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Corresponding Author:	Franziska Schneider-Warme Universitats-Herzzentrum Freiburg Bad Krozingen GmbH Freiburg, Baden-Württemberg GERMANY
Corresponding Author's Institution:	Universitats-Herzzentrum Freiburg Bad Krozingen GmbH
Corresponding Author E-Mail:	franziska.schneider.uhz@universitaets-herzzentrum.de
Order of Authors:	Ramona Angela Kopton
	Cinthia Buchmann
	Robin Moss
	Peter Kohl
	Rémi Peyronnet
	Franziska Schneider-Warme
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Cover Letter

Universitäts-Herzzentrum Freiburg • Bad Krozingen, und Medizinische Fakultät der Albert-Ludwigs-Universität Freiburg
Institut für Experimentelle Kardiovaskuläre Medizin • Elsässer Str. 2Q •79110 Freiburg

Dr. Franziska Schneider-Warme

Sektionsleiterin Optogenetik, Institut für experimentelle kardiovaskuläre Medizin
Head of Optogenetics Section, Institute for Experimental Cardiovascular Medicine
Telefon +49 761 270-63954
Telefax +49 761 270-63959

franziska.schneider@universitaets-herzzentrum.de

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Dear editors, dear Dr Berard,

Following your invitation to submit a research protocol, please find enclosed our manuscript entitled “Electromechanical Assessment of Optogenetically Modulated Cardiomyocyte Activity” to be considered for publication in Jove.

Optogenetics - the use of light-activated proteins to modulate and/or observe cellular behaviour - is a fast-developing technology first applied to neuroscience research just over a decade ago. To date, a number of studies have begun to transfer the optogenetic approach to cardiovascular research, with a focus on manipulation and monitoring of cardiomyocyte activity.

Here, we provide a protocol to characterize the electromechanical effects of activating light-gated chloride channels in rabbit isolated cardiomyocytes. The protocol includes cell isolation, culturing and adenoviral transduction, followed by patch-clamp recordings and carbon fibre measurements of cell mechanics to assess cardiomyocyte responses to light stimulation.

We think that a thorough characterization of light-induced effects in the target cells is an important prerequisite for efficient and informative optogenetic experiments in cardiac tissue and whole hearts. As our protocol can be generally applied to test different optogenetic actuators, we anticipate that it will be useful for many researchers using optogenetics in the heart.

With kind regards on behalf of all coauthors,

F. Schneider-Warme

Dr Franziska Schneider-Warme

TITLE:

Electromechanical Assessment of Optogenetically Modulated Cardiomyocyte Activity

AUTHORS AND AFFILIATIONS:

Ramona A. Kopton[1,2,3], Cinthia Buchmann[1,2], Robin Moss[1,2], Peter Kohl[1,2], Rémi Peyronnet[1,2], and Franziska Schneider-Warme[1,2]

[1] Institute for Experimental Cardiovascular Medicine, University Heart Center Freiburg–Bad Krozingen, Medical Center—University of Freiburg, Freiburg, Germany
[2] Faculty of Medicine, University of Freiburg, Freiburg, Germany
[3] Faculty of Biology, University of Freiburg, Freiburg, Germany

Corresponding Author:

Ramona A. Kopton
ramona.kopton@universitaets-herzzentrum.de

Email Address of Co-authors:

Cinthia Buchmann (cinthia.buchmann@universitaets-herzzentrum.de)
Robin Moss (kai.robin.moss@universitaets-herzzentrum.de)
Peter Kohl (peter.kohl@universitaets-herzzentrum.de)
Rémi Peyronnet (remi.peyronnet@universitaets-herzzentrum.de)
Franziska Schneider-Warme (franziska.schneider@universitaets-herzzentrum.de)

KEYWORDS:

Natural anion channelrhodopsin, GtACR1, *Guillardia theta*, heart, cardiomyocytes, optogenetics, action potential, cardiac electrophysiology, carbon fiber technique, contractility, force measurement, mechanics

SUMMARY:

We present a protocol for evaluating the electromechanical effects of GtACR1 activation in rabbit cardiomyocytes. We provide detailed information on cell isolation, culturing and adenoviral transduction, and on functional experiments with the patch-clamp and carbon-fiber techniques.

ABSTRACT:

Over the past two decades, optogenetic tools have been established as potent means to modulate cell-type specific activity in excitable tissues, including the heart. While Channelrhodopsin-2 (ChR2) is a common tool to depolarize the membrane potential in cardiomyocytes (CM), potentially eliciting action potentials (AP), an effective tool for reliable silencing of CM activity has been missing. It has been suggested to use anion channelrhodopsins (ACR) for optogenetic inhibition. Here, we describe a protocol to assess the effects of activating the natural ACR GtACR1 from *Guillardia theta* in cultured rabbit CM. Primary readouts are electrophysiological patch-clamp recordings and optical tracking of cardiomyocyte contractions, both performed while applying different patterns of light stimulation. The protocol includes CM isolation from rabbit heart, seeding and culturing of the cells for up to 4 days, transduction via

adenovirus coding for the light-gated chloride channel, preparation of patch-clamp and carbon fiber setups, data collection and analysis. Using the patch-clamp technique in whole-cell configuration allows one to record light-activated currents (in voltage-clamp mode, V-clamp) and AP (current-clamp mode, I-clamp) in real time. In addition to patch-clamp experiments, we conduct contractility measurements for functional assessment of CM activity without disturbing the intracellular milieu. To do so, cells are mechanically preloaded using carbon fibers and contractions are recorded by tracking changes in sarcomere length and carbon fiber distance. Data analysis includes assessment of AP duration from I-clamp recordings, peak currents from V-clamp recordings and force calculation from carbon fiber measurements. The described protocol can be applied to the testing of biophysical effects of different optogenetic actuators on CM activity, a prerequisite for the development of a mechanistic understanding of optogenetic experiments in cardiac tissue and whole hearts.

INTRODUCTION:

ChR-mediated photocurrents were first recorded in the eyespot of unicellular green algae[1,2]. Soon after genetic cloning and heterologous expression of *Chlamydomonas reinhardtii* ChR1 and ChR2, ChR were used as tools to alter the membrane potential in Xenopus oocytes and mammalian cells by light[3,4]. Cation non-selective ChR depolarizes the membrane of cells with a resting membrane potential that is negative to the reversal potential of ChR. They can thus be used to elicit AP in excitable cells, including neurons and CM, allowing optical pacing[5,6].

Complementary to cation ChR, light-driven proton, chloride and sodium pumps[7–9] have been used to inhibit neuronal activity[10–12]. However, the latter have limitations, requiring high light intensities and sustained illumination, as one ion is transported per absorbed photon. In 2014, two independent studies by Wietek et al. and Berndt et al. described the conversion of cation-conducting ChR into ACR via mutations in the channel pore[13,14]. One year later, natural ACR were discovered in the cryptophyte *Guillardia theta* (GtACR)[15]. As engineered ACR showed residual cation conductance, they were replaced by natural ACR, characterized by a large single-channel conductance and high light sensitivity[15]. GtACR were used to silence neuronal activity by polarizing the membrane potential towards the reversal potential of chloride[16,17]. Govorunova et al. applied GtACR1 to cultured rat ventricular CM and showed efficient photoinhibition at low light intensity levels that were not sufficient to activate previously available inhibition tools, such as the proton pump Arch[18]. Our group recently reported that GtACR1-mediated photoinhibition of CM is based on depolarization and that GtACR1 can also be used, therefore, for optical pacing of CM[19].

Here, we present a protocol for studying the electrophysiological and mechanical effects of GtACR1 photoactivation on cultured rabbit ventricular CM. We first describe cell isolation, culturing and transduction. Electrophysiological effects are measured using whole-cell patch-clamp recordings. Light-mediated currents at a given membrane voltage are assessed in V-clamp mode. Membrane potential dynamics are measured while electrically or optically pacing CM (I-clamp mode). Optical inhibition of electrically triggered AP is tested using sustained light application. Mechanical effects are measured using carbon fibers in combination with imaging-based tracking of sarcomere length. To do so, optically paceable cells are mechanically preloaded

by attaching two carbon fibers to the plasma membrane near opposite cell ends. Sarcomere length changes are recorded during optical or electrical pacing. Finally, photoinhibition is measured during electrical field stimulation of the cells, and generated forces are analyzed.

The protocol includes the following steps shown in the flowchart in **Figure 1**: rabbit deep anesthesia, thiopental overdose injection, heart excision, Langendorff-perfusion and tissue digestion, mechanical dissociation of the tissue to release cells, microscopic analysis of CM yield, culturing of CM, transduction with adenovirus type 5, followed by incubation and functional experiments.

[Place **Figure 1** here]

PROTOCOL:

All rabbit experiments were carried out according to the guidelines stated in Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes and approved by the local authorities in Baden-Württemberg (Regierungspräsidium Freiburg, X-16/10R), Germany).

1. Solutions for cell isolation

1.1. Prepare the solutions for the cell isolation with water of the following requirements (**Table 1**) and according to the ionic compositions listed in **Table 2**.

[Place **Table 1** here]

[Place **Table 2** here]

NOTE: $CaCl_2$ and $MgCl_2$ are added from 1 M stock solutions.

1.2. Adjust all solutions to pH 7.4 at 37 °C and check osmolarity.

NOTE: Dissolve the enzymes (Collagenase type 2 and Protease XIV) directly before heart excision. Oxygenate all solutions prior to use.

2. Preparation of the Langendorff-perfusion setup

NOTE: The used setup is custom-made. As depicted in **Figure 2**, the setup consists of three water jacketed reservoirs (1-3), one spiral counter-flow heat exchanger (4) and a water jacketed perfusion vessel (5).

[Place **Figure 2** here]

2.1. Switch on the pump of the water bath to circulate water at 38 °C in the heat exchange system

and preheat all solutions to 37 °C.

NOTE: The temperature at the outflow of (4) must be controlled and constant at 37 °C.

2.2. Fill the three reservoirs with the respective solution and wash each line (black) with the corresponding solution. Fill the main (blue) line at the end without air bubbles using solution (1).

NOTE: Oxygenate the solutions prior (10 min) and during use. Fill the line from reservoir (3) to the tap with Ca^{2+}-free cardioplegic solution.

2.3. Prepare a suture to tie the heart around the aorta at the cannula.

3. Cell isolation

3.1. Prepare the following syringes.

3.1.1. For sedation/anesthesia: Mix 0.5 mL/kg body weight esketamine hydrochloride (25 mg/mL) and 0.2 mL/kg body weight xylazine hydrochloride (2%).

3.1.2. Fill two syringes with 12 mL of NaCl solution (0.9%).

3.1.3. Prepare 6 mL of 12.5 mg/mL Na-thiopental, dissolved in 0.9% NaCl solution.

3.1.4. Fill 0.2 mL of esketamine hydrochloride (25 mg/mL) in a syringe.

3.1.5. Dilute 0.2 mL of Na-Heparin (5,000 IU/mL) in 1 mL of 0.9% NaCl solution (end-concentration 1,000 IU/mL).

3.2. Sedate/anaesthetize rabbits (9-10 weeks, New Zealand white rabbit, female or male, ~2 kg) via intramuscular injection of esketamine hydrochloride and xylazine hydrochloride (step 3.1.1).

NOTE: Rabbits need at least 10 min to be fully anaesthetized; exact duration depends on their body weight. Confirm anesthesia with the loss of the righting reflex.

3.3. Shave the chest and the ears where the veins are located.

3.4. Insert a flexible cannula into the ear vein, fix it with tape and flush it with 0.9% NaCl solution.

3.5. Inject 1 mL of Na-Heparin solution intravenously and flush with 0.9% NaCl solution.

3.6. Inject 0.2 mL of esketamine hydrochloride, flush again with 0.9% NaCl solution and inject thiopental until apnea.

NOTE: Rabbit should not respond to the pedal withdrawal reflex.

3.7. Open the chest at the left side and remove the pericardium.

3.8. Start the timer when the heart is excised and wash the heart twice in physiological saline solution.

NOTE: Use scissors with round tips to prevent accidental damage to cardiac tissue.

3.9. Cannulate the aorta in a bath with physiological saline solution and keep all tissue in solution. Switch on the Langendorff perfusion system (physiological saline solution (1), speed 24 mL/min).

3.10. Transfer the heart to the Langendorff perfusion setup, connect the aorta to the perfusate nozzle, and tightly tie the heart with the suture around the aorta to the cannula (< 1 min).

NOTE: Pre-fill the cannula with physiological saline solution, ensure that no air bubbles enter the cannula during transportation from the cannulation site to the Langendorff setup, connect bubble-free.

3.11. Perfuse the heart until all blood is washed out (2-3 min).

3.12. Switch to Ca^{2+}-free cardioplegic solution. Perfuse for 2 more min after the heart has stopped beating and switch to enzyme solution.

3.13. Decrease speed to 16 mL/min after 5 min of digestion. Start recirculating the enzyme solution, after 2 min from start of digestion, back into the reservoir.

3.14. When the tissue appears soft (40–50 min of digestion), cut the heart off the cannula and separate the left ventricle.

3.15. Release cells by mechanical dissociation (gently pulling apart the tissue with a pipette and a fine forceps to hold the tissue) in blocking solution.

3.16. Filter the cell suspension through a mesh (pore size of 1 mm^2) and centrifuge for 2 min at 22 x *g* (gravitational acceleration).

3.17. Remove the supernatant containing non-myocytes and re-suspend CM in blocking solution.

4. Culturing of CM

NOTE: Perform the following steps under sterile conditions.

4.1. Dilute Laminin (from Engelbreth-Holm-Swarm murine sarcoma basement membrane, 1 mg/mL) 1:10 in sterile phosphate buffered saline (without Ca^{2+}/Mg^{2+}) to a final concentration of 100 µg /mL.

4.2. Prepare culture medium in M199-Medium with the supplements as indicated in **Table 3**.

[Place **Table 3** here]

4.3. Sterile-filter solution (0.22 µm) and add 5% Fetal Bovine Serum.

4.4. For patch-clamp experiments autoclave coverslips ø 16 mm, thickness No. 0, coat them with 100 µg/mL laminin directly before culturing.

4.5. For carbon fiber experiments, coat the Petri dish surface with poly(2-hyroxyethyl methacrylate) (poly-HEMA, 0.12 g/mL in 95:5 EtOH:H_20).

NOTE: Cells do not 'stick' to poly-HEMA coated Petri dishes; this is crucial for their friction-less contraction in cell mechanics studies.

4.6. After re-suspended CM have settled (~10-15 min), remove the supernatant, and then re-suspend CM in culture medium.

4.7. Count CM with a Neubauer chamber and seed at a target density of 17,500 cells/mL either on laminin coated coverslips or in poly-HEMA coated Petri dishes.

4.8. Incubate cells at 37 °C, 5% CO_2 for 3-4 hours. Exchange the medium (37 °C) of coverslip seeded cells.

4.9. Add adenovirus (type 5) coding for GtACR1-eGFP at a multiplicity of infection (MOI) of 75 and start functional experiments after 48 hours.

NOTE: After transduction keep the cells in the dark. Use red illumination when working with blue or green light-activated proteins. A commercially available adenoviral delivery system (see **Table of Materials**) is used to clone the genes encoding GtACR1-eGFP into the adenoviral vector. The insert of interest, here GtACR1-eGFP, is PCR amplified and then combined with an adenoviral vector including a CMV promotor in an IN-Fusion Cloning reaction. The CMV (human cytomegalovirus) promotor is commonly used to drive overexpression of transgenes in mammalian cells. eGFP is an enhanced green fluorescent protein derived from *Aequorea victoria* with an excitation maximum of 488 nm and an emission maximum at 507 nm. Adenovirus (type 5) was externally produced at Charité – Universitätsmedizin Berlin, Institut für Pharmakologie, Berlin, Prof. Dr. Michael Schupp.

CAUTION: Adenoviral transduction is categorized as BSL-2 safety level work, and appropriate safety measures are legally required.

5. Functional experiments

NOTE: Recordings are performed using an inverted fluorescence microscope.

5.1. **Patch-clamp setup**

5.1.1. Use an amplifier in combination with an analogue-to-digital converter. Use a data acquisition software to record current and voltage data (see **Table of Materials**).

NOTE: The recorded data are digitized at 10 kHz and filtered at 5 kHz.

5.2. **Carbon fiber setup**

5.2.1. Filter the transmission light by a red-band-pass filter (630/20 nm) in the condenser to avoid coactivation of GtACR1. Use a camera to detect carbon fiber position and sarcomere length by tracking changes in optical contrast (carbon fibers appear as darker structures, overlaid on the striated cell pattern). A schematic representation of the setup is shown in **Figure 3**.

NOTE: Sarcomere length is calculated in real time using a fast Fourier transform (FFT) of the power spectrum of the striation pattern.

[Place **Figure 3** here]

5.3. **Timed illumination**

5.3.1. Provide light for fluorescence microscopy and activation of light-gated ion channels via an external custom-built LED control box, comprising three LEDs of different color (460 nm, 525 nm, 640 nm, see **Table of Materials**).

5.3.2. Modify the microcontroller and graphical user interface (GUI) code for the control box to allow control of the LED via external Time to Live (TTL) pulses, generated in data acquisition software protocols (see **Table of Materials**). Have TTL pulses transmitted to the LED control box via the digital-analogue-converter.

5.3.3. Have the LED to be driven and the number of pulses be chosen via the GUI. Upon receiving the command from the GUI the microcontroller starts a process on a new core. In this process the TTL input as well as a control switch set from the GUI will be continuously checked.

5.3.4. When the TTL input is positive, have the microcontroller turn the LED on and then resume checking the TTL input. Once the TTL signal returns to zero, have the microcontroller turn the LED off and reduce the number of pulses left by one. If at any point the control switch is false or the number of pulses is zero, have the microcontroller stop this process until a new command is received from the GUI.

5.3.5. Directly couple the LEDs into the backport of the microscope.

5.4. **Determination of light intensity in the object plane**

5.4.1. Measure the illuminated area with a stage micrometer (objective magnification 40x, A= 0.8 mm^2).

5.4.2. Use an optical power meter (see **Table of Materials**).

5.4.3. Define the settings for the experiments: excitation wavelength (525 nm), objective magnification (40x), excitation filter (530/20 nm) or mirror, and read out the light power [W] at various LED-input voltages.

5.4.4. Calculate the light intensity [W/mm^2] by dividing the light power [W] by the illuminated area [mm^2] (here: 0.8 mm^2).

NOTE: Measure the actual light power with the respective protocols in step 5.6 to check if short light pulse durations of 10 ms reach and long durations hold the set value (**Supplemental Figure 1**).

5.5. Preparation for patch-clamp experiments

5.5.1. Prepare the following external and internal solutions (**Table 4**; for water requirements see **Table 1**).

[Place **Table 4** here]

5.5.2. Adjust the osmolarity with glucose to 300 ± 5 mOsmol/L. Aliquot the internal solution and store at -20 °C.

NOTE: Keep the internal solution on ice for the day of the recording. Keep the external solution at room temperature. The here described patch-clamp solutions were based on previously used solutions and Cl^- concentration was changed to lower, more physiological levels[7]. For characterization of ion selectivity of the respective optogenetic actuator, we suggest to vary the concentrations of major ions (e.g., Cl^-, Na^+, K^+, H^+) in the extra- and intracellular solutions[19].

5.5.3. Take off the recording electrode from the pipette holder and remove the silver chloride layer from the silver wire with very fine sandpaper.

NOTE: Do this step at the beginning of each measuring day.

5.5.4. Connect the wire to the positive pole of a 1.5 V battery and immerse in 3 M KCl solution for silver chloride coating for 10 min.

NOTE: The negative pole is connected to a reference silver wire immersed in the 3 M KCl solution.

5.5.5. Prepare the measuring chamber: put silicon grease on the frame of the measuring chamber and place a coverslip (diameter: 50 mm, thickness No. 0) on the top of the frame that the chamber is sealed.

5.5.6. Put a reference silver/silver-chloride pellet electrode in the bath and connect it with the head stage.

5.5.7. Pull 1.7 - 2.5 MΩ patch pipettes from soda lime glass capillaries (outer diameter: 1.55 mm, inner diameter: 1.15 mm) with a micropipette puller (see **Table of Materials**).

5.5.8. Start data acquisition software and adjust the membrane test (pulse 10 mV for 10 ms, baseline 0 mV).

5.6. **Protocols for patch-clamp measurements**

5.6.1. Record photoactivation protocol in the V-clamp mode at a holding potential of −74 mV. Use light pulses of 300 ms.

NOTE: We suggest performing V-clamp recordings close to the resting membrane potential of cultured CM (established in I-clamp; in our hands between −79 mV and −77 mV both for transduced and non-transduced CM[19]). Freshly isolated cells show a mean resting membrane potential of -79 mV (**Supplemental Figure 2**, all values after correction for liquid junction potential).

5.6.2. Record AP in I-clamp mode at 0 pA.

5.6.2.1. For electrical pacing, inject current pulses of 10 ms (ramp from 0 pA to the set value within 10 ms), 0.25 Hz and find the threshold to elicit AP. Record AP by current injections of 50% more than the threshold.

5.6.2.2. For optical pacing use light pulses of 10 ms, 0.25 Hz at light intensity to elicit reliable AP.

5.6.3. Record photoinhibition in I-clamp mode at 0 pA. Elicit AP as described in step 5.6.2.1 and apply sustained light for 64 s at 4 mW/mm^2 after 15 electrically triggered AP.

NOTE: **Figure 6F** shows a photoinhibition protocol where during sustained light higher current injections are applied. Starting from 1.5 times the threshold (here: 0.7 nA) the injected current was increased in steps of 0.1 nA (final level: 2.2 nA). At all tested current amplitudes, sustained light application inhibited AP generation.

5.6.3.1. As a control experiment, pause electrical stimulation for 64 s without light application.

5.7. Patch-clamp experiments

NOTE: Perform the following experiments in the dark (red light can be used for blue/green light-activated tools).

5.7.1. Place coverslip with cells in measuring chamber with external solution and select fluorescent CM.

NOTE: eGFP-positive cells can be detected using a blue LED (460 nm) in combination with a band-pass excitation filter (450 nm – 490 nm), a 510 nm dichroic mirror and a 515 nm long-pass emission filter. If other fluorescent tags are used, use corresponding LED and fluorescence filter sets. If a high transduction efficiency is achieved (in our hands >99% with the GtACR1 adenovirus), there is no need to check eGFP fluorescence before the functional experiments; this avoids potential GtACR1 pre-activation.

5.7.2. Fill the patch pipette with internal solution. Ensure that there are no air bubbles in the tip.

5.7.3. Attach pipette to the pipette holder, inserting the recording silver-chloride coated silver wire in the internal solution.

5.7.4. After reaching the cell-attached configuration, switch to whole-cell mode in the data acquisition software with a holding potential of -74 mV. Rupture the membrane by gently applying negative pressure to access the whole-cell configuration. This is indicated by an immediate increase in the measured capacitance.

5.7.5. Run the protocols described in section 5.6.

5.8. Carbon fiber technique

5.8.1. Produce carbon fibers.

5.8.1.1. Use glass capillaries with the following parameters: outer diameter: 2.0 mm, inner diameter: 1.16 mm, length: 100 mm (see **Table of Materials**). Using a micropipette puller, pull the glass capillary into two pipettes of the same length (total taper length ~11 mm, **Figure 5**) to a final inner diameter of ~30 µm.

NOTE: Settings used for the first and second pull are 85.2 and 49.0, respectively (will depend on the puller, type and age of the filament).

5.8.1.2. Bend the pipettes up to 45° with a self-made micro forge using settings of 12 V, 24 A (see **Figure 4** for details of the pipette bending setup).

5.8.1.2.1. Align the capillary (2) on the red line in the orientation circle (5), keep the positioning of the capillary constant so the length of the bend part is always the same after the center of the orientation circle (radius of 4.5 mm).

5.8.1.2.2. Bend the capillary up to 45° (green line) by pushing down the tip of the capillary with the bender (3) and forge by heating-up the filament (4) until the capillary captures the 45° angle even after the bender is removed.

[Place **Figure 4** here]

5.8.1.3. Fit the carbon fibers (provided from Prof. Jean-Yves Le Guennec) into the fine tip of the glass capillary under a stereo microscope. Use fine forceps with soft tubing at the end to increase grip and decrease the risk of damaging the fibers.

NOTE: These fibers are characterized by microstructures, which increase the contact surface between fibers and cells, thereby improving adhesion[20].

5.8.1.4. Cut the carbon fibers at a length of 2 mm and use super glue (cyanoacrylate) to fix the fiber to the front section of the capillary.

NOTE: The longer the fibers are, the more they bend upon application of the same force.

[Place **Figure 5** here]

5.8.2. Calibrate carbon fibers.

5.8.2.1. Calibrate the carbon fibers using a force transducer with a sensitivity of 0.05 mN/V and a force range of 0 - 0.5 mN (see **Table of Materials**).

NOTE: This setup is custom-made in order to measure compression instead of "pull".

5.8.2.2. Attach the capillary with the carbon fiber to a holder that is controlled by a micromanipulator and a piezo motor.

5.8.2.3. Place the tip of the fiber in contact with the force sensor, but without producing any force and move the piezo motor in steps of 10 µm (total movement of 60 µm) towards the sensor and read out the measured voltage (*E*) in Volt.

NOTE: Make sure the force transducer is contacted by the very tip of the free end of the carbon fiber.

5.8.2.4. Repeat these measurements three times.

5.8.2.5. Use Formula 1 to calculate the force for each piezo position ($\Delta E \triangleq$ difference of measured voltage between the piezo motor steps):

$$F[\mu N] = \Delta E[V] \cdot \frac{sensitivity\ [\mu N\ V^{-1}]}{gain} \quad \text{(Formula 1)}$$

NOTE: The sensitivity of the force transducer is dependent on the model of the transducer (here: 0.05 mN/V = 50 µN/V). The gain can be set at the controller.

5.8.2.6. Plot the force [µN] against the piezo position. The slope corresponds to the fiber stiffness [µN/µm].

5.8.3. Record force of contracting CM.

NOTE: Perform the following experiments in the dark (red light can be used for blue/ green light-activated tools).

5.8.3.1. Coat the surface of the measuring chamber with poly-HEMA. Fill the measuring chamber with external bath solution and put a few drops of the cultured cell suspension in the chamber (step 4.9).

5.8.3.2. Attach capillaries with carbon fibers to the stage micromanipulator. Select GtACR1-expressing CM by checking the ability to induce contractions by application of short green-light pulses. Align the carbon fibers near-horizontally to the surface of the measuring chamber.

5.8.3.3. Lower the first fiber onto the cell surface. Attach the second fiber parallel to the first fiber at the other end of the CM. The ideal alignment is near-perpendicular to the cell axis.

NOTE: Attach the fiber by gently pushing the cell to the bottom surface. Release the pressure before attaching the second fiber. Do not stretch the cell by attaching the second fiber.

5.8.3.4. After both fibers are attached on the cell, lift the fiber, so the cell has no contact to the chamber surface anymore and is able contract without any frictions with the chamber.

5.8.3.5. Focus the sarcomeres in the data acquisition software (see **Table of Materials**) and set the sarcomere length tracking window (**Figure 7A I (3)**) between the fibers.

NOTE: Resulting FFT power spectrum (**Figure 7A I (2)**) shows ideally one sharp peak, representing the average sarcomere length.

5.8.3.6. Track fiber bending using the edge detection module. Set the detection areas with the red and green window and define a threshold (red and green horizontal line) at the first derivative of the light intensity trace (**Figure 7A III**).

5.8.3.7. Start to optically pace the cell at 0.25 Hz (if possible, try faster pacing rates) and track sarcomere length and fiber bending.

NOTE: Fiber holder position, LED trigger and electric stimulation pulses are controlled via the data acquisition software (see **Table of Materials**).

5.8.3.8. After recording at least 15 optically elicited contractions, field-stimulate the cell electrically (see **Table of Materials**). Find the threshold to elicit contractions and record by applying 1.5 times of the threshold voltage.

5.8.3.9. For the inhibition protocol, apply electrical stimuli to elicit contractions and then expose to sustained light of 64 s (at various light intensities).

6. Data analysis

6.1. Patch-clamp recordings

NOTE: Correct all recorded and command voltages for the liquid junction potential after the experiment. Determine liquid junction potential in the data acquisition software by using the tool junction potential calculator (for the stated patch-clamp solutions in **Table 4**: 14.4 mV at 21 °C). Subtract the liquid junction potential from the recorded/command voltage.

6.1.1. For I-clamp AP recordings, check electrical pacing versus optical pacing. Calculate the AP duration (APD) at 20 and 90% repolarization with a custom-written script (**Supplemental Materials**). Determine resting membrane potential and AP amplitude.

NOTE: Determine the APD as described in Wang K. et al.[21] The script to load .abf files is generally accessible via the following link: https://de.mathworks.com/matlabcentral/ fileexchange/22114-fcollman-abfload. Average APD values for at least 6 AP.

6.1.2. For V-clamp photoactivation, check if baseline is at 0 pA. If not adjust baseline to zero. Analyze the recorded current triggered by 300 ms light pulses at -60 mV. Transfer the data to data analysis software and determine the peak and average stationary current.

6.2. Carbon fiber experiments

6.2.1. Contraction recordings during optical pacing: Load the recorded data in the data acquisition software and read out the baseline and maxima of the carbon fiber bending and the sarcomere length changes.

NOTE: Average values for 10 contractions of a stable recording.

6.2.2. Measure the cell width and calculate the cross-sectional area of the cell assuming an elliptical cross-section (**Figure 7B II**).

NOTE: The formula for the area of an ellipse is $A=\pi \cdot a \cdot b$ (Formula 2) where a is the distance from the center to the vertex and b is the distance from the center to the co-vertex. In our case this means a = (width of the cell)/2 and b = (thickness of the cell)/2. According to Nishimura et al.[22] the thickness of CM can be estimated to be one third of the cell width so that A=

572 $\pi \cdot (1/2) \cdot \text{width} \cdot (1/2) \cdot \text{thickness} = \pi \cdot (1/4) \cdot \text{width} \cdot (1/3) \cdot \text{width} = \pi \cdot (1/12) \cdot \text{width}^2$.
573
574 6.2.3. Calculate the end-systolic force (F):
575 $$F[\mu N\ mm^{-2}] = \frac{(baseline(fibre)[\mu m] - peak(fibre)[\mu m]) \cdot stiffness(fibre)[\mu N\ \mu m^{-1}]}{cross\ section\ area(cell)[mm^2]}$$ (Formula 3)
576
577 6.2.4. Calculate the end-systolic cell deformation (ESD):
578 $$ESD\ [\%] = \frac{baseline(sarcomere\ length)[\mu m] - peak(sarcomere\ length)[\mu m]}{baseline(sarcomere\ length)[\mu m]} \cdot 100\%$$ (Formula 4)
579
580 NOTE: Further contractile parameters can be analyzed: resting sarcomere length, time to peak,
581 time to 90% relaxation, fractional sarcomere shortening, maximum velocity of contraction and
582 relaxation (see software acquisition manual).
583
584 **REPRESENTATIVE RESULTS:**
585 GtACR1-eGFP was expressed in cultured rabbit CM (**Figure 6 insert**) and photocurrents were
586 measured with the patch-clamp technique. Photoactivation of GtACR1 shows large inward
587 directed currents at -74 mV. In **Figure 6A** peak current (I_P) at 4 mW/mm^2 is 245 pA. AP were
588 triggered either electrically (**Figure 6B**) or optically (**Figure 6C**) with current injections 1.5 times
589 the threshold, or short depolarizing light pulses of 10 ms, respectively. Analyzing APD values,
590 electrically paced CM show an APD20 of 0.24 ± 0.08 s and an APD90 of 0.75 ± 0.17 s, whereas
591 optically paced CM show an APD20 of 0.31 ± 0.08 s and an APD 90 of 0.81 ± 0.19 s (SE, n=5, N=2,
592 in the here presented example APD 20 $_{electrical}$ = 0.17 s; APD 20 $_{optical}$ = 0.27 s and APD 90 $_{electrical}$ =
593 0.61 s; APD 90 $_{optical}$ = 0.0.68 s; **Figure 6D**). Optically paced CM show a slower AP onset (**Figure**
594 **6D**). CM activation was inhibited upon sustained illumination (for 64 s, 4 mW mm^{-2}) by polarizing
595 the membrane potential towards the reversal potential of chloride, here -58 mV (**Figure 6E**).
596 Higher current injections than 1.5 times the threshold do not elicit AP generation (**Figure 6F**).
597 Generated peak forces were determined from carbon fiber bending (**Figure 7B,C,E**). The CM
598 generated 232 µN/mm^2 upon electrical pacing (**Figure 7B**) and 261 µN/mm^2 following optical
599 pacing (**Figure 7C**). Prolonged green-light pulses inhibit contractions (**Figure 7E**). Following optical
600 inhibition for 64 s, reoccurring contractions generate a lower contractile force, and force values
601 recover towards baseline after ~10 contractions (pacing at 0.25 Hz, **Figure 7D**) in keeping with
602 diastolic calcium loss from rabbit CM.
603
604 [Place **Figure 6** here]
605
606 [Place **Figure 7** here]
607
608 [Place **Table 5** here]
609
610 **FIGURE AND TABLE LEGENDS:**
611 **Figure 1: Flowchart of the protocol used to obtain electrically and optically paceable CM.** Hearts
612 are excised from rabbits 9-10 weeks old, and cardiac tissue is digested while being perfused using
613 a Langendorff setup. Cells are released by mechanical agitation. The CM yield is counted under a

microscope. CM are cultured, transduced with adenovirus type 5 and functional experiments are performed 48-72 h post-transduction.

Figure 2: Langendorff-perfusion setup optimized for rabbit cell isolation. (1-3) Water jacketed reservoirs with (1) physiological saline solution, (2) Ca^{2+}- free cardioplegic solution and (3) enzyme-containing cardioplegic solution. (4) Spiral counter-flow heat exchanger and (5) water jacketed collecting tank. The inflow of the water jacketed system is the spiral heat exchanger (temperature of solutions leaving the perfusion cannula at the end of the heat exchanger should be constant at 37 °C), followed by the perfusion vessel and the three reservoirs. All solutions are oxygenated (dashed line).

Figure 3: Scheme depicting experimental setup for carbon fiber measurements. (Drawing is not at scale)**.** Two carbon fibers are attached on a cell and their position is controlled by a piezo positioner. The pacer is used for electrical field stimulation. Multi-color LEDs are coupled into the epifluorescence port of the inverted microscope for illumination of cells in the object plane. LED power is controlled via a dedicated control box, which receives digital pulses via the digital output of the digital-analogue-converter (DAC). The DAC communicates via analogue output with the fluorescence system interface. A black-and white camera (774 pixels by 245 lines) for cellular imaging is connected to the computer to track sarcomere length and carbon fiber bending.

Figure 4: Pipette bending setup. (1) The micromanipulator on the left side is used to control the position of the capillary, and a second micromanipulator on the right is used to bend it. (2) Capillary. (3) Bender. (4) Microforge. (5) Orientation circle.

Figure 5: Pipette with carbon fiber.

Figure 6: Representative patch-clamp recordings of electrically and optically paced/ inhibited CM. (**A**) Representative photocurrent at -74 mV using a light pulse of 300 ms, 4 mW/mm^2. I_P indicates the peak current. The insert shows a GtACR1-eGFP positive cell. (**B**) Representative AP recording at 0 pA using a current ramp of 10 ms, 0.6 nA to electrically pace the CM. (**C**) Representative AP recording at 0 pA using light pulses of 10 ms, 0.4 mW/mm^2. (**D**) Top graph shows the overlay of the 10th AP of electrically (blue) and optically (green) activated CM. AP were aligned by the maximum change in membrane potential (dV/dt max). In the bottom graph is the difference of membrane potential between optically and electrically triggered AP shown ($E_{optical}$ – $E_{electrical}$). (**E**) Electrically triggered AP were inhibited under sustained light of 64 s, 4 mW/mm^2. (**F**) AP are inhibited by higher current injections than 1.5 times the threshold (from 0.7 nA in steps of 0.1 nA to 2.2 nA) under sustained light.

Figure 7: Representative data from carbon fiber recordings of optically and electrically paced/inhibited CM. (**A**) Display in the data acquisition software. Image (I) shows the measured CM with the window for calculating sarcomere length. Cell width is labelled in orange. (1) Range of relevant frequencies. (2) FFT power spectrum shows the frequency of the sarcomere spacing on the cell. The average sarcomere length is then calculated from peak frequency. (3) Sarcomere length tracking window. (4) Intensity trace. (5) The intensity trace multiplied by a Hamming

window is the windowed intensity trace. Schema (II) shows the elliptical cross-section of the cell width in orange and thickness in dashed blue. Image (III) shows the position of the carbon fibers with the respective detection boxes, left in red and right in green. (6) Intensity trace. (7) First derivative of intensity trace (see data acquisition software manual). (**B**) Representative trace of electrically elicited contractions. Panel (I) shows the sarcomere length shortening, panel (II) the distance between the two carbon fibers. (**C**) Representative trace of optically elicited contractions (525 nm, 0.25 Hz, 10 ms, 6 mW/mm^2). Panel (I) shows the sarcomere length shortening, panel (II) the distance between the two carbon fibers. (**D**) Generated peak force from contraction 1 to 11 after a pause caused by inhibition of AP generation. (**E**) Representative trace of optical inhibition of contractions under sustained illumination (525 nm, 64 s, 6 mW/mm^2). Panel (I) shows the sarcomere length shortening, panel (II) the length between the two carbon fibers.

Supplemental Figure 1: Actual light intensity measurements with optical power meter. (A) Measurement of 10 ms light pulses at 4 $mW\ mm^{-2}$. **(B)** Measurement of sustained illumination of 64 s at 4 mW/mm^2.

Supplemental Figure 2: Freshly isolated CM and their structural change in culture. (**A**) Action potential recording of a freshly isolated CM (APD20 of 1.11 ± 0.34 s, APD90 of 1.96 ± 0.32 s, n=7, N=2). Mean resting membrane potential of -79.3 ± 0.8 mV (n=7, N=2). (**B**) Carbon fiber recording of an electrically paced freshly isolated CM. Mean peak force of 205 ± 78 $\mu N/mm^2$ (n=7, N=2). (**C**) Confocal image of a freshly isolated CM (I); untransduced (II) and transduced (III) CM after 48 hours in culture.

Supplemental Material: MatLab script to determine APD and resting membrane potential.

Table 1: Water requirements.

Table 2: CM isolation solutions.

Table 3: Cell culture medium.

Table 4: Patch-clamp solutions.

Table 5: List of abbreviations.

DISCUSSION:

Whereas optogenetic tools enable modulation of excitable cell electrophysiology in a non-invasive manner, they need thorough characterization in different cell types (e.g. CM) to allow one to choose the best available tool for a specific experimental design. The patch-clamp technique is a standard method for assessing cellular electrophysiology. In the whole cell configuration, it allows one to record photo-activated currents across the plasma membrane or temporal changes in membrane voltage following light stimulation/inhibition. Optogenetic manipulation of electrical excitation also affects CM contractions. We use sarcomere tracking

and carbon fiber-assisted force measurements to quantify the effects of optical interrogation on the mechanical activity of myocytes.

We describe a protocol to characterize the basic effects of a light-gated chloride channel, GtACR1, in CM. As model system, we chose rabbit CM, as their electrophysiological characteristics (e.g., AP shape and refractory period) resemble those observed in human CM more closely than rodent CM. Moreover, rabbit CM can be cultured for several days, long enough for adenoviral delivery and expression of GtACR1-eGFP. Notably, isolated CM change their structural properties in culture over time, including rounding of cell endings and gradual loss of cross-striation, T-tubular system and caveolae[23,24]. In line with this, functional alterations have been reported in cultured CM: depolarization of the resting membrane potential, prolongation of the AP and changes in cellular Ca^{2+} handling. For review of cellular adaptations in culture, please see Louch et al.[25]. **Supplementary Figure 2** shows exemplary AP and contraction measurements of freshly isolated CM for comparison with those observed in cultured CM (**Figure 6, Figure 7**) using the here presented protocol.

Whole-cell patch-clamp recordings enable direct measurements of photocurrent properties (e.g., amplitudes and kinetics) and light-induced changes in membrane potential or AP characteristics at high temporal resolution. However, such recordings have several limitations: Firstly, the cytosol is replaced by the pipette solution in whole-cell recordings, which is advantageous to control ionic electrochemical gradients, but has the intrinsic disadvantage of washing-out cellular organelles, proteins and other compounds, thus potentially affecting cellular electrical responses. Secondly, side effects like activation of additional ion channels resulting from non-physiologically long depolarization (e.g., slow time constants of light-gated ion channels) are difficult to assess as our method only allows one to detect changes in APD, but not to conduct direct measurements of ionic concentrations in electrophysiologically relevant cell compartments. This could be done with fluorescent indicators (e.g., Ca^{2+} sensors) or ion-selective electrodes. Further characterization may include light intensity titrations, determination of pH-dependency, photocurrent kinetics at different membrane potentials, and recovery kinetics during repetitive light stimulation.

In contrast to patch-clamp recordings, single cell force measurements enable analysis of cellular contractions of intact myocytes without affecting their intracellular milieu. Secondary effects on ion concentrations (e.g., Ca^{2+}) can be indirectly assessed by determining generated force amplitude and dynamics (e.g., maximum velocity of contraction and relaxation; here not analyzed). Force measurements with the carbon fiber technique have an advantage over freely contracting cells as they provide direct information on passive and active forces in pre-loaded cells (i.e., in conditions that are more similar to the in situ or in vivo settings). Mechanical preloading is especially important when analyzing cellular contractility, as stretch affects force production and relaxation[26,27].

Optogenetic approaches allow for spatiotemporally precise manipulation of the cellular membrane potential, both in single cardiomyocytes and intact cardiac tissue. Classically, ChR2, a light-gated cation non-selective channel, has been used for depolarization of the membrane

potential, whereas light-driven proton and/or chloride pumps were used for membrane hyperpolarization. Both groups of optogenetic actuators require high expression levels, as ChR2 is characterized by an intrinsically low single-channel conductance and light-driven pumps maximally transport one ion per absorbed photon[28]. Furthermore, prolonged activation of ChR2 in cardiomyocytes may lead to Na^+ and/or Ca^{2+} overload, and light-driven pumps may change trans-sarcolemmal H^+ or Cl^- gradients[29,30]. In search for alternative tools for optogenetic control of cardiomyocyte activity, we recently tested the natural anion channelrhodopsin GtACR1, characterized by a superior single-channel conductance and higher light sensitivity compared to cation ChR such as ChR2. We found that GtACR1 activation depolarizes CM and can be used for optical pacing and inhibition, depending on the light pulse timing and duration. An additional advantage of using ACR instead of cation ChR might be the more negative reversal potential of Cl^- compared to Na^+, reducing artificially introduced ion currents. As we have previously shown, optical pacing with GtACR1 may lead to action potential prolongation as a result of the slow component of GtACR1 channel closure, which could be overcome by using faster GtACR1 mutants[19]. However, action potential prolongation is much less pronounced when using a lower, more physiological intracellular Cl- concentration (see **Figure 6**). Moreover, GtACR1-mediated inhibition by prolonged illumination results in profound membrane depolarization, which again could activate secondary Na^+ and Ca^{2+} influx, thereby altering the activity of voltage-gated channels. In our measurements, we find that AP and contraction parameters recover to baseline within 40 s after a light-induced inhibition for 1 min (see Kopton et al. 2018, **Figure 6**, **Figure 7**). Light-gated K^+ channels offer a potent alternative for silencing CM without affecting the CM resting membrane potential[31].

In future we would like to quantitatively compare different optogenetic tools for their potential to inhibit cardiac activity. To this end, we test a variety of light-gated ion channels including ACR, ChR2 and red-shifted ChR variants[32], as well as hyperpolarizing actuators such as halorhodopsin or the light gated adenylyl cyclase bPAC in combination with the potassium channel SthK (PAC-K)[31].

The here presented protocol can be used for in-depth characterization of the electromechanical properties of CM. It is principally applicable also to CM from other species, and to CM isolated from diseased myocardium. Optical stimulation allows one to pace CM at different frequencies, and different preloads can be tested during carbon fiber contraction experiments. An interesting experiment would be to use low-intensity illumination for subthreshold depolarization, to mimic gradual increase in the resting membrane potential, as can be observed during the development of cardiac tissue remodeling during disease progression. Finally, functional measurements could be combined with Ca^{2+} imaging for further insight into excitation-contraction coupling, or with pharmacological interventions to evaluate the effects of different drugs on CM activity.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure1

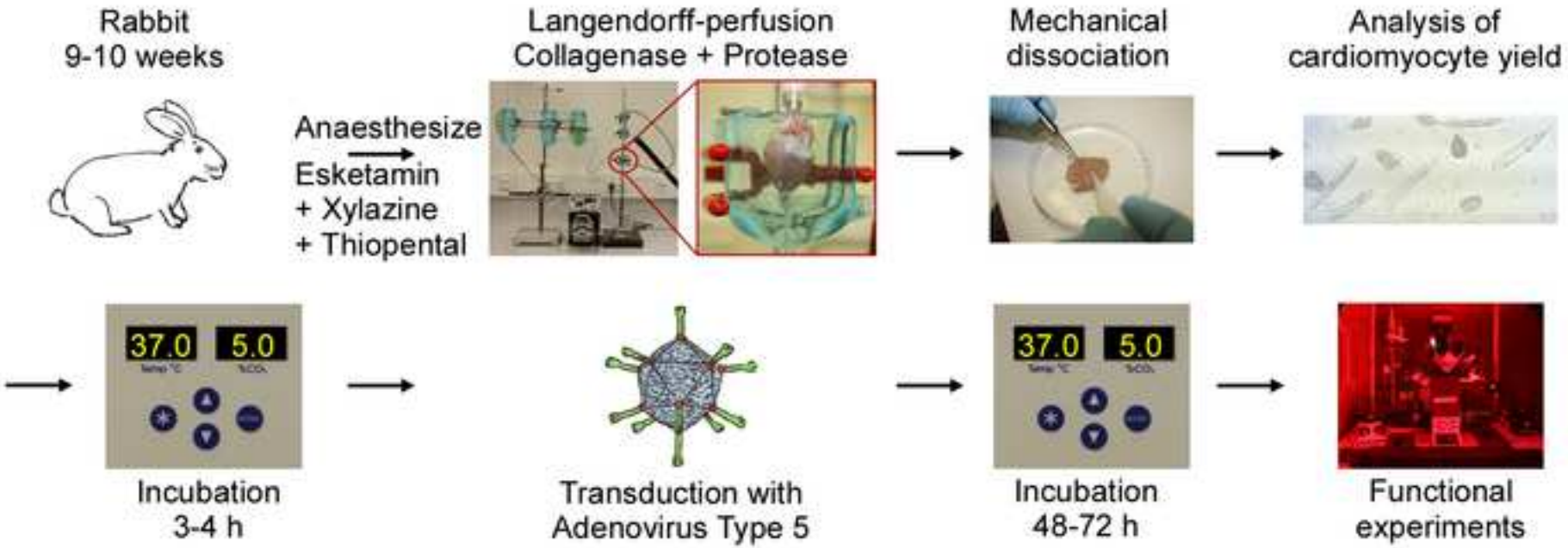

Figure2

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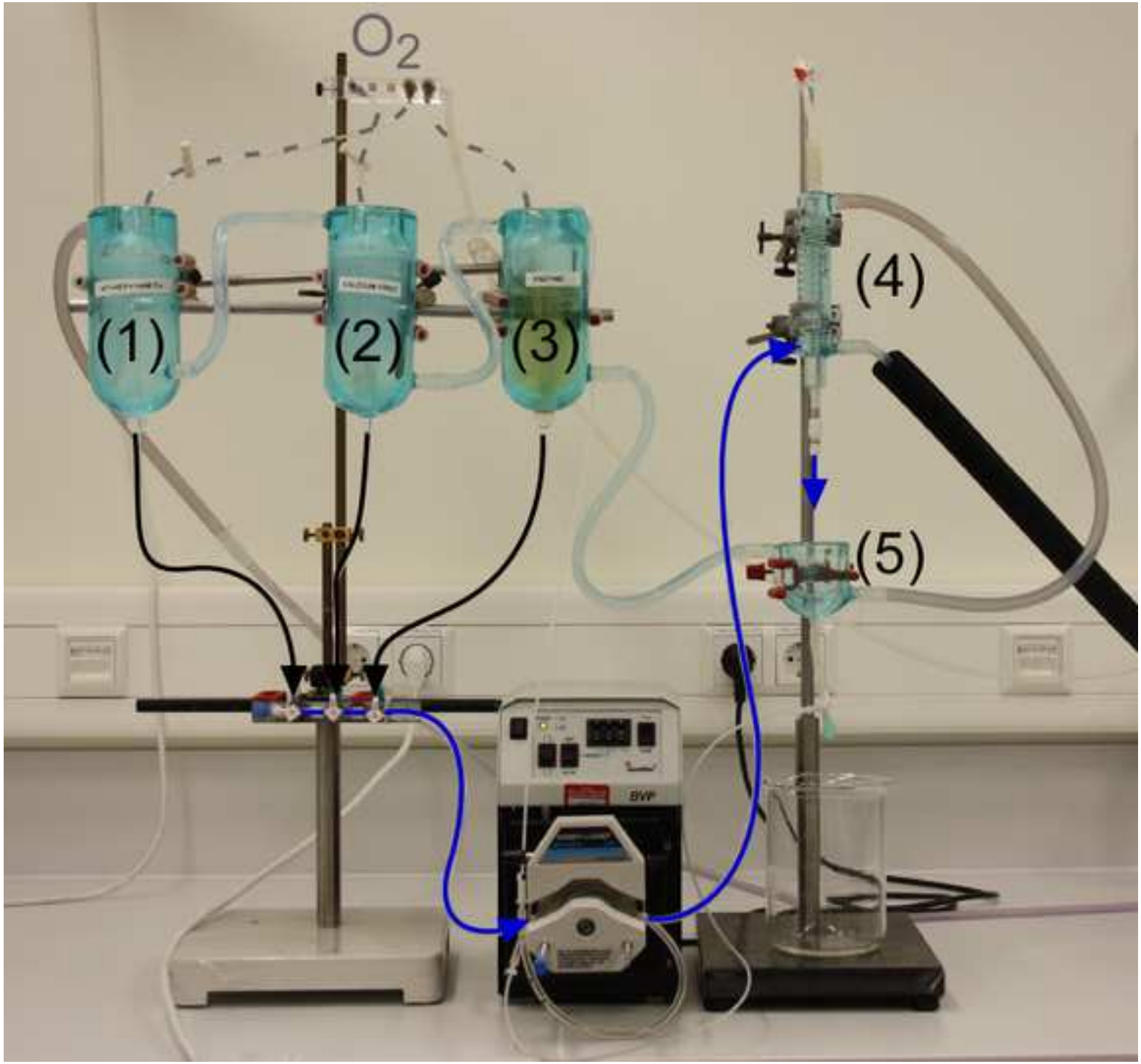

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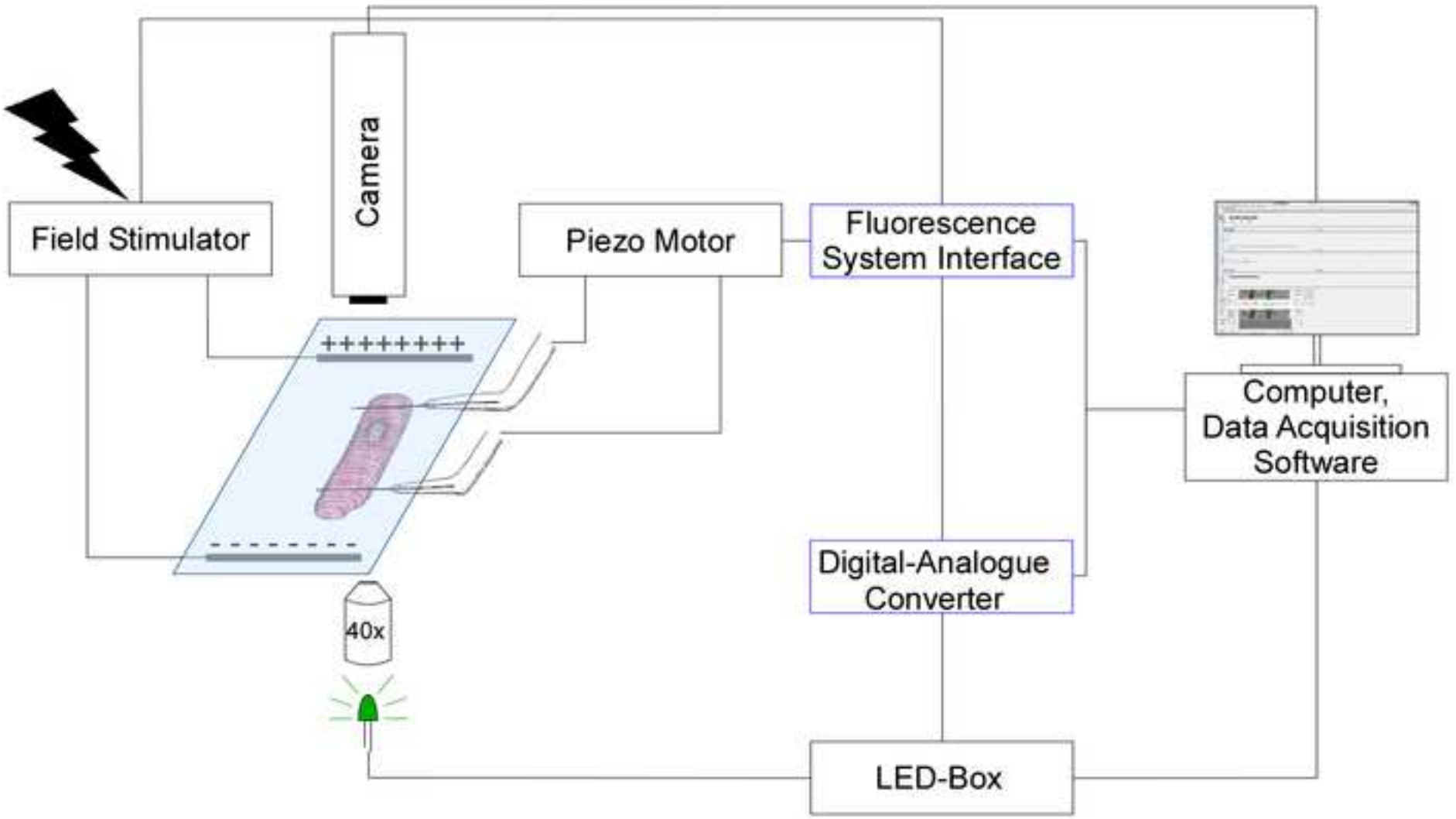

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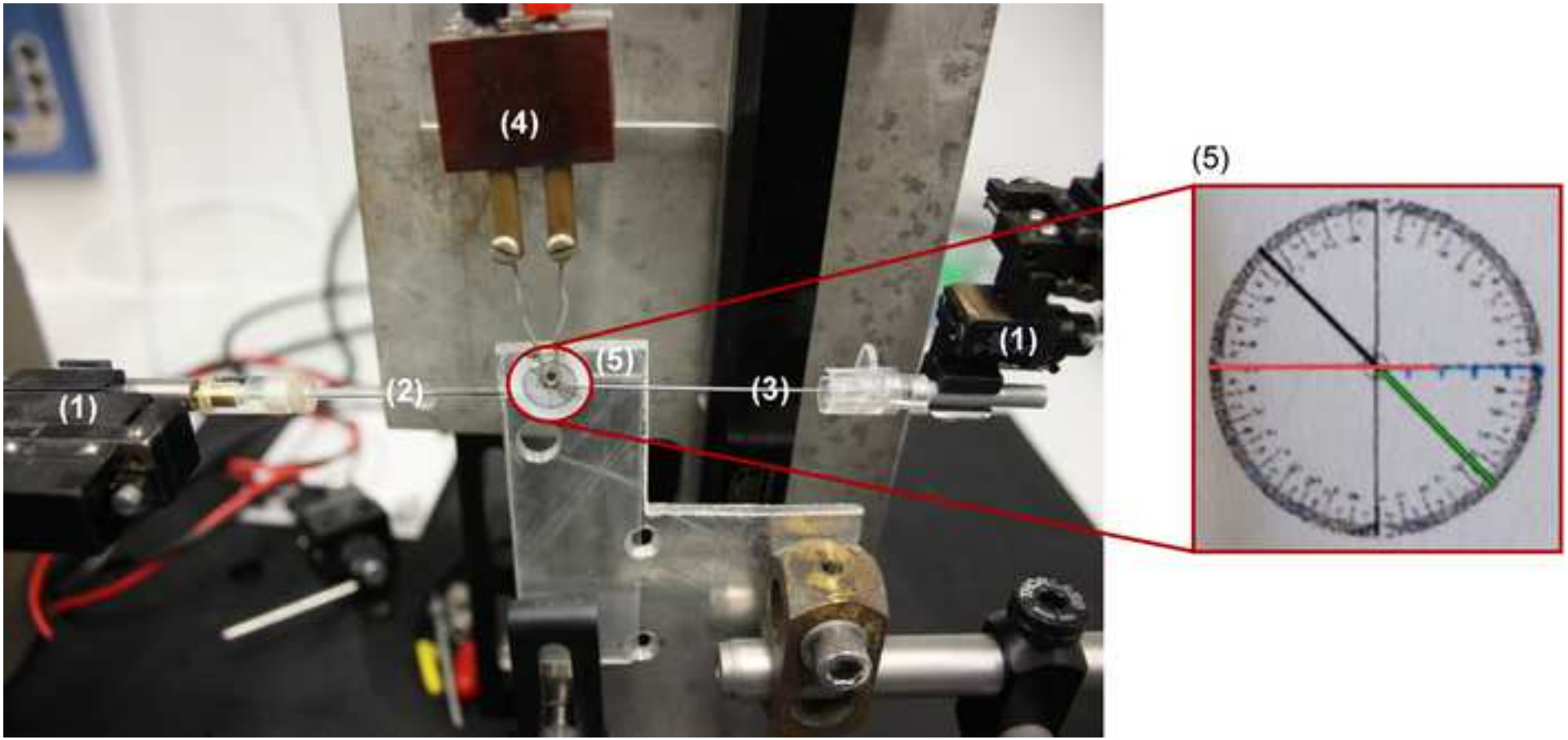

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Figure6

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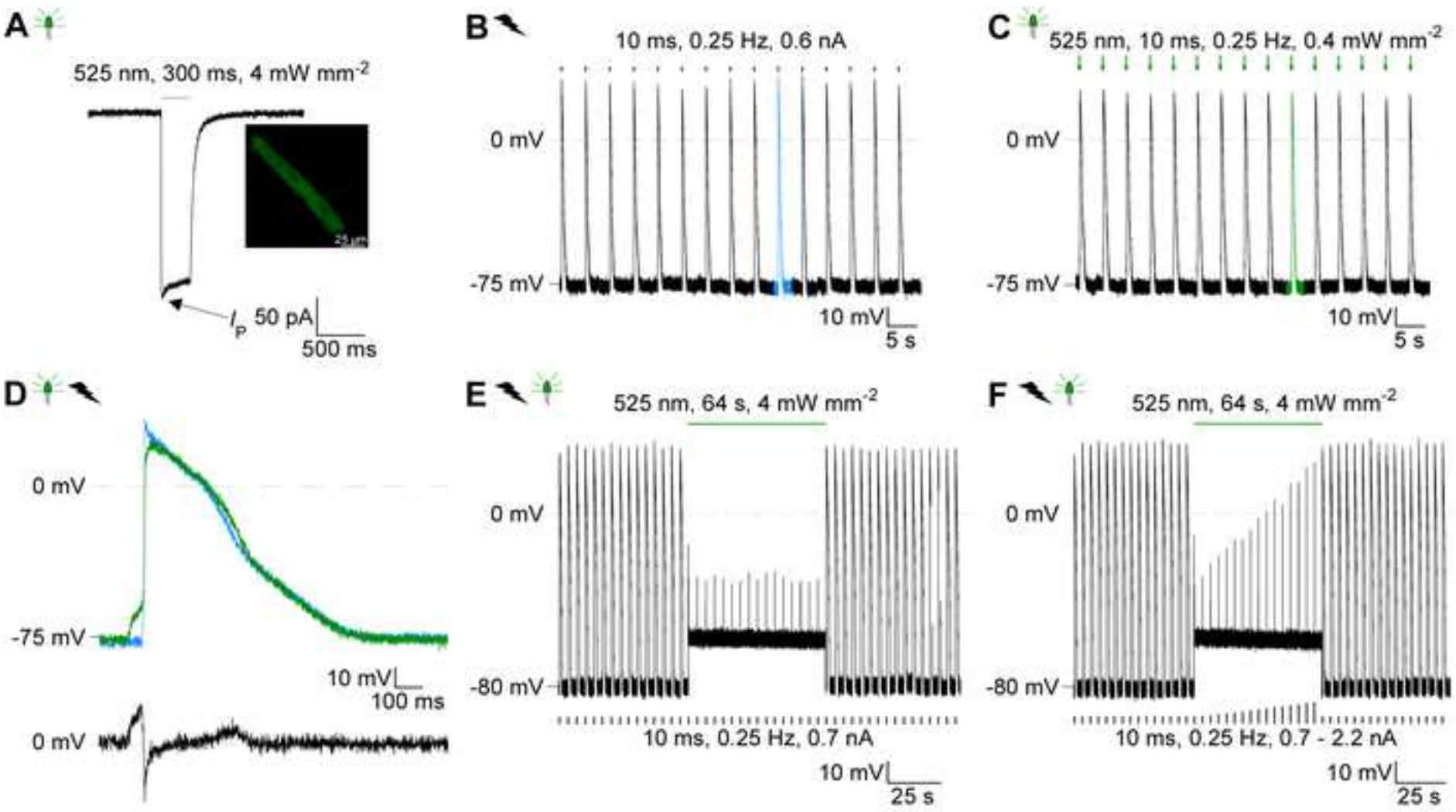

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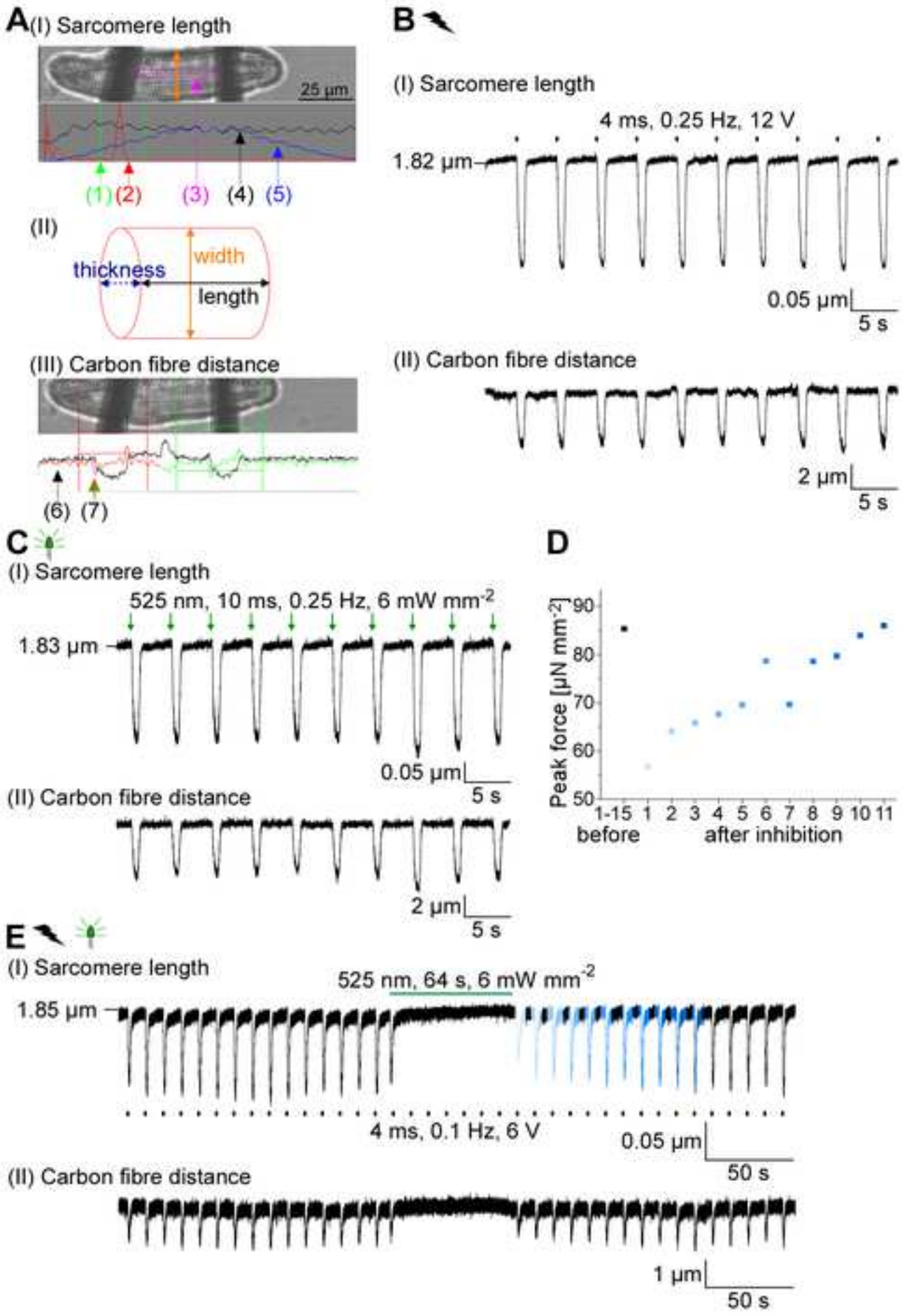

	Water requirements
Conductivity [µS cm^{-1}] at 25 °C	0.055
Pyrogen [EU mL^{-1}]	< 0.001
Particle (size > 0.22 µm) [mL^{-1}]	≤ 1
Total organic carbon [ppb]	< 5
Microorganisms [CFU mL^{-1}]	≤ 1
RNase [ng mL^{-1}]	< 0.01
DNase [ng mL^{-1}]	< 4

Table2

	Physiological saline solution (1)	Ca^{2+}- free cardioplegic solution (2)	Enzyme solution (3)	Blocking solution
NaCl [mM]	137	137	137	137
KCl [mM]	4	14	14	14
HEPES [mM]	10	10	10	10
Creatine [mM]	10	10	10	10
Taurine [mM]	20	20	20	20
Glucose [mM]	10	10	10	10
$MgCl_2$ [mM]	1	1	1	1
Adenosine [mM]	5	5	5	5
L-Carnitine [mM]	2	2	2	2
$CaCl_2$ [mM]	1	-	0.1	0.1
Na-Heparin [IU L^{-1}]	5000	-	-	-
EGTA [mM]	-	0.096		
Collagenase type 2, 315 U mg^{-1} [g L^{-1}]	-	-	0.6	-
Protease XIV [g L^{-1}]	-	-	0.03	-
Bovine serum albumin [%]	-	-	-	0.5
Osmolarity [mOsmol L^{-1}]	325 ± 5	345 ± 5	345 ± 5	345 ± 5

	Cell culture medium in M199-Medium
Creatine [mM]	5
L-Carnitine hydrochloride [mM]	2
Taurine [mM]	5
Na-Pyruvat [mM]	1
Insulin (bovine pancreas) [U L^{-1}]	0.25
Cytosine-β-D-arabinofuranoside [mM]	0.01
Gentamycin [mg mL^{-1}]	0.05

Table4

	External bath solution	Internal pipette solution
NaCl [mM]	140	-
KCl [mM]	5.4	11
$CaCl_2$ [mM]	1	-
$MgCl_2$ [mM]	2	2
Glucose [mM]	10	-
HEPES [mM]	10	10
K-Aspartate [mM]	-	119
Mg-ATP [mM]	-	3
EGTA [mM]	-	10
pH	7.4 (NaOH)	7.2 (KOH)
Osmolarity (adjust with Glucose) [mOsmol L^{-1}]	300 ± 5	300 ± 5

A	area
ACR	anion channelrhodopsin
AP	action potential
APD	action potential duration
CFU	colony forming unit
ChR	channelrhodopsin
CM	cardiomyocyte
eGFP	enhanced green fluorescent protein
ESD	end systolic cell deformation
EU	endotoxin units
F	force
FFT	fast Fourier transform
GtACR	*Guillardia theta* anion channelrhodopsin
GUI	graphical user interface
I-clamp	current-clamp
IU	international units
MOI	multiplicity of infection
poly-HEMA	poly(2-hyroxyethyl methacrylate)
V-clamp	voltage-clamp

Name of Material/Equipment	Company	Catalog Number	Comments/ Description
Adenosine	Sigma-Aldrich, St. Louis, Missouri, United States	A9251-100G	
Adeno-X Adenoviral System 3 CMV	TaKaRa, Clontech Laboratories, Inc., Mountain View, California, USA		
Amplfier	AxonInstruments, Union City, CA, United States	Axopatch 200B	
Aortic cannula	Radnoti		4.8 OD x 3.6 ID x 8-9 L mm
Bovine serum albumin	Sigma-Aldrich, St. Louis, Missouri, United States	A7030-50G	
$CaCl_2$	Honeywell Fluka, Muskegon, MI, USA	21114-1L	
Carbon fibers	provided from Prof. Jean-Yves Le Guennec		BZ:00
Collagenase type 2, 315 U mg^{-1}	Worthington, Lakewood, NJ, USA	LS004177	
Coverslip ø 50 mm, Thickness No. 1	VWR International GmbH, Leuven, Belgium	631-0178	Borosilicate Glass
Coverslips ø 16 mm, Thickness No. 0	VWR International GmbH, Leuven, Belgium	631-0151	Borosilicate Glass
Creatine	Sigma-Aldrich, St. Louis, Missouri, United States	C0780-50G	
Cytosine-β-D-arabinofuranoside	Sigma-Aldrich, St. Louis, Missouri, United States	C1768-100MG	
Digitizer Axon Digidata	Molecular Devices, San José, CA, United States	1550A	
Digitizer Axon Digidata	Molecular Devices, Sunnyvale, CA, United States	1550B	
EGTA	Carl Roth GmbH + Co. KG, Karlsruhe, Germany	3054.3	
Esketamine hydrochloride, Ketanest S 25 mg mL^{-1}	Pfizer Pharma PFE GmbH, Berlin, Germany	PZN-07829486	
Fetal Bovine Serum	Sigma-Aldrich, St.Louis, Missouri, United States	F9665	
Filter (530/20)	Leica Microsystems,Wetzlar, Germany	11513878	BZ:00
Filter (530/20)	Leica Microsystems,Wetzlar, Germany	11513878	
Filter (630/20)	Chroma Technology, Bellows Falls, Vermont, United States	227155	
Filter (630/20)	Chroma Technology, Bellows Falls, Vermont, United States	227155	

Fluorescence System Interface	IonOptix, Milton,MA United States	FSI-800	2.0 OD x 1.16 ID x 100 L mm
Force Transducer System	Aurora Scientific Inc., Ontario, Canada	406A	
Gentamycin (50 mg mL^{-1})	Gibco, Life Technologies, Waltham, MA, USA	15750-037	
Glass capillaries for force measurements	Harvard Apparatus, Holliston, Massachusetts, United States	GC200F-10	
Glucose	Sigma-Aldrich, St. Louis, Missouri, United States	G7021-1KG	
Griffin Silk, Black, 2 m Length, Size 3, 0.5 mm	Samuel Findings, London, UK	TSGBL3	
Headstage	AxonInstruments, Union City, CA, United States	CV203BU	
Heparin-Sodium, 5,000 IU mL^{-1}	Braun Melsungen AG, Melsungen, Germany	PZN-03029843	
HEPES	Sigma-Aldrich, St. Louis, Missouri, United States	H3375-1KG	
Incubator	New Brunswick, Eppendorf, Schönenbuch, Switzerland	Galaxy 170S	
Insulin (bovine pancreas)	Sigma-Aldrich, St. Louis, Missouri, United States	I6634-50MG	
Interface	Scientifica, Uckfield, UK	1U Rack, 352036	
Interface National Instruments	National Instruments, Budapest, Hungary	BNC-2110	
K-aspartate	Sigma-Aldrich, St. Louis, Missouri, United States	A6558-25G	
KCl	VWR International GmbH, Leuven, Belgium	26764.260	1 mg mL^{-1}
KOH	Honeywell Fluka, Muskegon, MI, USA	35113-1L	
Laminin from Engelbreth-Holm-Swarm murine sarcoma basement membrane	Sigma-Aldrich, St. Louis, Missouri, United States	L2020-1MG	
Langendorff-perfusion set-up	Zitt-Thoma Laborbedarf Glasbläserei, Freiburg, Germany		Custom-made
Langendorff-pump	Ismatec, Labortechnik-Analytik, Glattbrugg-Zürich, Switzerland	ISM444	
L-Carnitine hydrochloride	Sigma-Aldrich, St. Louis, Missouri, United States	C9500-25G	
LED 525 nm	Luminus Devices, Sunnyvale, CA, United States	PT-120-G	
LED 525 nm	Luminus Devices, Sunnyvale, CA, United States	PT-120-G	
LED control box	Essel Research and Development, Toronto, Canada		
LED control software	Essel Research and Development, Toronto, Canada		

LED control system	custom-made		
LED control system	custom-made		
M199-Medium	Sigma-Aldirch, St.Louis, Missouri, United States	M4530	
Mesh: Nylon Monodur filter cloth	Cadisch Precision Meshes Ltd		800 µm holes, 1 m wide
Mg-ATP	Sigma-Aldrich, St. Louis, Missouri, United States	A9187-1G	
$MgCl_2$	Sigma-Aldrich, St. Louis, Missouri, United States	63069-500ML	
Microcontroller	Parallax Inc., Rocklin, California, United States	Propeller	
Micropipette Puller	Narishige Co., Tokyo, Japan	PP-830	
Micropipette Puller	Narishige Co., Tokyo, Japan	PC-10	
Microscope inverted	Leica Microsystems, Wetzlar, Germany	DMI4000B	
Microscope inverted	Leica Microsystems, Wetzlar, Germany	DMI4000B	
Motorised Micromanipulator	Scientifica, Uckfield, UK	PatchStar	
MyoCam-S camera	IonOptix, Dublin, Ireland		
MyoCam-S camera Power	IonOptix, Milton, MA, United States	MCS-100	
MyoPacer Field Stimulator	IonOptix Cooperation,Milton, MA, United States	MYP100	
NaCl	Fisher Scientific, Loughborough, Leics., UK	10428420	
NaCl-Solution 0.9%, Isotone Kochsalz-Lösung 0.9%	Braun Melsungen AG, Melsungen, Germany	3200950	
NaOH	AppliChem GmbH, Darmstadt, Germany	A6579	without Ca^{2+}/Mg^{2+}
Na-pyruvat	Sigma-Aldrich, St. Louis, Missouri, United States	P2256-100MG	
Neubauer chamber	VWR International GmbH, Leuven, Belgium	717806	
Optical power meter	Thorlabs, Newton, NJ, United States	PM100D	
Phosphate Buffered Saline	Sigma-Aldrich, St. Louis, Missouri, United States	D1408-500ML	
Piezo Motor	Physik Instrumente (PI) GmbH & Co. KG, Karlsruhe, Germany	E-501.00	
Poly(2-hydroxyethyl methacrylate)	Sigma, Poole, UK	192066	
Protease XIV from Streptomyces griseus	Sigma-Aldrich, St. Louis, Missouri, United States	P5147-1G	
Rabbit, New Zealand White	Charles River	Strain Code: 052	

Scissors	Aesculap AG, Tuttlingen, Germany	BC774R	Bauchdeckenschere ger. 18cm
Silicone Grease	RS Components, Corby, UK	494-124	
Silicone Grease	RS Components, Corby, UK	494-124	
Silver wire	A-M Systems, Sequim, WA, United States	787500	Silver, Bare 0.015'', Coated 0.0190'', Length 25 Feet
Soda lime glass capillaries	Vitrex Medical A/S, Vasekaer, Denmark	160213 BRIS, ISO12772	1.55 OD x 1.15 ID x 75 L mm
Software Axon pClamp	Molecular Devices, San José, CA, United States	Version 10.5	
Software Axon pClamp	Molecular Devices, San José, CA, United States	Version 10.5	
Software IonWizard	IonOptix, Dublin, Ireland	Version 6.6.10.125	
Software MatLab2017	The MathWorks, Inc.		
Software MatLab2017	The MathWorks, Inc.		
Stage micrometer	Graticules Optics LTD, Tonbridge, UK	1 mm	
Stage micrometer	Graticules Optics LTD, Tonbridge, UK	1 mm	
Sterile filter, 0.22 µm	Merck, Darmstadt, Germany	SLGP033RB	
Taurine	Sigma-Aldrich, St. Louis, Missouri, United States	T0625-500G	
Thiopental Inresa 0.5 g	Inresa Arzneimittel GmbH, Freiburg, Germany	PZN-11852249	
Xylazine hydrochloride, Rompun 2%	Bayer Vital GmbH, Leverkusen, Germany	PZN-01320422	

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Title of Article:	Electromechanical Assessment of Optogenetically Modulated Cardiomyocyte Activity (protocol related to Kopton et al. Frontiers in Physiology 20181)
Author(s):	Ramona A. Kopton, Cinthia Buchmann, Peter Kohl, Rémi Peyronnet, and Franziska Schneider-Warme

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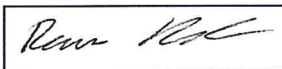
CORRESPONDING AUTHOR

Name:	Ramona A. Kopton
Department:	Institute for Experimental Cardiovascular Medicine
Institution:	University Heart Center Freiburg Bad Krozingen, Faculty of Medicine, University of Freiburg
Title:	
Signature:	
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Dear Dr Steindel, dear editors.

We thank you for providing the editorial comments and five quite detailed reviews. We have revised the manuscript according to the many suggestions.

Most importantly, we have

1) Repeated all patch-clamp recordings with an intracellular chloride concentration of 15 mM and adjusted the protocol accordingly;
2) Included an additional Supplementary Figure 1 showing the measured light intensities when applying the light stimulation protocol; and
3) Added a Supplementary Figure 2 wherein we show electrophysiology and contraction data from freshly isolated cardiomyocytes, as well as confocal images comparing freshly isolated to cultured myocytes.

For the detailed responses to all suggestions, please see below. We are looking forward to receiving your comments.

On behalf of all co-authors,

Kind regards, Franziska Schneider-Warme

Editorial comments:
General:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
2. Please ensure that the manuscript is formatted according to JoVE guidelines–letter (8.5" x 11") page size, 1-inch margins, 12 pt Calibri font throughout, all text aligned to the left margin, single spacing within paragraphs, and spaces between all paragraphs and protocol steps/substeps.

All text has now been aligned to the left and spaces have been added between all paragraphs and steps/substeps.

3. Please remove references from the title/abstract.

The reference in the abstract has been removed.

4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please limit the use of commercial language from your manuscript (and figures) and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: Axopatch, Axon Digidata, Clampex, MyoCam-S, IonWizard, MyoPacer etc.

Commercial language has been deleted and replaced with generic terms.

Protocol:
1. For each protocol step/substep, please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.

This has been corrected. Each step or substep has now just 2-3 actions.

Specific Protocol steps:
1. 3.2: How is anesthesia confirmed?

Anesthesia is confirmed with losing the righting reflex. This has now been added to the manuscript.

2. 5.1: Please rewrite the long paragraphs here as a series of substeps in the imperative and/or 'Notes'.

Section 5.1 is now written in a series of substeps in the imperative form and some parts are put in the notes.

3. 5.4.4: Are you intending to film all of 5.3? They should be highlighted if so.

We don't want to show in the film how to set up the protocols. We just want to show the application of the protocols how we stated it in section 5.4.

4. 6.1.1: Can you include such a script as supplemental material?

The script to load .abf files is generally accessible under the following link:
https://de.mathworks.com/matlabcentral/fileexchange/22114-fcollman-abfload

The script to determine action potential duration is now attached in the supplemental material. Examples of the command are included in the manuscript.

Figures and Tables:
1. Please cite all Figures and Tables in the manuscript text; currently, Figures 2, 3 and 4 and Table 5 are not.

We now cite Figure 2-4 in the text. Table 5 is the list of used abbreviations, we are currently not sure how to cite this Table in the main text.

2. Please remove titles from the Figures themselves.

This has now been corrected.

3. Currently you have 2 files per figure; there should only be one per figure.

This has now been corrected.

4. Figure 7: The labels for panels D and E have apparently been swapped.

This has now been corrected.

Table of Materials:
1. Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.

All materials are stated in the table of materials.

Reviewers' comments:
Reviewer #1:

Manuscript Summary:
The manuscript by Kopton et al. describes the protocol to introduce GtACR1 to isolated adult rabbit cardiomyocytes for studying its electrophysiological and mechanical effects. The methodology is potentially innovative and interesting.

Major Concerns:
Although the cell shown in Figure 7B barely keeps rod shape, the edge is dull and it looks no longer intact. Of course it has been reported that culture of rod shaped viable cardiomyocyte is challenging, therefore, this work may be more improved and useful if authors clarified the effects of 48-72h culture on electrophysiological and mechanical functions (need to compare AP shape and contractility between freshly isolated cells and 48-72h cultured cells). Otherwise it may be difficult for readers to distinguish effects of cell culture and optogenetic interventions when they use this protocol.

We thank the reviewer for his positive feedback on the manuscript. Regarding the raised concern, we agree that cardiomyocytes slowly change their structural and functional phenotype when being cultured for several days. However; culturing is crucial for transduction of cardiomyocytes with genes encoding optogenetic actuators. We have optimized the protocol to minimize the time for the cells to be cultured by using adenoviral transduction resulting in expression of the ion channels within 24 h. If edges (membrane) were not intact, calcium would go in and the cell would over contract and dye. Electrophysiological and contractility experiments show that cells are functional implying that the sarcolemma must be, at least, not leaky. We have now discussed the limitations of myocyte culture in the discussion and included literature comparing the behavior of freshly isolated cardiomyocytes to those in culture. We have also added a Supplementary Figure comparing action potentials, contractions and confocal images between freshly isolated and cultured cardiomyocytes (Supplementary Figure 2).

Minor Concerns:
Figure 7D. Typo in the unit of the Peak Force. Shouldn't it be micro"N" mm^-2?

This has now been corrected.

Reviewer #2:

Manuscript Summary:
The manuscript by Kopton et al. entitled "Electromechanical Assessment of Optogenetically Modulated Cardiomyocyte Activity" presents a step-by-step protocol for the assessment of anion channelrhodopsin (ACR) functionality in cultured rabbit cardiomyocytes. ACRs are recently discovered algal proteins that have been used in >20 studies to control neuronal activity, but their potential for cardiology applications have not yet been fully developed. This submission will definitely help the readers interested in harnessing ACRs for their research, and will likely be also relevant for a more general audience in the field of cardiology who struggle for the improvement of their laboratory techniques. I recommend this manuscript for publication if several issues listed below can be resolved in the revised version.

We thank the reviewer for the positive feedback.

Major Concerns:

Line 61: "ChR-mediated photocurrents were first recorded in the eyespot of unicellular green algae2,3."

The first report of photoelectrical events in green algae that later were found to be channelrhodopsin-mediated is the following paper: Litvin F.F., Sineshchekov O.A., Sineshchekov V.A. (1978) Nature, 271, 476-478. Please add this reference to your list and remove reference 3: kinetic characteristics of the slow proton current measured in algae do not match those of currents generated by channelrhodopsins in heterologous systems; the origin of the former remains unclear.

Thank you for this comment. This has now been corrected according to your suggestion.

Lines 290-291: "at a holding potential of -60 mV"

Does this value correspond to the resting membrane potential of rabbit cardiomyocytes? Please provide a reference and/or own data. Please note that the resting potential of, for example, healthy neonatal rat ventricular cardiomyocytes was -68 mV (Gaughan J.P., Hefner C.A., Houser S.R. (1998) Am. J. Physiol. 275:H577-H590). Also, I have not found in the manuscript any explanation of whether voltage values were corrected for liquid junction potentials, and if so, how exactly this correction was done. Neglecting the liquid junction potentials may lead to substantial errors in e.g. estimation of the reversal potentials.

In our previous studies we measured an average resting membrane potential between -63 mV and -65 mV (corresponds to -77 mV to -79 mV after correction for liquid junction potential) for both non-transduced and transduced rabbit CM (for details see Supplementary Figure 1E of Kopton et al. Frontiers in Physiology, 2018). In line with these values, we suggest to perform voltage-clamp experiments at -60 mV (-74 mV).

We have now included the following clarification in the protocol: "NOTE: We suggest performing V-clamp recordings close to the resting membrane potential of cultured CM (established in I-clamp; in our hands between −79 mV and −77 mV both for transduced and non-transduced CM[19]). Freshly isolated cells

show a mean resting membrane potential of -79 mV (Supplemental Figure 2, all values after correction for liquid junction potential).

Liquid junction potential was now determined calculated via the Clampex software. For the internal buffer with a chloride concentration of 15 mM the liquid junction potential was calculated to be 14.4 mV at 21 °C. The stated values in the protocol are now corrected for the liquid junction potential.

We have now included the following paragraph in the manuscript: NOTE: Correct all recorded and command voltages for the liquid junction potential after the experiment. Determine liquid junction potential in the data acquisition software by using the tool junction potential calculator (for the stated patch-clamp solutions in Table 4: 14.4 mV at 21°C). Subtract the liquid junction potential from the recorded/command voltage.

Table 4 Patch clamp solutions:

For patch clamp recording, the Authors use the pipette solution with 52 mM Cl-. The intracellular Cl- activity in the rabbit papillary muscle was estimated (by using Cl--selective microelectrodes) to be in the range 14-18 mM (Baumgartner C.M. and Fozzard H.A. (1981) Am. J. Physiol. 241:C121-C129; Caille J.P., Ruiz-Ceretti E. and Schanne O.F. (1981) Am. J. Physiol. 240:C183-C188). As the Cl- gradient determines the direction of ACR currents at a given voltage, the Authors should explain their choice of the intracellular solution, ideally by referring to a study which reported the intracellular Cl- activity in their preparation.

We originally used a pipette solution that has previously been used by others to characterize ChR-mediated currents in cardiomyocytes to allow for direct comparison of our experiments with those previously reported (Bruegmann, Nature methods, 2010). We agree that the used Cl^- concentration is higher than the estimated Cl^- concentration in cardiomyocytes. In our recent work, we tested different Cl^- concentrations in the pipette solution, but found very similar results (inward currents via GtACR1) also at 4 mM and 15 mM intracellular Cl^- (Kopton, Frontiers in physiology, 2018).

We have now added the following statement to the manuscript: "The here described patch-clamp solutions were based on previously used solutions and Cl^- concentration was changed to lower, more physiological levels[7]. For characterization of ion selectivity of the respective optogenetic actuator, we suggest to vary the concentrations of major ions (e.g. Cl^-, Na^+, K^+, H^+) in the extra- and intracellular solutions[19]. "

As a response to your feedback and the feedback from reviewer Figure 6 has now been exchanged to recordings performed with an internal Cl^- concentration of 15 mM. The composition of the solution has also been changed accordingly.

Minor Concerns:

Lines 72-73: "natural ACR were discovered" and thereafter.

Please add "s" at the end (i.e., "ACRs") to indicate the plural form of the noun to make it consistent with that of the verb.

Thank you for this stylistic advice. We usually use abbreviations without a plural-s, as the plural form should not change the abbreviation per se. Also, we already introduced anion channelrhodopsins (ACR) in the plural form.

Line 171 and thereafter:
Several sentences are highlighted yellow by the Authors. Do I understand correctly that this is just a typo?

The yellow highlights are according to the Jove guidelines and indicate the parts of the protocol that will be filmed for preparation of the video.

Reviewer #3:

Manuscript Summary:
The manuscript by Kopton et al. shows the effect of activating the natural anion channel rhodopsin GtACR1 from Guillardia theta in cultured rabbit cardiomyocytes using electrophysiological patch-clamp recordings and mechanical carbon fibre measurements. The authors successfully used the protocol to record light-activated currents and action potential using patch-clamp techniques and recorded the contraction of preloaded cardiomyocytes using carbon fibres in GtACR1-expressing cardiomyocytes. This protocol could thus be applied to test the effect of different optogenetic actuators on cardiomyocyte activity. Although the protocol is interesting, I have several major and minor concerns to improve your manuscript as follows:

We thank the reviewer for the positive feedback.

Major Concerns:

(1) The authors should discuss the advantages and disadvantages of recording light-activated current and action potential in cardiomyocytes using GtACR1 in the discussion section.

I am not exactly sure if I understand this correctly. In addition to discussing limitations of the patch-clamp technique, we have now added a paragraph on the disadvantages when using cultured cardiomyocytes for current and action potential recordings (see response to reviewer 1). We have also added a section discussing GtACR1-mediated optical pacing and inhibition:

Optogenetic approaches allow for spatiotemporally precise manipulation of the cellular membrane potential, both in single cardiomyocytes and intact cardiac tissue. Classically, ChR2, a light-gated cation non-selective channel, has been used for depolarization of the membrane potential, whereas light-driven proton and/or chloride pumps were used for membrane hyperpolarization. Both groups of optogenetic actuators require high expression levels, as ChR2 is characterized by an intrinsically low single-channel

conductance and light-driven pumps maximally transport one ion per absorbed photon[28]. Furthermore, prolonged activation of ChR2 in cardiomyocytes may lead to Na^+ and/or Ca^{2+} overload, and light-driven pumps may change trans-sarcolemmal H^+ or Cl^- gradients[29,30]. In search for alternative tools for optogenetic control of cardiomyocyte activity, we recently tested the natural anion channelrhodopsin GtACR1, characterized by a superior single-channel conductance and higher light sensitivity compared to cation ChR such as ChR2. We found that GtACR1 activation depolarizes CM and can be used for optical pacing and inhibition, depending on the light pulse timing and duration. An additional advantage of using ACR instead of cation ChR might be the more negative reversal potential of Cl^- compared to Na^+, reducing artificially introduced ion currents. As we have previously shown, optical pacing with GtACR1 may lead to action potential prolongation as a result of the slow component of GtACR1 channel closure, which could be overcome by using faster GtACR1 mutants[19]. However, action potential prolongation is much less pronounced when using a lower, more physiological intracellular Cl- concentration (see Figure 6). Moreover, GtACR1-mediated inhibition by prolonged illumination results in profound membrane depolarization, which again could activate secondary Na^+ and Ca^{2+} influx, thereby altering the activity of voltage-gated channels. In our measurements, we find that AP and contraction parameters recover to baseline within 40 s after a light-induced inhibition for 1 min (see Kopton et al, 2018 and Figures 6/7). Light-gated K^+ channels offer a potent alternative for silencing CM without affecting the CM resting membrane potential[31].

(2) The authors should describe other types of experiments in cardiomyocytes to which this protocol can be applied in addition to the mentioned application in the discussion section.

The here presented protocol can be used for in-depth characterization of the electromechanical properties of CM. It is principally applicable also to CM from other species, and to CM isolated from diseased myocardium. Optical stimulation allows one to pace CM at different frequencies, and different preloads can be tested during carbon fibre contraction experiments. An interesting experiment would be to use low-intensity illumination for subthreshold depolarization, to mimic gradual increase in the resting membrane potential, as can be observed during the development of cardiac tissue remodeling during disease progression. Finally, functional measurements could be combined with Ca^{2+} imaging for further insight into excitation-contraction coupling, or with pharmacological interventions to evaluate the effects of different drugs on CM activity.

Minor Concerns:

(1) Paragraph 5.5.3.3 on page 10 describes the CF attachment on cardiomyocytes. However, the explanation was incomplete. The authors should add a statement describing how to set the second fibre.

We have now corrected the following statements: Lower the first fibre onto the cell surface. Attach the second fibre parallel to the first fibre at the other end of the CM. The ideal alignment is near-perpendicular to the cell axis.

NOTE: Attach the fibre by gently pushing the cell to the bottom surface. Release the pressure before

attaching the second fibre. Don't stretch the cell by attaching the second fibre.

After both fibres are attached on the cell lift the fibre, so the cell has no contact to the chamber surface anymore and is able contract without any frictions with the chamber.

(2) Figure 7 illustrates representative data from carbon fibre measurements. Several points were unclear or showed errors.
(i) The authors should change the order of (A) and (B). The first figure should be (B).

This has been implemented as suggested.

(ii) Scheme B (II) might be rather difficult to understand. Please correct scheme (II) by adding the direction of the cell.

The scheme has been rotated and is now aligned corresponding to image of the cell.

(iii) The legends of (D) and (E) are placed in the wrong order. Please correct these mistakes.

This has been corrected.

Reviewer #4:

Manuscript Summary:
Within this MS the authors describe a detailed protocol to investigate Cl- conducting light sensitive ion channels in cardiomyocytes. This is a controversial topic within the recent years as the Nernst potential of CL- is quite special in cardiomyocytes and thus this approach leads to depolarization in cardiomyocytes in contrast to the observed hyperpolarization in neurons. This has been nicely demonstrated by the same group in a recent publication and a detailed protocol will provide better basis for this discussion. In that regard, the protocol can still be improved in some points:

We thank the reviewer for the positive feedback and the suggestions to improve the protocol.

Major Concerns:

1. What is the reason for the chosen CL- concentration in the internal and external solution. The Nernst potential will be in this case around -27mV. However in adult cardiomyocytes it seems according to the literature be a little bit lower ~-50 mV. Please discuss this very critical point in more detail.

We agree that the original Cl^- concentration was higher than the physiological concentration in CM. The experiments of Figure 6 have therefore been repeated using a lower Cl^- concentration in the pipette solution (15 mM). This has been accordingly revised in the protocol and table with solutions. Please also see response to reviewer 2.

2. One other crucial part is illumination through the microscope with defined light intensities at cellular level. It could be helpfull for researchers new within this field to elaborate more on the following points: a) how to triggert he LEDs? In this case it is not clear how the authors encoded the light intensity with TTL signals only. Why didn't they chose to use an analog trigger encoding time and light power?

We used a TTL pulse to encode the time the LED is switched on. The light power was independently controlled via the LED box using a custom-written Matlab script.

b) Within this custom build LED setup: How is heating of the LED controlled? Temperature has a dramatic effect on the resistance of the LED and likewise on the actual light power.

We have now measured the light intensity in the object plane for light durations of 10 ms and 64 s (see Supplemental Figure 1). In these measurements, we show that the light stimulation is stable both for repetitive short light pulses and for prolonged illumination.

c) How did the authors control that the voltage (LED) and light intensity (cellular level) are really within the linear range at the desired light intensity? Normally using objectives with high magnification light intensity is far too high and LED have to be driven at very low voltages far below the linear range.

We agree with the reviewer that the voltage and light intensity might be below the linear range. We instead propose to record the light intensity as a function of voltage at small intervals. We determined the light intensity at a given voltage before each experimental series to know the exact light intensity in the object plane for each optical stimulation experiment. The respective sentence on the linear relationship has now been deleted.

3. Why did the authors use the 525nm LED to excite eGFP? The excitation max of eGFP is much lower. Why did the authors chose to tag it with eGFP although imaging will than coexcite the channel.

We thank the reviewer for this correction. In fact, we used a 460-nm blue LED in combination with a 450 nm – 490 nm band-pass excitation filter, a 510 nm dichroic mirror and a 515 nm long-pass emission filter for eGFP visualization.

We agree that the blue LED also activates GtACR1, but this would equally be the case when using fluorescent reporter proteins with green or yellow excitation. As we achieved more than >99% of transduction efficiency with the GtACR1 adenovirus, we did not usually check fluorescence before functional experiments to avoid pre-activation of the channel. If needed, fluorescence was checked after the functional experiments.

We have revised the respective paragraph as follows: eGFP-positive cells can be detected using a blue LED (460 nm) in combination with a band-pass excitation filter (450 nm – 490 nm), a 510 nm dichroic mirror and a 515 nm long-pass emission filter. If other fluorescent tags are used, use corresponding LED and fluorescence filter sets. NOTE: If you achieve a transduction efficiency (in our hands >99% with the GtACR1 adenovirus), you do not need to check eGFP fluorescence before the functional experiments; this avoids potential GtACR1 pre-activation.

4. How did the authors illuminate the cells for contraction measurements if room light already activates the channel. Can it be excluded that imaging light excites the channel?

All experiments were performed under red light. In line, a red band-pass filter 630/20 was used for the transmission light during the contraction measurements.

We have added the following statement: Filter the transmission light by a red-band-pass filter (630/20 nm) in the condenser to avoid coactivation of GtACR1.

5. When comparing the APs which have been electrically or optically excited, how did the authors get rid of the stimulation artefacts from electrical pacing. Is the big difference in AP upstroke due to the artefact correction?

We did not correct for the stimulation artifact. Generally, we agree that stimulation artifacts may overlay AP upstroke. We minimized artifacts by only pacing at 50% above threshold for electrical stimulation.

6. Can the activity of CM be blocked by light at any given strength of the electrical stimulation or only at 1.5x supramaximal?

We additionally performed this experiment and it is now included in Figure 6 F. AP generation is also inhibited under sustained light at higher currents injection as permanent depolarization is inactivating Na^+ channels. Current injections were increased to almost 5x above threshold.

7. Why did the authors illuminate in voltage clamp only for 10ms and loose thereby the information on desensitiztation and steady state currents?

We have now exchanged the graph with a light pulse of 300 ms. Steady state currents appear in this trace with prolonged illumination.

Minor Concerns:

1. When and how is anaesthesia depth controlled?

This has been added to the protocol:

In substep 3.2: Confirm anaesthesia with the loss of the righting reflex.

In substep 3.6: Rabbit shouldn't respond to the pedal withdrawl reflex.

2. l 181: what is the real percentages of non myocytes and myocytes in the supernatant and in the pellet? How effective is this step?

We agree that this step may result in approximately 10% of cardiomyocytes remaining in the supernatant and approx. 10% of non-myocytes in the pellet. However, the resulting culturing protocol ensures high purity of cardiomyocytes on laminin-coated coverslips. We have not observed a notable amount of non-myocytes growing on the coverslips in our experiments so far.

3. How is the speed for perfusion chosen, do you recommend to check once the resulting pressure?

The speed of perfusion was optimized when establishing the protocol. Pressure curves have been recorded, but we cannot provide any additional information as pressure immediately decreases very early after the beginning of the enzyme perfusion while the tissue is still very stiff.

Reviewer #5:

Manuscript Summary:
The paper of Kopton et al. describes the detailed protocol that combines electrophysiological patch-clamp recordings and the unique carbon fiber technique to study the effects of GtACR1 photoactivation on electrophysiological and mechanical activity in cultured rabbit ventricular cardiomyocytes. The protocol is well-organized, well-illustrated and highly useful for biophysical measurements, but has some minor concerns.

Thank you for this positive evaluation and the very critical look at the details of the contraction experiments. This is very much appreciated.

Minor Concerns:

1. "mechanical carbon fibre measurements" in the Abstract is not clear

This has been revised to: “optical tracking of cardiomyocyte contractions”

2. It is mentioned in the Abstract that contractions are recorded by sarcomere length tracking. But authors also record the distance between carbon fibers, not only the changes in SL

We have changed the respective statement to: […]contractions are recorded by tracking changes in sarcomere length and carbon fibre distance.

3. 5.5. 5.5. There are no references of (1-5) to fig 2./fig 4

These cross-references have now been added.

4. Fig 5 - It easier to understand the scale when a scale bar is added directly to an image

These have now been added in Figure 5, 6 and 7.

5. Authors write measurement units in different styles, e.g. uN/mm2 or uN*mm-2 that could be confusing

Thank you for this advice. We have now unified the use of units.

6. I recommend to use "V" instead of "E" in formula 1 to avoid confusion with the common abbreviation of electric field

We agree with the reviewer that this might be confusing, but as we use "V" as unit for the voltage and explain in the bracket (ΔE - difference of measured voltage) we believe that this should be clear in this context.

7. A sensitivity of 406A force transducer in 5.5.2.1. is 0.05 mN/V

Thank you. This has been revised.

8. The measurement units are given only for F and deltaE in formula 1.

The missing unit has been added.

9. 6.1. Do authors analyze AP amplitude or resting potential?

We agree that these are important parameters that we usually determine. We have added the following statement: "Determine resting membrane potential and AP amplitude."

10. It is not determined in the text how authors estimate carbon fibre bending

The following substep has been added: Focus the sarcomeres in the data acquisition software (see Materials Table) and set the sarcomere length tracking window (Figure 7 A I (3)) between the fibres. NOTE: Resulting FFT power spectrum (Figure 7 A I (2)) shows ideally one sharp peak, which represents the average sarcomere length.

Track fibre bending using the edge detection module. Set the detection areas with the red and green window and define a threshold (red and green horizontal line) at the first derivative of the light intensity trace (Figure 7 A III).

Start to optically pace the cell at 0.25 Hz (if possible, try faster pacing rates) and track sarcomere length and fibre bending.
NOTE: Fibre holder position, LED trigger and electric stimulation pulses are controlled via the data acquisition software (see Materials Table).

11. 6.2.3, 6.2.4 Formulas are not numbered

This has now been added.

12. 6.2.3. stiffness [uN/um, not uN] will be in the numerator (i.e. Iribe et al., 2014)

Thank you. This has now been corrected.

13. Do authors calculate the end systolic cell deformation not as the relative cell shortening? (baseline-peak)/baseline)*100%)

This has now been revised.

14. 5.5.3.4. Stimulation frequency is not given.

We usually pace at 0.25 Hz at RT. This has now been added to the 5.5.3.4.

15. Results section. How is cell peak force determined from SL?

This has been deleted. The paragraph is now stated in the following:

Generated peak forces were determined from carbon fibre bending.

16. Fig. 7. D, E. Panels does not correspond to the caption.

This has now been corrected.

Supplemental Figure1

Click here to access/download;Supplemental File (Figures, Permissions, etc.);SupplementalFigure1.psd