers can be identified from their loose tissue composition
172 and a nonaligned fiber orientation compared to myocardium of the epicardial layers
173
174 3.1.3. For optimal vibratome processing, cut rectangular tissue blocks of approximately 8 mm x 8
175 mm x 8 mm with a scalpel from a larger tissue specimen. For smaller biopsies skip this step and
176 move to agarose embedding.

177

3.2. Embedding cardiac tissue into low-melting-point agarose

3.2.1. 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.

3.2.2. 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.

3.2.3. 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.

3.2.4. 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.

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

3.3. Slicing the myocardium

3.3.1. Mount an unused razor 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).

3.3.2. 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).

3.3.3. Fix the agarose block to the specimen holder with a thin layer of cyanoacrylate glue and gentle pressure.

3.3.4. 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.

3.3.5. 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.

3.3.6. Check cardiomyocyte alignment under a standard light microscope.

4. Tissue digestion

4.1. Place the heat plate on the lab shaker and warm it to 37 °C. Start the lab shaker at 65 rpm.

4.2. 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.

4.3. 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.

4.4. Add calcium chloride ($CaCl_2$) to the collagenase containing solution (prepared in step 4.3) to a final concentration of 5 µM.

4.5. 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.

4.6. 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.

4.7. 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.

4.8. 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.

4.9. Wash 2x with 2 mL of prewarmed solution 1 (37 °C).

4.10. 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.

4.11. 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.

4.12. 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

5.1.   Dissociate the digested tissue slices with forceps by carefully pulling the fibers apart.

5.2.   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.

5.3.   Check for the liberated rod-shaped cardiomyocytes under the light microscope.

6. Reintroduction of physiological calcium concentration

6.1.   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.

6.2.   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

7.1. 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

309 centrifuge tube and allow the cells to sediment for 10 min at 35 °C, then aspirate 700 µL of the
310 supernatant and discard, resuspend the cells, transfer them back to a 35 mm tissue culture dish
311 and proceed with step 7.2.
312
313 7.2. Add 700 µL of solution 3 to the cells, resume agitation on the heat plate and incubate for
314 10 min.
315
316 7.3. Repeat steps 7.1 and 7.2.
317
318 7.4. Transfer the solution to a 15 mL centrifuge tube and allow the cardiomyocytes to sediment
319 for a minimum of 10 min and a maximum of 30 min at room temperature or spin at 50 x g for
320 1 min. Remove the supernatant completely and resuspend in modified Tyrode's solution or the
321 desired experimentation buffer.
322
323 NOTE: Cardiomyocytes can be stored in modified Tyrode's solution (**Table 1**) for several hours at
324 37 °C and 5% CO_2 before use.
325
326 7.5. Verify the cell quality with a standard light microscope at 40x and 200x magnification.
327
328 NOTE: Around 30–50% of the cardiomyocytes should be rod-shaped, smooth without membrane
329 blebs, and display clear cross-striations. Only 5–10% of the viable cells should show spontaneous
330 contractions.
331
332 **REPRESENTATIVE RESULTS:**
333 To verify isolation efficiency, the protocol was used with rat myocardium and the resulting
334 number of viable myocytes was compared with the numbers obtained by isolation via coronary
335 perfusion and by isolation from small tissue chunks (chunk isolation, **Figure 2**). Chunk isolation
336 and isolation from tissue slices were performed from the same hearts. For the isolation via
337 coronary perfusion, however, the whole heart was used. Coronary perfusion yielded
338 predominantly rod-shaped and cross-striated cardiomyocytes. With isolations from myocardial
339 slices a lower proportion of rod-shaped cells were observed, but the total number was still high.
340 In contrast, after isolation from tissue chunks, only few rod-shaped cells were recovered (**Figure
341 2A**). Flow cytometry was used to compare the results quantitatively. Due to their relatively large
342 size and cross-striation, cardiomyocytes typically show large values for both forward and side
343 scatter. These properties were used to count rod-shaped myocytes with a flow-cytometric cell
344 analyzer, applying a simple gating scheme that discriminated rod-shaped from hypercontracted
345 and rounded cardiomyocytes. (**Supplemental Figure 1**). Representative dot plots are depicted in
346 **Figure 2B.** Statistical analysis showed distinctively higher counts in the cardiomyocyte gate CM1
347 for isolation from slices than from tissue chunks (41.5 ± 11.9 vs. $7.89 \pm 3.6\%$, respectively; n =3
348 isolations; $p < 0.05$, paired two-tailed t-test). As expected from visual inspection, coronary
349 perfusion yielded the highest counts, with $71.0 \pm 9.4\%$ (n = 3 isolations; $p < 0.05$, unpaired two-
350 tailed t-test) (**Figure 2C**). Thus, the isolation of cardiomyocytes from tissue slices does not reach
351 the yield obtained by coronary perfusion, but results in a considerably larger number of rod-
352 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

397 small biopsies (as little as 40 mg) from child hearts (**Supplemental Figure 4**).
398
399 To demonstrate that human cells isolated with the described protocol can be subjected to
400 structural analysis, they were stained with alpha-actinin, the EC coupling proteins L-type calcium
401 channel (LTCC) and ryanodine receptor (RyR), as well as the cell membrane and nuclei, according
402 to a staining method described recently in rat myocytes[17] (**Figure 4**). Alpha-actinin staining
403 revealed a dense, regular z-line pattern and a mean sarcomere length of 1.92 ± 0.06 μm in resting
404 cardiomyocytes (n = 4, **Figure 4A**). LTCC and RyR showed clear clusters and were, as expected,
405 colocalized near the cell membrane and t-tubules (**Figure 4B**).
406
407 The potentiometric dye FluoVolt[18] and the calcium sensitive dye Fluo-4[17] were used to
408 demonstrate that human cardiomyocytes isolated with the described protocol were excitable
409 and could be used for studies on cellular electrophysiology or excitation-contraction coupling
410 (**Figure 5**). Action potential (AP) shape and duration were analyzed (**Figure 5A,B**). The values of
411 50% and 90% AP duration (APD_{50}: 400.6 ± 41.1 ms, APD_{90}: 748.9 ± 56.6 ms, n = 10 cells) were in
412 the range reported by others for ventricular cardiomyocytes from failing human hearts[4,6,7,9]. The
413 calcium transients recorded by confocal line scanning of Fluo4-loaded cells (**Figure 5C,D**) showed
414 a clear upstroke after stimulation and an acceptable signal-to-noise ratio. The calcium transient
415 amplitude (maximum F/F_0) was 2.9 ± 0.5 (n = 31 cells). Cardiomyocyte shortening by contraction
416 can be seen in the example from the deflection of the lower cell border, which had a mean of
417 4.6 ± 0.8% (n = 31 cells).
418
419 **FIGURE AND TABLE LEGENDS:**
420 **Figure 1: Workflow of the isolation protocol from human cardiac tissue slices.** Tissue blocks or
421 biopsies from human myocardium (top left) are embedded in low-melting-point agarose and 300
422 μm thick slices are generated with the oscillating blade of a vibratome (top middle). The slices
423 are transferred to a culture dish and removed from the surrounding agarose (top right). Tissue
424 digestion is carried out at ~35 °C and 65 rpm on a heated and agitated plate (middle, left) and
425 includes: (step 4.8) Incubation in nominally calcium-free solution supplemented with proteinase,
426 (step 4.10) Digestion of the extracellular matrix with collagenase, (step 5) Dissociation and
427 liberation of individual cardiomyocytes with forceps and pipetting. (step 6) The extracellular
428 calcium concentration is raised slowly with adaptation intervals of 5 min from 5 μM to 1.5 mM.
429 (step 7) Stepwise removal of the mechanical uncoupler 2,3-butanedione monoxime (BDM). Rod-
430 shaped and cross-striated human cardiomyocytes are retrieved.
431
432 **Figure 2: Cardiomyocyte yields of myocardial slice, perfusion, and chunk isolation**. (**A**)
433 Representative light micrographs of cardiomyocytes isolated either via coronary perfusion
434 (Perfusion), from vibratome-cut tissue (Myocardial slices), or from minced tissue (Tissue chunks).
435 (**B**) Respective representative flow cytometry dot plots from cardiomyocyte isolations described
436 in **A**. Side scatter area (SSC-Area) and forward scatter area (FSC-Area) were used as indicators of
437 cellular granularity and size, respectively. The gating scheme of the gate CM1 for cardiomyocytes
438 is indicated by the black line and the respective fractions of total counts in percent are displayed.
439 (**C**) Mean ± standard error of the fractions of cardiomyocytes (CM1) assessed by flow-cytometric
440 analysis as described in **B** from n = 3 cell isolations per group. The isolation from myocardial slices

441 and tissue chunks was performed from the same hearts (paired two-tailed t-test). Isolation by
442 perfusion was performed from whole hearts of different animals and compared by the unpaired
443 two-tailed t-test. *p < 0.05.

444

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

455

456 **Figure 4: Microstructural characterization of human cardiomyocytes after isolation**. (**A**)
457 Representative confocal microscopic image after immunostaining of alpha-actinin (green) and
458 nuclear stain with 4',6-diamidin-2-phenylindol (DAPI, blue). The sarcomere length was
459 $1.92 \pm 0.06\,\mu m$ (n = 4 myocytes), determined by analyzing the Fourier spectrum. (**B**)
460 Representative confocal microscopic images of a fixed human ventricular cardiomyocyte
461 coimmunostained for L-type calcium channel (LTCC) and cardiac ryanodine receptor (RyR). The
462 overlay of RyR and LTCC (overlay) and the staining of the extracellular matrix with wheat germ
463 agglutinin (WGA, magenta) is also shown. Nuclei were stained with DAPI (blue).

464

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

477

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

479

480 **Supplemental Figure 1: Validation of the cardiomyocyte (CM1) gating scheme.** Dot plots from
481 flow cytometry, measuring forward- and side-scatter area (FSC-A, SSC-A) indicating size and
482 granularity of the detected cells in a control sample (CTRL) from perfusion-isolated rat
483 cardiomyocytes and after incubation of cardiomyocytes from the same preparation for 15 min at
484 37 °C in modified Tyrode's solution containing 100 mM calcium (calcium overdose). The high

485 extracellular calcium causes hypercontraction and rounding of rod-shaped cardiomyocytes,
486 resulting in a reduction of cells (87.6% vs. 6.00%) in the defined gate (CM1).
487
488 **Supplemental Figure 2: Cardiomyocyte characteristics isolated by perfusion or from slices.** Cell
489 length (**A**) and cell width (**B**) were determined from rat cardiomyocytes fixed directly after
490 isolation via perfusion or from myocardial slices by microscopy (n = 19 and n = 21 cells,
491 respectively). The sarcomere length (**C**) was determined from microscopic images after
492 immunostaining with alpha-actinin and Fourier analysis (n = 24 and n = 23 cells, respectively). The
493 mean activation time (**D**), the maximum F/F_0 (**E**) and the relative shortening (**F**) were assessed
494 from Fluo-4 loaded cardiomyocytes isolated by perfusion or from myocardial slices and
495 responsive to electrical stimulation (n = 100/31 myocytes in D, n = 104/25 myocytes in E and n =
496 42/7 myocytes in F). The fraction of rod-shaped cardiomyocytes (**G**) that contracted upon
497 electrical stimulation, assessed by counting the total number of rod-shaped cells and the
498 contractile cells in ten fields of view at 200x magnification in a standard light microscope was
499 assessed for perfusion and slice isolation (n = 452/276 cells, respectively). (**H**) Analysis as
500 performed in G but after 48 h of cultivation (n = 371/329 cells, respectively).
501
502 **Supplemental Figure 3: Human cardiomyocyte isolation with low yield.** Representative light
503 microscope image of myocytes from an isolation with predominantly hypercontracted (blue) or
504 rounded cells (black) and only few rod-shaped and striated cells (white).
505
506 **Supplemental Figure 4: Cardiomyocyte isolation from infant cardiac biopsies.** (**A**) Image of an
507 endomyocardial biopsy (wet weight approximately 40 mg) obtained during corrective surgery for
508 tetralogy of Fallot in an infant. (**B**) Myocardial slice from the tissue shown in **A**, embedded in
509 agarose. (**C**) Cardiomyocytes isolated from a myocardial slice as shown in **B**.
510
511 **Supplemental Table 1: Human patient data.** A list of the number of samples from human
512 patients included in this study is given. Samples were categorized according to surgery types, age,
513 disease etiology, and transportation time, with the respective total number of samples and the
514 number of successful myocyte isolations.
515
516 **DISCUSSION:**
517 Although the isolation of living cardiomyocytes was established more than 40 years ago and is
518 still a prerequisite for many experimental approaches in cardiac research, it remains a difficult
519 technique with unpredictable outcomes. Cardiomyocyte isolation via perfusion of the coronary
520 arteries with enzyme solution is commonly used for hearts of small animals and yields large
521 numbers of viable cells. However, this requires a relatively complex system and expertise.
522 Furthermore, most human tissue samples are not suited for this method, due to their small size
523 or the absence of coronary artery branches, which makes new methods for the isolation of
524 human cardiomyocytes desirable. The protocol described here is based on the atraumatic
525 generation of thin cardiac tissue slices, which are then subjected to enzymatic digestion. It can
526 be applied to myocardial specimens from animal hearts and human myocardium such as apical
527 cores from left-ventricular assist-device implantation, endomyocardial biopsies from septal
528 myectomy, or explanted hearts (see **Supplemental Table 1**), and reliably produces sufficient

529 amounts of viable, calcium-tolerant cardiomyocytes that can be used for subsequent
530 experiments, like cardiomyocyte cultivation[17].

531

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

549

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

573

574   The described protocol uses a solution that contains high potassium and only traces of sodium
575   for digestion. This solution gave the highest yields of rod-shaped, cross-striated cardiomyocytes.
576   For enzymatic digestion, however, it required a higher concentration of collagenase (4 mg/mL)
577   compared to digestion in normal Tyrode's solution (1.5 mg/mL). High extracellular sodium may
578   lead to intracellular sodium overload, exacerbating the negative effects of the calcium paradox,
579   thus reducing cell yields[27]. Therefore, it is suggested to use solutions with high potassium and
580   low sodium. A high extracellular magnesium concentration is frequently employed in
581   cardiomyocyte isolation protocols[3,28], and has been shown to reduce ischemia-reperfusion
582   injury[29,30] and improve cardiac function after extended periods of cold ischemia[31]. Therefore, the
583   solution applied here also contains a high concentration of magnesium (10 mM). However, it was
584   shown that excitability, contraction force, and conduction velocity may be reduced by high
585   magnesium concentrations[32]. Although these effects were shown to be reversible, effects on
586   isolated cardiomyocytes cannot be excluded. Thus, using magnesium at physiological
587   concentrations (1–2 mM), might result in a lower overall cell yield but improve excitability.

588

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

601

602   The generation of high-quality myocardial slices requires a high precision vibratome. The need
603   for a high-precision tissue slicer or vibratome is a possible drawback of the method, because it is
604   not easily available at every laboratory. The device used for this study is described in more detail
605   elsewhere[14,15]. Different devices have been used successfully to generate myocardial slices by
606   other groups[33–35]. Hence, if the settings of advancing speed, blade amplitude, and oscillation
607   frequency can be met and a horizontal blade orientation with a z-deflection below 0.1 μm is
608   available, successful slicing and isolation should be feasible with other vibratomes. In addition,
609   the tissue slicing is elaborate and significantly prolongs the duration of the protocol
610   (approximately 1–2 h). The use of BDM as a mechanical uncoupling agent has protective effects
611   by reducing cellular energy exposure, ischemic injury, and hypercontraction[26,36,37]. Although the
612   negative inotropic effect of BDM was shown to be quickly reversible after washout[38,39], it is not
613   clear if BDM may have unknown consequences on cardiomyocyte function[40]. This study shows
614   that cells can be isolated with high yields after tissue storage times of up to 36 hours and that
615   human cardiomyocytes can be cultured for up to three days after isolation with this method[17].
616   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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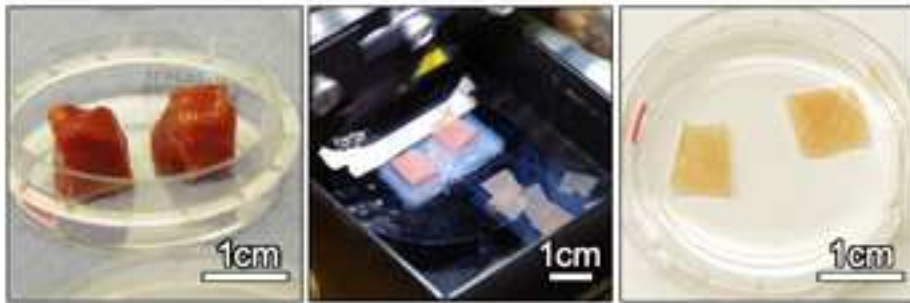740 *Translational Science.* **1**, 155–167 (2016).
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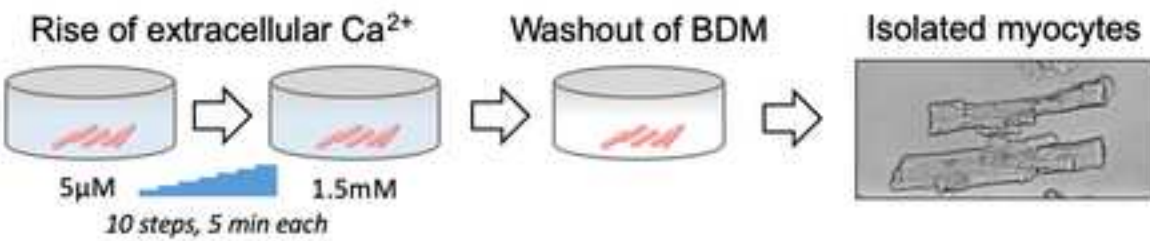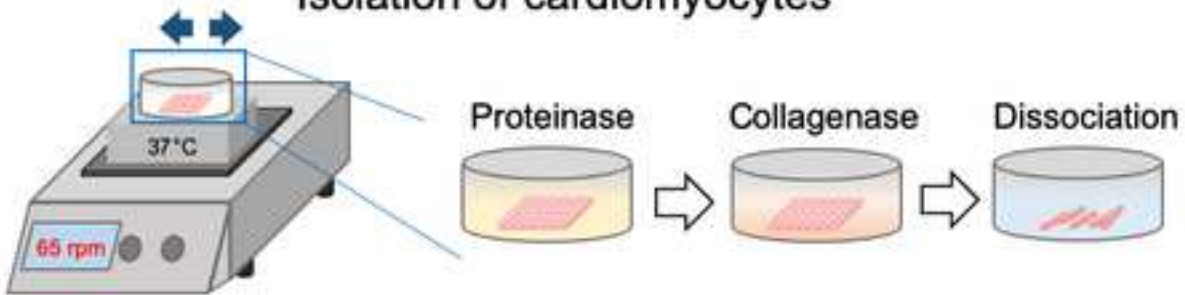747 38.   Horiuti, K. et al. Mechanism of action of 2, 3-butanedione 2-monoxime on contraction of
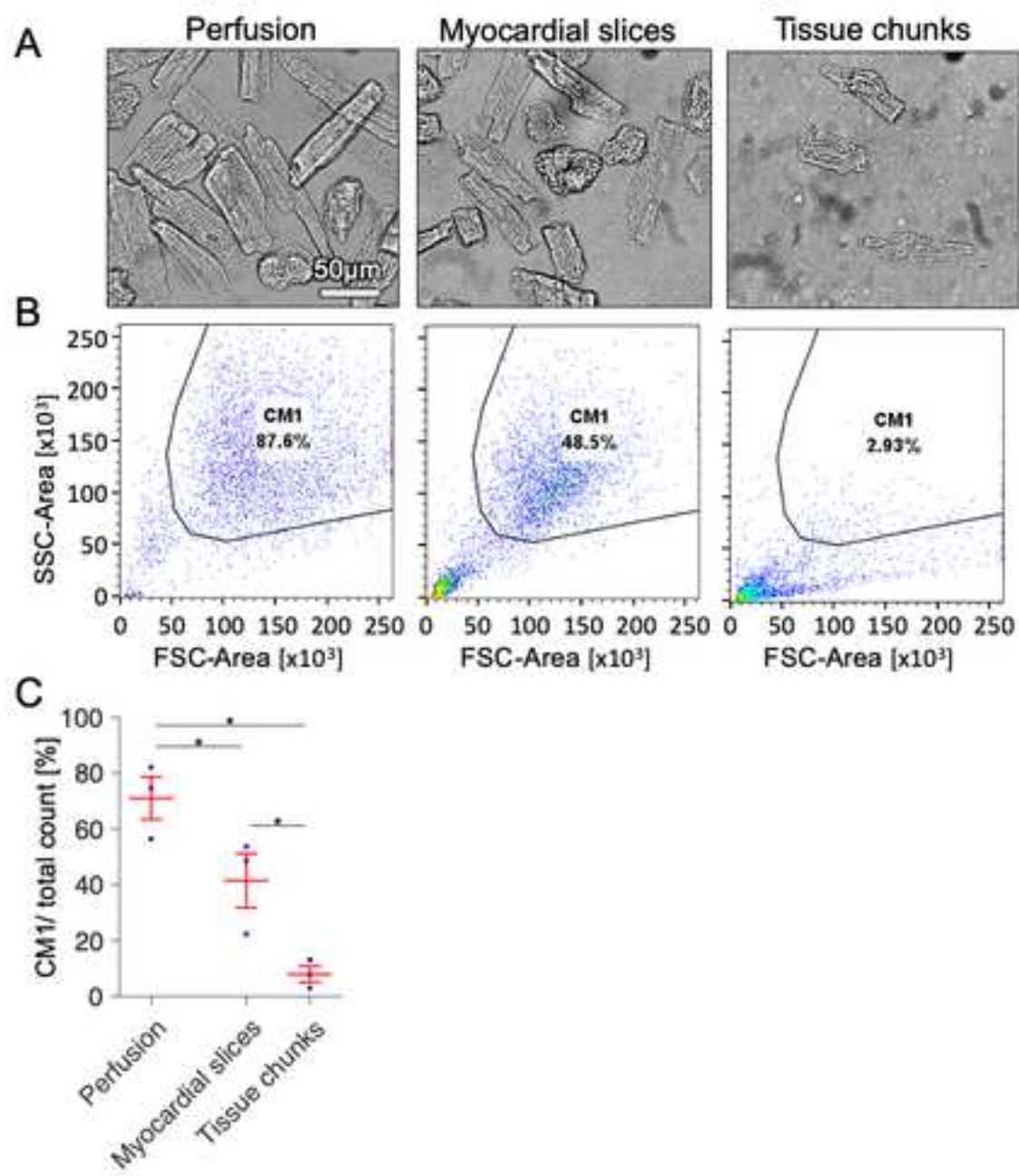748 frog skeletal muscle fibres. *Journal of Muscle Research and Cell Motility.* **9**, 156–164 (1988).

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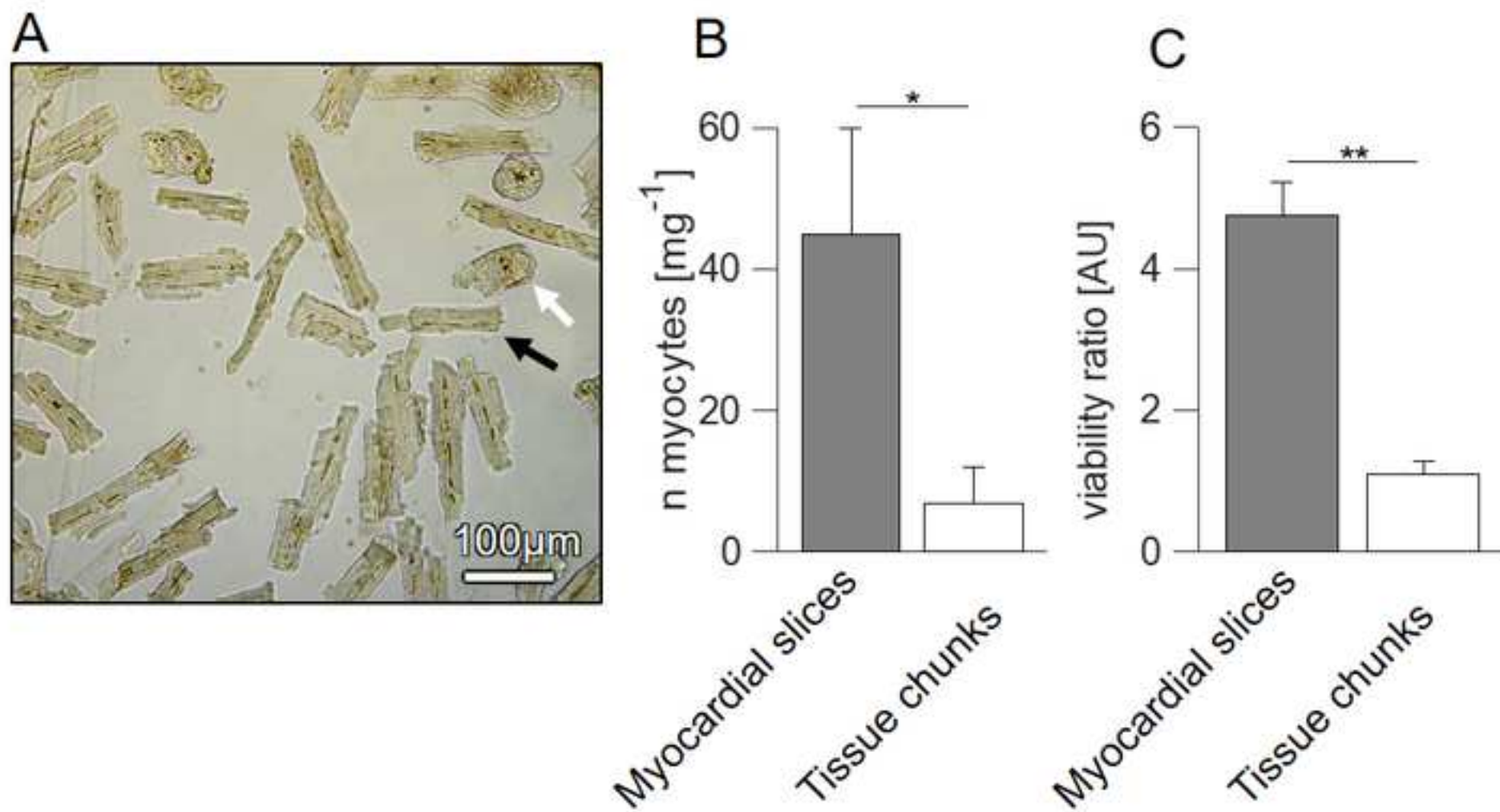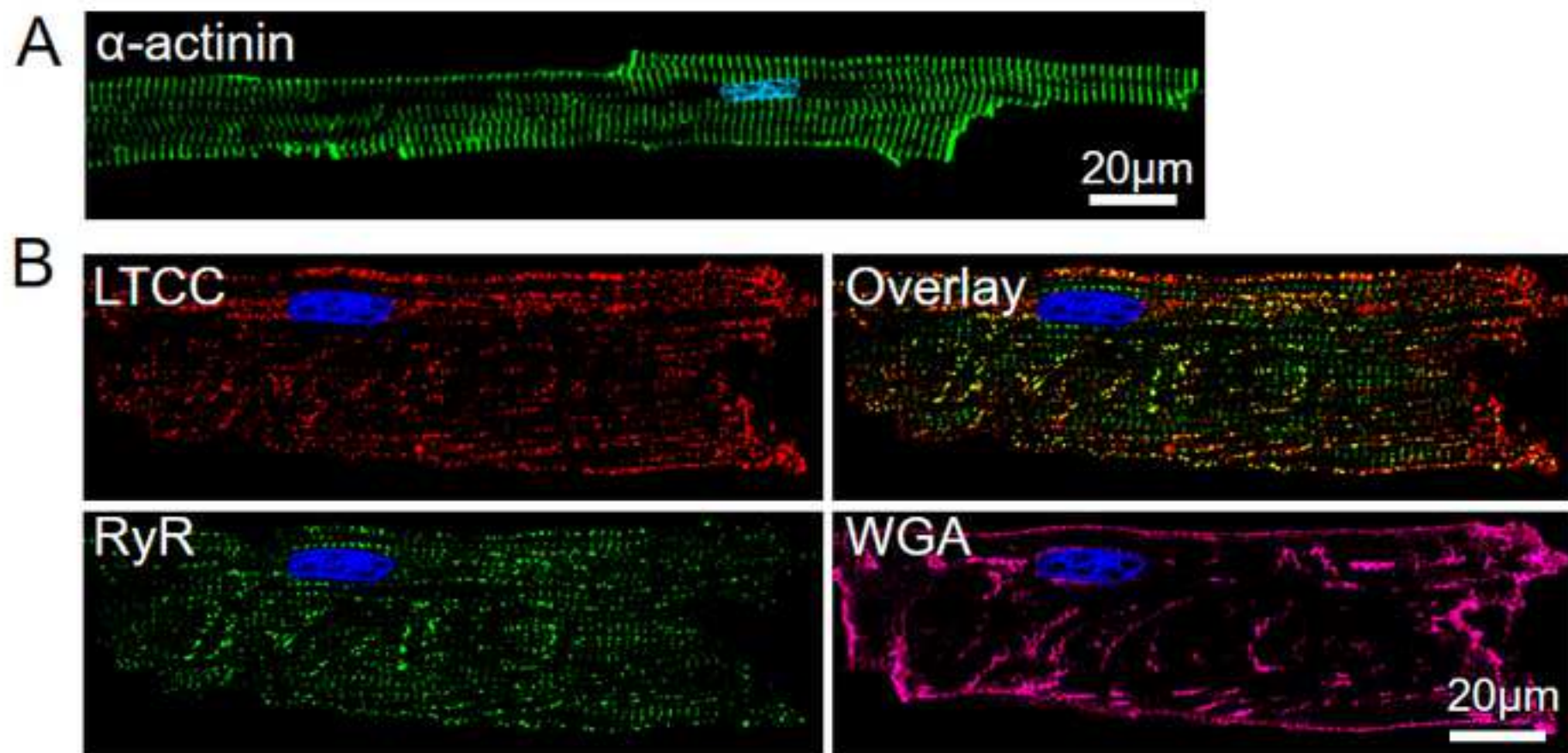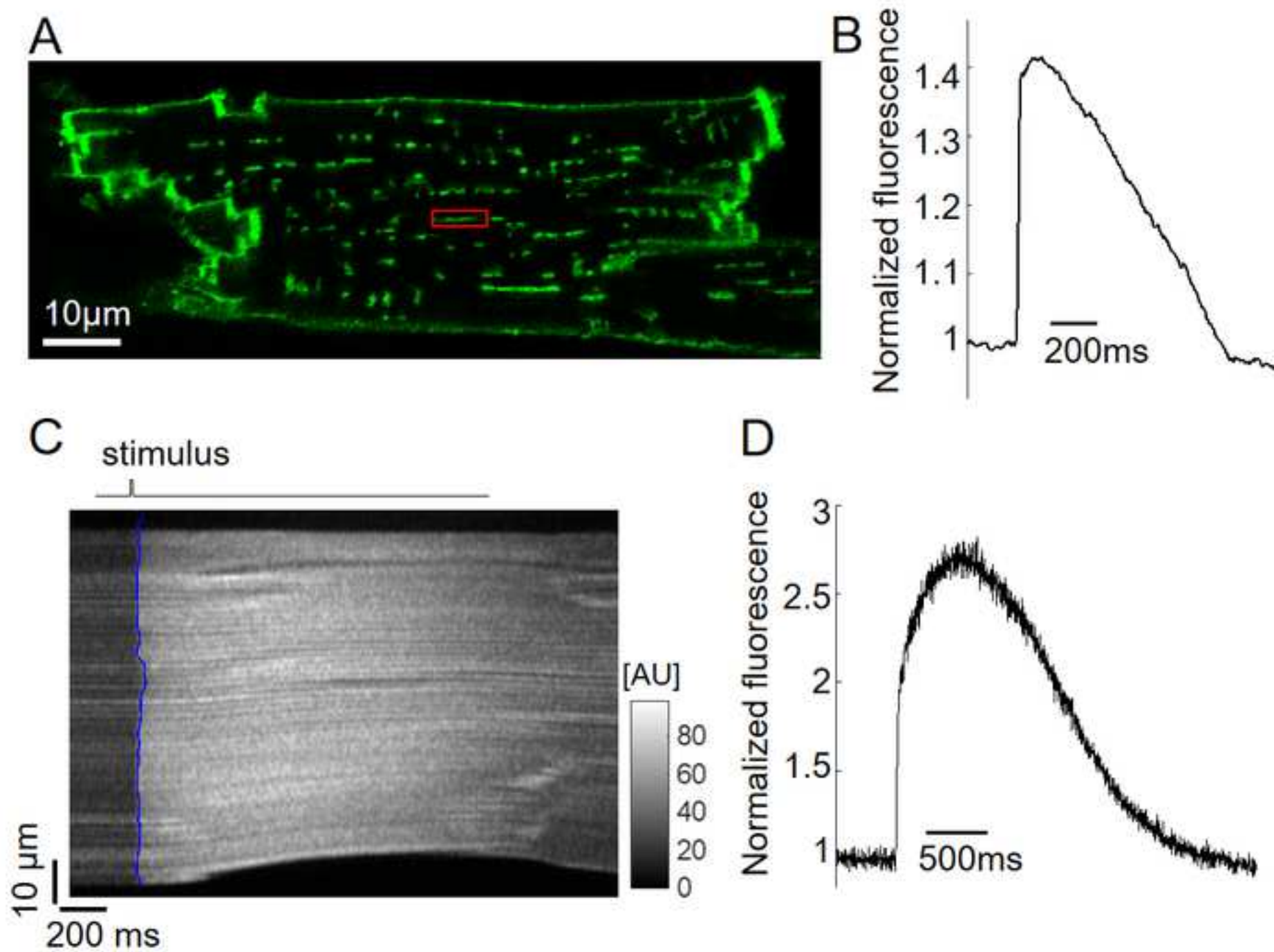
765

Figure 1

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Generation of myocardial slices

Isolation of cardiomyocytes

Figure 2

A. Perfusion — Myocardial slices — Tissue chunks (50μm scale bar)

B. Flow cytometry plots: SSC-Area [x10³] vs FSC-Area [x10³]
- Perfusion: CM1 87.6%
- Myocardial slices: CM1 48.5%
- Tissue chunks: CM1 2.93%

C. CM1/ total count [%] for Perfusion, Myocardial slices, Tissue chunks

Figure3

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Figure4

Figure5

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Table 1

Name	Composition in mmol/L	Supplements
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
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
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
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
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	

pH	Required volume in mL
7.3 with KOH	300
7.3 with KOH	300
7.3 with KOH	300
7.3 with NaOH at 5 % CO_2	100
7.3 with NaOH	500

Table of Materials

Name of Material/ Equipment	Company	Catalog Number
Chemicals		
2,3-butanedionemonoxime	Carl Roth	3494.1
Bovine serum albumin	Carl Roth	163.2
$CaCl_2$	Carl Roth	5239.2
Creatine monohydrate	Alfa Aesar	B250009
Glucose	Merck	50-99-7
HEPES	Carl Roth	9105.3
KCl	Carl Roth	P017.1
KH_2PO_4	Carl Roth	3904.2
L-glutamic acid	Fluka Biochemica	49450
Low melting-point agarose	Carl Roth	6351.5
$MgCl_2$ x6H2O	Carl Roth	A537.1
$MgSO_4$	Sigma Aldrich	M-7506
NaCl	Carl Roth	9265.1
$NaHCO_3$	Carl Roth	8551.2
Paraformaldehyde	Sigma Aldrich	P6148
Taurine	Sigma Aldrich	T8691
Dyes		
Di-8-ANEPPS	Thermo Fisher Scientific	D3167
Fluo-4 AM	Thermo Fisher Scientific	F14201
FluoVolt	Thermo Fisher Scientific	F10488
Enzymes		
Collagenase CLS type I	Worthington	LS004196
Collagenase CLS type II	Worthington	LS004176
Protease XIV	Sigma Aldrich	P8038
Proteinase XXIV	Sigma Aldrich	P5147
Material		
Cell analyzer (LSR Fortessa)	BD Bioscience	649225
Centrifuge tube, 15 mL	Corning	430790
Centrifuge tube, 50 mL	Corning	430829
Compact shaker	Edmund Bühler	KS-15 B control

Disposable plastic pasteur-pipettes	Carl Roth	EA65.1
Forceps	FST	11271-30
Heatblock	VWR	BARN88880030
Nylon net filter, 180 µm	Merck	NY8H04700
TC Dish 100, Standard	Sarstedt	83.3902
TC Dish 35, Standard	Sarstedt	83.3900
TC Dish 60, Standard	Sarstedt	83.3901
Vibratome (VT1200S)	Leica	1491200S001

Comments/Description
Purity>99%
Used for human tissue at 4 mg/mL (activity: 280 U/mg)
Used for rat tissue at 1.5 mg/mL (activity 330 U/mg)
Used for rat tissue at 0.5 mg/mL (activity ≥ 3.5 U/mg)
Used for human tissue at 0.5 mg/mL (activity: 7-14 U/mg)
Agitation direction: horizontal

For cell trituration use only pipettes with an inner tip diameter ≥2 mm

Includes VibroCheck for infrared-assisted correction of z-deflection

Reviewer #1:

Manuscript Summary:

In this study entitled "Isolation of human ventricular cardiomyocytes from vibratome-cut myocardial slices" the authors describe an efficient method to isolate functional cardiomyocytes (CM) from human and animal myocardial slices. The authors compare this approach with currently gold standard methods such as coronary perfusion and tissue-chunk digestion.

The results show that myocardial slices are indeed a valuable and useful alternative for CM isolation and that CM isolation from myocardial slices is more efficient than isolation from tissue-chunks. The protocol is well written and easy to follow and it will indeed help other groups to replicate this approach and expand the utilization of this method.

We thank the reviewer for the positive comments on our manuscript.

Major Concerns:

I do not have any major concern

Minor Concerns:

Below is a list of minor changes that could further improve the manuscript:

1. The vibratome settings for the z-vibration are not indicated (in other paper it is indicated <1µm).

With the mentioned infrared-assisted device to measure z-deflection, we minimized the value to below 0.1 µm. The cutting angle of the blade was 15° We added these details to the protocol.

2. Paragraph 339-346 some more info related to Ca2+ transients and action potentials should be included (time to peak, t50, t90, decay rate...)

We performed additional analyses determining the time to peak, the time to 50% and 90% repolarization of action potentials (APD_{50}, APD_{90}) recorded from human cardiomyocytes. In addition, we determined the maximal amplitude of the calcium transient F/F_0 of human cardiomyocytes and the relative shortening. The respective values are now given in the text of the results part.

3. Fig 1 please better indicate the re-equilibration steps with the actual steps indicated in the manuscript

The individual steps are now numbered according to the protocol. We have also added more detail on the re-addition of calcium (Step 7).

4. Fig 2 the graph could show the individual values for the reader to appreciate the variability between samples.

The graph was changed, now showing the individual datapoints of the experiments.

5. Discussion 432-439: in order to isolate CM with coronary perfusion a Langendorff system is often required. This is an expensive and complex system. Myocardial slices (as indicated in the limitations) is also time consuming and requires specific skill and access to a vibratome, still it should be emphasised that new and simpler methods for human specimens are needed.

The requirement of new methods for human cardiomyocyte isolation is now emphasized at the beginning of the discussion.

6. Line 448 Watson et al (Nat Prot 2017) have shown that in myocardial slices cell damage is limited to the surface of the preparation. This supports the author's justification for improved CM number. This paper should also be acknowledged as the only detailed protocol for myocardial slice preparation available in the literature.

We thank the reviewer for pointing out the importance of the reference. We now reference the publication of Watson et al 2017 and emphasized its importance as it provides a very nice and detailed protocol for myocardial slice preparation.

7. Line 524 long term systems for myocardial slice in vitro culture are briefly mentioned. The bibliography however is quite old. In 2019 three papers have shown novel biomimetic culture systems able to better recapitulate tissue physiology and limit in vitro tissue remodelling (PMID:31092830, PMID:30671746 and PMID:21972180). These are superior to previous methods and they should be cited.

We added the corresponding references, referring to the recent improvements and optimizations of the culture conditions for myocardial slices.

8. In the protocol mechanical sheer stress from the pipette is proclaimed harmful. The introduction of a step to pull apart the slice with a forceps, could also lead to harmful mechanical stress. This could be made an optional step.

We agree that every mechanical intervention may cause shear stress and, consequently, cardiomyocyte damage. However, from our experience a dissociation of the tissue is a prerequisite for sufficient liberation of cardiomyocytes and should not be indicated as an optional step. Nevertheless, we acknowledge, that dissociation by forceps should be done gently and with great care. Therefore, we now explained this issue in more detail in the "Note".

9. Point 8.4 the minimum time for CM to sediment is indicated. It would be informative to also have the max time for the CM to left in the tube.

We now provide a maximum time value of 30 min for sedimentation, which should not be exceeded due to limited oxygen supply.

10. In the discussion the advantage of cell isolation is clearly described. It would also be interesting to mention the possibility to isolate other cell types such as cardiac fibroblasts, endothelial cells etc. (with an adapted protocol). This will further expand the utilization of this approach.

We agree that this could be an additional application of our protocol. We now mention that, in principle, and with adequate modifications, the protocol could be adapted to isolate cardiac

fibroblasts, endothelial cells or macrophages from myocardial slices. For protocol modifications and cultivation of isolated cells we refer to an earlier publication (Eghbali-Webb, M. & Agocha, A. E. Novel Methods in Molecular and Cellular Biochemistry of Muscle (eds. Pierce, G. N. & Claycomb, W. C.) 195–198 (Springer US, 1997). doi:10.1007/978-1-4615-6353-2_19.)

Reviewer #2:

Manuscript Summary:

The study from Fiegle et al presents a myocardial slice-based method for the isolation of ventricular cardiomyocytes from rats and humans. The method appears promising, but some additional characterization on the quality and function of the isolated cells is required to properly assess the utility of the method.

The rationale for this study is sound and presented clearly; indeed, human cardiomyocyte isolations are challenging, particularly for groups without easy access to cardiac surgical facilities or to very large pieces of myocardium that can be perfused through the coronary vasculature. Improved methodologies for small chunks of human tissue are needed (and the "tissue chunk method" attempted by many in the past is very limited, as the authors confirm here).

The paper is well written - the methods are detailed, user friendly, and seem reasonable. The comparisons that the authors do make are clear and analyzed appropriately. However, the very limited functional characterization of the slice-isolated cells restricts the ability of the reader to discern just how useful this approach may be, particularly when compared to the gold standard of isolation via tissue perfusion.

Major Concerns:

 1. The flow cytometry-based quantification of percentage of rod-shaped myocytes in figure 2 is useful and clearly demonstrates the utility of perfusion vs. slice vs. chunk approaches in rats. The quantification of cell yields and viability for slice vs. chunk methods for human isolations in figure 3 is also useful. However, cell viability does not necessarily ensure cell quality for functional assays or cell culture, and the structural/functional characterizations in figures 4 and 5 are limited to single representative examples that are not particularly informative. Using the rat as an easily accessible benchmarking tool, the authors should compare other key metrics of cell morphology and function between perfusion and slice isolated cardiomyocytes. This is partly motivated by the apparent difference in cell quality suggested from the images in Figure 2A. These metrics should include at least cell length, width, resting sarcomere length, percent excitable cells (rod shaped cells that respond to field stimulation) and fractional shortening.

We recognize that Figure 2A was somewhat misleading because the images for "Perfusion" and "Myocardial slice" were acquired at different magnification. This might have contributed to the perception of differences in myocyte quality. For this reason, we acquired new photographs providing more representative example images. These were recorded with the same lens and magnification (see Figure 2B). In addition to the representative examples shown in Figure 4 and Figure 5, we now also provide statistical data recorded from human cardiomyocytes from action potential measurements (TTP: 59.18 ± 7.07 ms, APD50: 400.58 ± 41.06 ms, APD90: 748.87 ± 56.62 ms n =10 cells) and calcium transients (maximal F/F_0 2.9 ± 0.5 AU, relative shortening 4.6 ± 0.8 %, n=31 cells, respectively) in the results part.

To address the quality of the isolated rat cardiomyocytes, we added an additional figure (new Supplemental Figure 2) where we show cardiomyocyte metrics (length and width), assessed by microscopy of fixed cells, isolated either via perfusion or from myocardial slices. Length and width did not differ significantly (length: 110.0 ± 5.4 μm vs 99.4 ± 3.2 μm, p=0.08, width: 33.9 ± 1.9 μm vs 30.6 ± 1.2 μm, p=0.08, for n=19 and 21 cells, respectively). In addition, we analyzed the sarcomere length in alpha-actinin stained cardiomyocytes by Fourier analysis and found no significant difference

(1.62 ± 0.04 μm vs 1.68 ± 0.04 μm, p=0.28, n = 24 and 23 cells for perfusion vs myocardial slice respectively). Furthermore, we compare the mean activation time, maximal signal of the calcium transient (F/F$_0$), and fractional shortening. These parameters were assessed by confocal calcium imaging after Fluo-4 loading as described previously (Seidel et al. 2019, BRIC, PMID 31673803).

For the experiments, addressing the fraction of contractile cardiomyocytes please see the response to point 2. below.

2. Ideally, these assessments would also be carried out in 48hr cultured cells to demonstrate that slice-isolated cells are amenable to cell culture. Perfusion-isolated rat cardiomyocytes hold up very well in short term culture, importantly allowing high quality gene transfer studies followed by functional assessments. As an example for why such a demonstration is needed, viability from mouse cell isolations can be quite high (>80%) and remain high after 24-48 hrs in cell culture, but these cells are notoriously (and mysteriously) difficult to field stimulate after time in culture (although a few can always be found), and are thus typically not considered acceptable for gene transfer studies. Additional data showing that the slice-isolated cells are healthy, functional, and can (or cannot) maintain their function in culture would greatly strengthen the appeal of this approach and add clarify its usefulness.

We performed an additional experiment using rat hearts and compared the fraction of rod-shaped cardiomyocytes that respond to electrical field stimulation directly after isolation and after 48 hours of cultivation. From the new Supplemental Figure 2, G, H it can be appreciated that 62.1 ± 2.9 % of the cardiomyocytes isolated from myocardial slices are responding to electrical excitation with contraction. After 48 hours of cultivation 31.5 ± 2.3 % of the cardiomyocytes still responded to stimulation. Although, these values were higher in myocytes isolated by perfusion (fresh: 74.3 ± 4.2 %, and 48 hours: 41.9 ± 3.6 %, p<0.05), the difference of approx. 10% seems to be well acceptable. In conclusion, the contractility and survival of cardiomyocytes isolated from tissue slices is sufficient for downstream functional experiments and cultivation. We would also like to refer to our recent publication (Seidel et al. 2019, BRIC, PMID 31673803), where we kept human cardiomyocytes isolated with the described protocol in culture for up to three days.

Minor Concerns:

1. I could not find any patient details on the human tissue utilized for these studies, other than to say they were from LVAD cores or explanted hearts. Were the explants non-failing or failing hearts? Did the success of isolation (the authors mention that 21 out of 27 were successful, what is the benchmark of success?) correlate with etiology? Others have found that LVAD cores are more difficult to isolate from then LV free wall, did their success with isolation depend on the tissue location? These additional details will help the user. Also, LVAD cores have been a challenge for many groups, and thus demonstrating convincingly that their slice method can yield high quality cells from LVAD cores will benefit the field and should be robustly demonstrated and highlighted.

We now provide a table listing the total number human specimens used in this study. As stated in "Table 3: Patient data", we successfully isolated cardiomyocytes from 8 of 8 LVAD cores. In addition, there was no prominent difference of isolation success when regarding disease etiologies. However, we found that transportation times above 36 hours generally reduce the probability of a successful outcome of the experiment. An experiment was defined as successful, when the isolation yielded sufficient viable cardiomyocytes to perform the mentioned downstream experiments (more than

approx. 100 rod-shaped myocytes per dish). To emphasize the possibility to isolate cardiomyocytes from LVAD cores we mentioned the origin of human specimens again in the discussion.

Reviewer#3:

Manuscript Summary:

In the present study Dr. Fiegle et al. explain a protocol to isolate human (and rat) ventricular myocytes from vibratome-cut slices in order to perform calcium handling experiments with freshly isolated myocytes. Human myocytes isolation is always an interesting technique, specially if the myocytes can be used for electrophysiological experiments. The protocol is well described and it allows to obtain good quality cells. However, I have some major concerns:

Major Concerns:

- the authors mention that digestion time is quite variable and recommend to increase time of digestion of enzymes concentrations. This is really unprecise. If all these conditions must be tested in "each laboratory" as the authors say and with each human tissue samples (because its variavility from patient to patient is very high) it seems unlikely to be able to reproduce the protocol just by reading this paper. What are the specific timings for every step of the protocol? How many tests are needed to find the right conditions for every tissue in different laboratories? Also, what happens with small biopsies? Although in the picture of Fig 1 there are 2 cubes of 1x1x1 cm many biopsies would be by large much smaller and that means perhaps just 1 test per tissue…

We thank the reviewer for his/her positive feedback. As we mention in the protocol Collagenase digestion can be quite variable, which does not allow for a general statement for a definite digestion time. However, we used different batches of collagenases and repeatedly observed that a digestion time between 30-40 min gave the best results. Furthermore, human myocardial tissue itself is quite variable. We noticed that fibrotic samples from old patients tended to require longer digestion times (40 min), whereas samples from children required less (30 min). Rat myocardial slices, which show much less variability, usually required 30 min. Following the steps provided in the protocol should lead to a successful outcome. Variation of the digestion time between 30-40 min could however slightly increase the yields of viable cardiomyocytes. If enough sample material is present, it is feasible to perform multiple experiments in parallel to quickly approach the best conditions. Indeed, for very small biopsies, as shown in Supplemental Figure 4, this is impossible as only a small number of slices can be generated. We now describe, that a regular checkup of the tissue texture during the digestion is also recommended. This makes it possible to determine the optimal digestion time also when only little sample material is available.


- What is the advantage of the agarose inclusion and posterior vibratome-cut other than the transport? So, what is the advantage of the proposed agarose inclusion method for the isolation it self? Because if it is just the transport it seems a very complicated method to allow transport of tissues which could be transported in cold cardioplegic solution as for example custodiol

Embedding the cardiac tissue into agarose is only required for cutting on a vibratome. It improves the mechanical stability of the tissue block and ensures myocardial slices of uniform thickness (We refer to recent articles, which describe the generation of myocardial slices in detail accordingly (Fischer, C. et al. Nature Communications **10**, (2019), PMID: 30631059; Watson, S. A. et al. Nature Protocols **12**, 2623–2639 (2017), PMID: 29189769.
However, we do not recommend to embed the tissue in agarose for transportation because the agarose might compromise diffusion of oxygen and nutrients. Transportation should be carried out with the tissue being submerged in cold cutting solution immediately after excision from the heart.

We added a more detailed description in the paragraph "Transportation of cardiac tissue" to clarify this issue.

Supplemental Figure 3

100μm
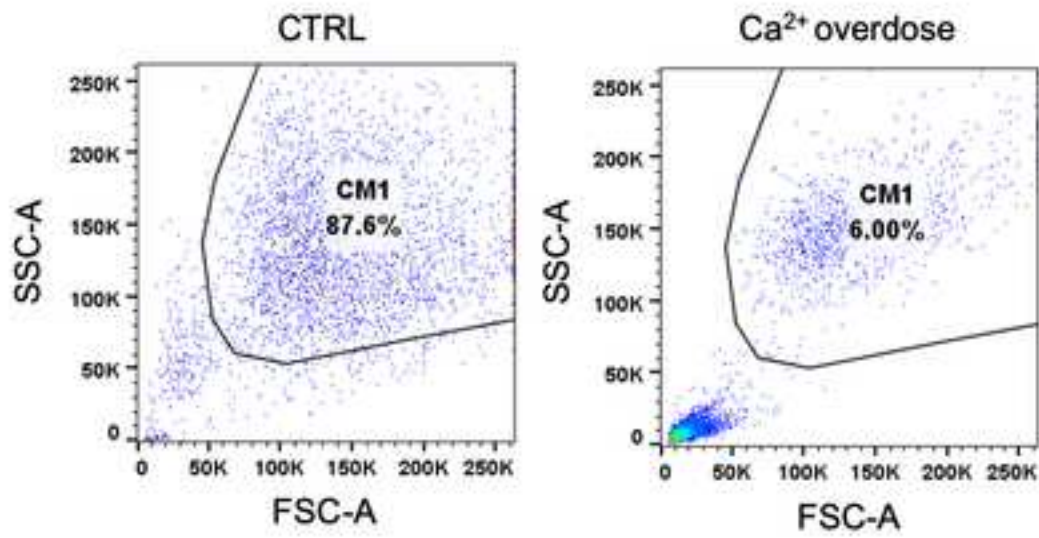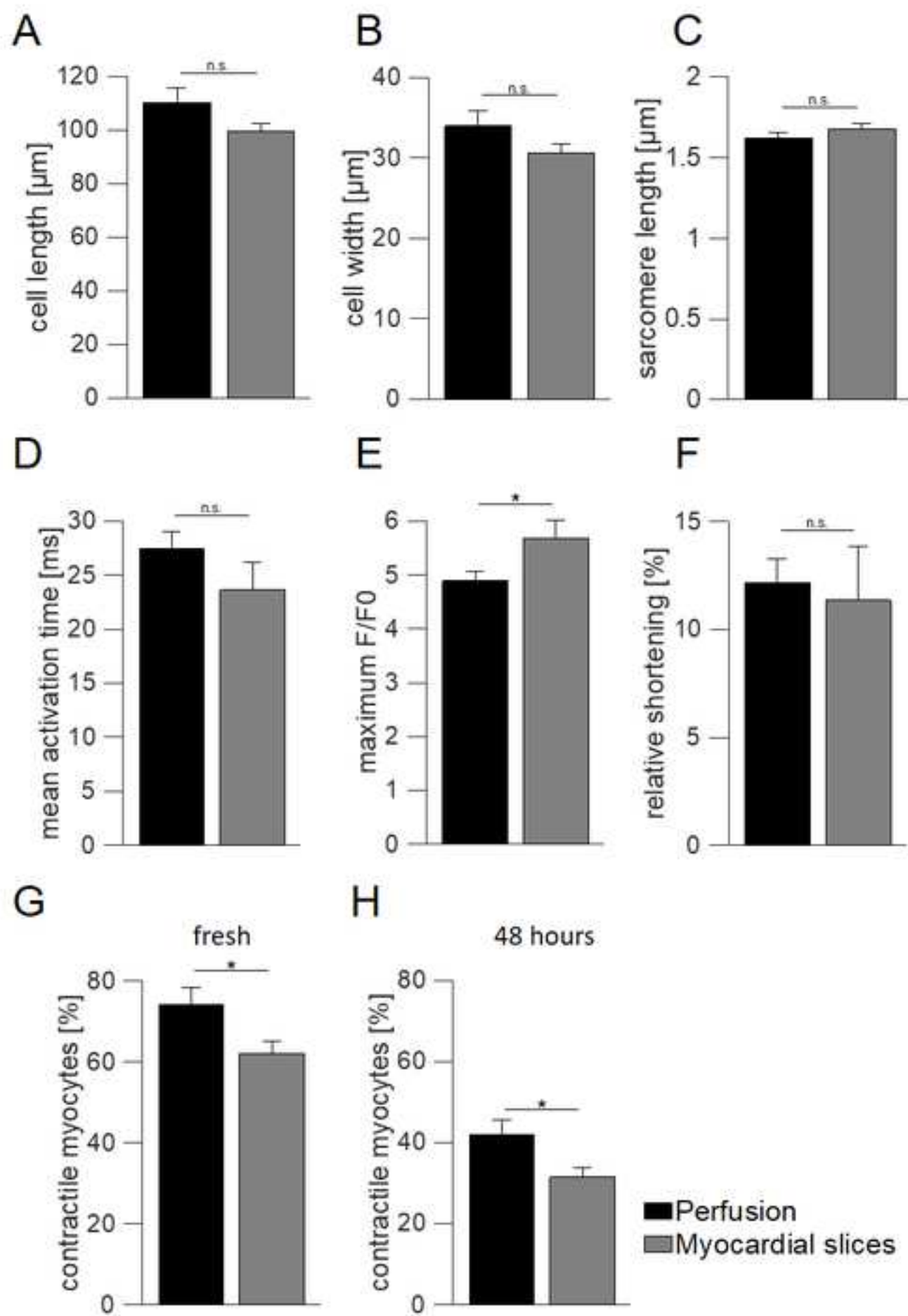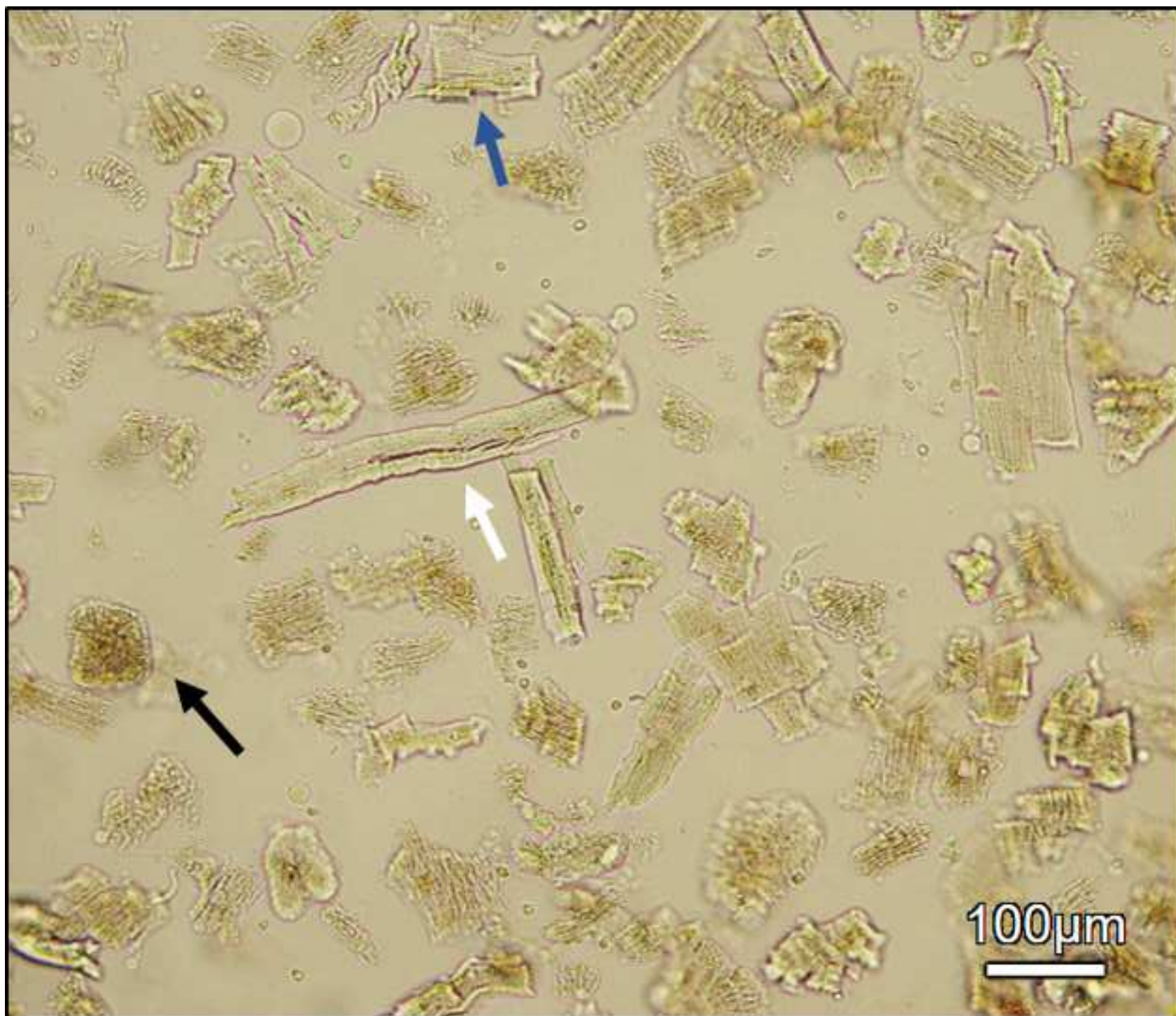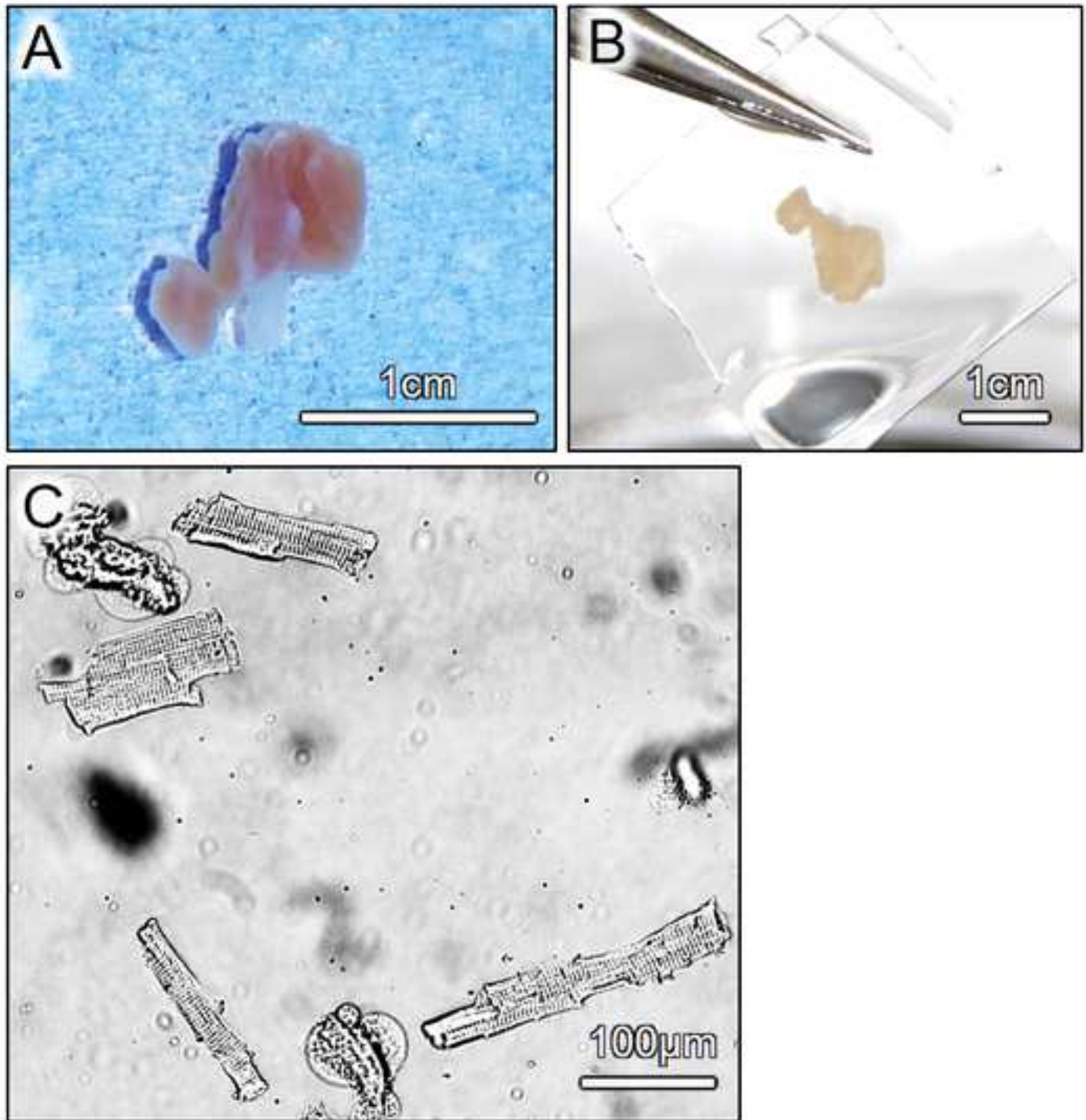
	number of samples	of which successful
total	30	23
surgery		
Left-ventricular assist device implantation,	8	8
Heart transplantation, HTX	17	12
septal myectomy	3	1
Fallot corrective surgery	2	2
age		
adult	24	19
children	6	4
type of cardiomyopathy		
Ischemic cardiomyopathy, ICM	7	7
Non-ischemic cardiomyopathy, NICM	23	16
transportation time		
< 16 hours	10	7
> 16 hours	20	16

% successful
77%
100%
71%
33%
100%
79%
67%
100%
70%
70%
80%