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Corresponding Author:	Inês Pires, Ph.D. Unidade de Investigacao Cardiovascular, Departamento de Cirurgia e Fisiologia Porto, Porto PORTUGAL
Corresponding Author's Institution:	Unidade de Investigacao Cardiovascular, Departamento de Cirurgia e Fisiologia
Corresponding Author E-Mail:	ipires@med.up.pt
Order of Authors:	Patricia Gonçalves-Rodrigues
	Joao Almeida-Coelho
	Alexandre Gonçalves
	Flávio Amorim
	Adelino F Leite-Moreira
	Ger Stienen
	Inês Pires, Ph.D.
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TITLE:

In Vitro Assessment of Cardiac Function Using Skinned Cardiomyocytes

AUTHORS AND AFFILIATIONS:

Patrícia Gonçalves-Rodrigues[1]*, João Almeida-Coelho[1]*, Alexandre Gonçalves[1], Flávio Amorim[1], Adelino F Leite-Moreira[1], Ger JM Stienen[2], Inês Falcão-Pires[1]

*Both authors contributed equally

[1]Unidade de Investigação Cardiovascular, Departamento de Cirurgia e Fisiologia, Faculdade de Medicina, Universidade do Porto, Portugal

[2]Kilimanjaro Christian Medical University College, Department of Physiology, Moshi, Tanzania

Corresponding author:

Inês Falcão Pires

ipires@med.up.pt

Email Addresses of Co-authors:

Patrícia Rodrigues (rodrigues13patricia@gmail.com)

João Almeida Coelho (jac@med.up.pt)

Alexandre Gonçalves (alexandregoncalvs@gmail.com)

Flávio Amorim (flaviojramorim@gmail.com)

Ger Stienen (g.stienen@amsterdamumc.nl)

Adelino F. Leite-Moreira (amoreira@med.up.pt)

KEYWORDS:

skinned myocytes, skinned cardiomyocytes, myocardium, biopsies, permeabilized cardiomyocytes, cardiac function, myofilaments

SUMMARY:

This protocol aims to describe step-by-step the technique of extraction and assessment of cardiac function using skinned cardiomyocytes. This methodology allows measurement and acutemodulation of myofilament function using small frozen biopsies that can be collected from different cardiac locations, from mice to men.

ABSTRACT:

In this article, we describe the steps required to isolate a single permeabilized ("skinned") cardiomyocyte and attach it to a force-measuring apparatus and a motor to perform functional studies. These studies will allow measurement of cardiomyocyte stiffness (passive force) and its activation with different calcium (Ca^{2+})-containing solutions to determine, amongst others: maximum force development, myofilament Ca^{2+}-sensitivity (pCa_{50}), cooperativity (nHill) and the rate of force redevelopment (ktr). This method also enables determination of the effects of drugs acting directly on myofilaments and of the expression of exogenous recombinant proteins on both active and passive properties of cardiomyocytes. Clinically, skinned cardiomyocyte studies

highlight the pathophysiology of many myocardial diseases and allow in vitro assessment of the impact of therapeutic interventions targeting the myofilaments. Altogether, this technique enables the clarification of cardiac pathophysiology by investigating correlations between in vitro and in vivo parameters in animal models and human tissue obtained during open heart or transplant surgery.

INTRODUCTION:

Traditionally, assessment of myocardial mechanical properties has been attempted mostly in multicellular preparations, such as papillary muscles and trabeculae[1,2]. Multicellular cardiac muscles strips include a heterogeneous population of cells, including contractile cardiomyocytes with an unknown pattern of orientation and force generation, electrical activity and stress/strain distributions as well as a surrounding connective tissue matrix[3,4]. A preparation without collagen and containing a single cardiomyocyte would allow measurement of sarcomere length and cross-bridge contractile properties in a very precise and controlled manner[5,6]. Therefore, over the last four decades, several methodologies were developed allowing investigating the mechanical, contractile, and relaxation properties of a single cardiomyocyte[6,7]. The contractile function of these cells is strongly dependent on sarcomere length and cross-bridge cycling kinetics[3]. Thus, it is desirable to investigate muscle function directly in single isolated cardiac cells, considering that it allows assessing sarcomere length and performance as well as cross-bridge function and contractile properties. However, isolating and attaching functional cardiomyocytes with a reasonable optical sarcomere resolution while recording force measurement at the µN level is still challenging and evolving[3,6]. Other challenges are the logistics that need to be installed to isolate cardiomyocytes from freshly collected biopsies. The unpredictability of human biopsies collection, for instance, may jeopardize the feasibility of the experiments.

Moreover, ethical concerns regarding the Replacement, Reduction and Refinement of animal experimentation for scientific procedures (principles of the 3Rs) have promoted study changes at the cellular and tissue level, preferably in human biopsies, or in smaller animal samples. Indeed a progressive refinement of methodologies to assess cardiac function in vitro on a smaller level of complexity allows proper integration of the results to the whole body and translate them to the clinical scenario[7]. Altogether, using samples stored at -80 °C to extract cardiomyocytes may be an appealing alternative.

The myocardial tissue is cut into small pieces and homogenized with a mortar and a pestle. The result of this homogenization is a suspension of skinned bundled and isolated cells with varying degrees of sarcolemmal damage, wherein the myoplasm is exposed to the bathing medium and all the cellular components are washed out. Structures such as the myofibrils that are further away from the sarcolemma are preserved. Thus, sarcomere shortening and functional properties associated with the myofibrillar apparatus are kept intact and can be recorded[8,9].

The cardiomyocyte force measurement system consists of an electromagnetic motor, used to adjust cardiomyocyte length, and a force transducer, that measures isometric cardiomyocyte contraction. A permeabilized, or skinned, cardiomyocyte is placed in an experimental chamber containing a relaxing solution ($[Ca^{2+}] < 10$ nM) and silicon-glued to 2 thin needles: one attached

to the motor and the other to the force transducer. An optical system is used to determine cardiomyocyte morphology and sarcomere length. The experimental protocol often consists of a series of force recordings upon buffer solutions containing different Ca^{2+} concentrations, the determination of actin-myosin cross-bridge kinetics and the measurement of the passive tension of the mounted cardiomyocytes at pre-defined sarcomere lengths (**Figure 1**). Isolation of permeabilized cardiomyocytes from myocardial samples frozen in liquid nitrogen (and subsequently stored at -80 °C) is a technique that utilizes cellular mechanics and protein biochemistry for measuring maximal Ca^{2+}-activated (active) force per cross-sectional area (T_{active}, $kN.m^{-2}$), Ca^{2+}-independent (passive) tension ($T_{passive}$, $kN{\cdot}m^{-2}$), myofilaments Ca^{2+}-sensitivity (pCa_{50}), cooperativity (nHill), the rate of force redevelopment (ktr) as well as sarcomere length dependencies of T_{active}, $T_{passive}$, pCa_{50}, nHill and ktr.

The goal of this protocol is to illustrate and update the cardiomyocyte force measurement system as a reliable procedure to assess the functional mechanical properties of single skinned cardiomyocytes isolated from frozen samples from different species.

PROTOCOL:

All animal experiments comply with the Guide for the Care and Use of Laboratory Animals (NIH Publication no. 85–23, revised 2011) and the Portuguese law on animal welfare (DL 129/92, DL 197/96; P 1131/97). The competent local authorities approved this experimental protocol (018833).

1. Stock solution preparation (Table 1)

1.1. Prepare 1,000 mL of relaxing solution for cardiomyocytes' isolation (RELAX-ISO) by following instructions in **Table 2**. Dissolve the reagent above in ≈500 mL and adjust the pH to 7.0 with KOH. Adjust the final volume to 1000 mL.

1.1.1. Distribute RELAX-ISO in 50 mL tubes. Store at -20 °C.

1.2. Prepare 250 mL of **activating solution** by following the instructions in **Table 3**. Dissolve the reagents above in ≈100 mL of ultra-pure water. Adjust the pH to 7.1 with 5 M KOH at 15 °C.

NOTE: Usually, it is necessary to add a significant amount of KOH to reach the desired pH. Put the volumetric balloon in a box with ice to cooldown the solution to 15 °C.

1.2.1. Adjust the final volume to 250 mL. Agitate this solution continuously with a magnetic stirrer until the moment of mixing it with the relaxing solution.

1.3. Prepare 100 mL of **relaxing solution** by following the instructions in **Table 4**. Dissolve the reagents above in ≈50 mL of ultra-pure water. Adjust the pH to 7.1 with KOH 5 M at 15 °C.

1.3.1. Usually, it is necessary to add a significant amount of KOH to reach the pH of 7.1. Place

the volumetric balloon in a box filled with ice to cool down the solution to 15 °C. The ionic strength of the solutions used during the measurements amounted to 180 mM.

1.3.2. Adjust the final volume to 100 mL. Agitate this solution continuously with a magnetic stirrer until the moment of mixing it with activating solution.

1.4. Mix activating and relaxing solutions in the proportions presented in **Table 5** to obtain pCa solutions between 5.0 and 6.0.

1.4.1. Always keep relaxing and activating solutions agitating while mixing both.

1.4.2. Aliquot each solution to 2 mL microtubes. Aliquot the remaining relaxing solution in 2 mL microtubes. Aliquot the remaining activating solution in 2 mL microtubes. Store all the microtubes at -20 °C.

1.5. Prepare a different batch of pCa solution (4.5 to 6.0) for each protocol.

2. Calibration of the force transducer

NOTE: The calibration of the force transducer is a routine procedure that should be performed every couple of months or whenever it is suspected to be de-calibrated. The force transducer is highly sensitive and is easily broken. It should be gently handled in every step of its usage, including calibration, gluing of the cardiomyocyte and cleaning.

2.1. Detach the force transducer from the rest of the apparatus.

2.2. With the help of a clamp, place the force transducer horizontally in such a way that the needle points downward in the same orientation that the cardiomyocyte will develop force. This will facilitate hanging a series of masses with known weights (elastic band, suture or pin).

NOTE: Check the characteristics of the force transducer before proceeding to this step to check the scale factor [mg/volt] and to avoid excessive weight on the transducer. For the force transducer model, the scale factor is 50 (50 mg correspond to 1 volt) and we use 5 weights between 12.5 and 250 mg.

2.3. Turn the force transducer on and let it warm up for 30 min.

2.4. Start by hanging the lighter mass on the force transducer and registering the corresponding voltage measured at FORCE OUT.

2.4.1. Repeat this procedure for up to five weights.

2.5. Plot force applied to the force transducer (load) versus voltage and check for linearity.

2.6. If there is no linearity, adjust the zero and gain potentiometers in the circuit board of the transducer. Check its specific instruction for further information.

2.6.1. Turn the zero potentiometer until the output voltage reads 0.0 V.

2.6.2. Hand a medium weight on the transducer needle and adjust the gain potentiometer to read the corresponding voltage (for instance, 50 mg correspond to 1 V). Remove the weight and re-adjust the zero potentiometer to 0.0.

2.6.3. Repeat step 2.6.2 until the output with and without the weight are correct.

2.7. Mount the force transducer back into the apparatus.

3. Setting the experimental apparatus

3.1. Thaw one vial each of the activating, 4.5, 5.0, 5.2, 5.4, 5.6, 5.8, 6.0 and relaxing solutions and maintain them on ice.

NOTE: ATP and PCr are labile compounds that should be maintained at cold temperatures.

3.2. Prepare the microscope, testing apparatus and associated computer for use (**Figure 1**).

3.3. Adjust the temperature so that the in-chamber thermometer reads 15 °C. Perform all experiments at this temperature except for kinase and phosphatase incubations (20 °C).

3.4. Turn on the force-transducer and the motor.

4. Extraction and permeabilization of skinned cardiomyocytes

4.1. Defrost 50 mL of RELAX-ISO solution.

4.2. Turn on the centrifuge and fast cool it up to 4 °C.

4.3. Thaw 3-5 µg of a myocardial sample in a Petri dish filled with 2.5 mL of RELAX-ISO solution.

4.4. Cut the tissue in small pieces with a scalpel blade (**Figure 2**). Cut the sample in a precise way to avoid unnecessary cells damage.

4.5. Transfer the 2.5 mL of RELAX-ISO solution with the tissue to a Potter-Elvehjem glass with a cut pipette tip.

4.6. Mechanically disrupt the tissue with a grinder at a rotation speed of 30-40 rpm. Press the tissue 3 times for 2 s each to obtain a good cell suspension.

4.7. Prepare 10% Triton in RELAX-ISO solution (250 µL of Triton with 2.25 mL of RELAX-ISO) in a 15 mL tube and add this solution to the cell suspension.

4.8. Gently mix by inverting the tube 3 times.

4.9. Incubate at room temperature for 1 min and 4 min on ice.

4.10. Wash out the Triton by adding RELAX-ISO up to the top of the 15 mL tube; gently mixing (inverting 3 times the tube) and finally spinning down the cells in an angled centrifuge (1 min at 348 x *g*). Remove the supernatant up to 3 mL above the cell pellet.

NOTE: Remove the supernatant gently to avoid disturbing the cell pellet. Still, some cells in supernatant will be inevitably lost.

4.11. Repeat the step 4.10 at least 4 times or until no more bubbles produced by Triton residues are observed.

NOTE: The more wash-out steps are made, the more cells are lost with the discarded supernatant.

4.12. In the last washout, remove the supernatant up to a volume of 5-10 mL of cell suspension.

5. Selecting and gluing the skinned cardiomyocyte

5.1. Put a cell suspension drop on a coverslip on top of a glass slide in the microscope slide holder (**Figure 1**).

5.2. Select a single rod-shaped cardiomyocyte with a good striation pattern and size (**Figure 2**).

5.3. Find the needle tips of the force-transducer and the motor using the lowest magnification of the inverted microscope.

5.4. Rotate the coverslip to position the selected cardiomyocyte horizontally so that its ends are aligned with the needle of the force transducer and the motor (**Figure 2**).

5.5. Place a thin line of glue on the side of the coverslip with the help of a swab tip (**Figure 2**).

5.6. Immerse the needle tips of the force transducer and the motor into the glue line to create a glue halo around both tips.

NOTE: Steps 5.6 – 5.10 are accomplished through careful use of the motorized micropositioners.

5.7. Quickly move the needle tips close to the focal plane of the cardiomyocyte.

5.8. Move the needle tip of the force transducer down so that it glues to one edge of the cardiomyocyte.

5.9. Repeat this procedure with the tip of the motor and the other extremity of the cell.

NOTE: This procedure must take less than 2-3 min as the glue starts to cure very fast.

5.10. After 5-8 min, lift the needles ≈15 µm to avoid gluing the cell to the coverslip. This is done by moving up both micromanipulators simultaneously.

5.11. Let the glue cure. This procedure can last from 15 to 45 min, depending on the type of glue. In our case, the cardiomyocyte is adequately glued after ≈15 min.

6. Recording force measurements of active, passive and Ca^{2+} sensitivity

6.1. Fill the first experimental well with the relaxing solution (55-100 µL in the experimental apparatus) and the second experimental well with activating solution.

6.2. Using the camera software, place the region of interest (ROI) in an area of the cardiomyocyte with a clear pattern of striation.

NOTE: For cardiac myocytes, the operating sarcomere length varies between 1.8 and 2.2 µm, and the optimal sarcomere length is around 2.15 µm.

6.3. Measure the distance between the two extremes of the cardiomyocyte (from the motor to the transducer glue halo, **Figure 3**) after the optimal sarcomere length has been set (2.2 µm). Record the value as myocyte length in the software.

6.4. Measure cardiomyocyte width and depth, the latter with the aid of a prism mirror placed perpendicular to the cell.

NOTE: A powerful, external light source will be required to visualize the cell through the prism. In case there is no prism, and assuming that cardiac cells have an elliptical shape, cardiomyocyte depth can be inferred as 70% of cardiomyocyte width.

6.5. Calculate cross-sectional area (CSA, mm^2) assuming an elliptical shape of the cardiomyocyte.

$$CSA = \pi * \left(\frac{width}{2*1000}\right) * \left(\frac{depth}{2*1000}\right)$$

6.6. Gently move the microscope stage so that the cell moves from the coverslip to the well containing relaxing solution on the back of the stge.

NOTE: This procedure can easily damage the cell. Before moving the cell, gently move the needles up a bit more. Avoid removing the cell out of the solution.

6.7. Select the protocol in software that contains two cell shortening (80% of its initial length), that will occur when the cell is emerged in Ca^{2+} solution and in relaxing solution, respectively (**Figure 1, Supplementary File**).

NOTE: First "Slack" of the cell will be performed within activating solution and the second "Slack" within relaxing solution. By doing this, calculate the total force (F_{total}) of the cell from the 1st and the passive force ($F_{passive}$) of the cell from the 2nd. Use the formula to calculate active force, F_{active}= F_{total}- $F_{passive}$. The cell is shortened 80% in order to detach all cross-bridges record force.

6.8. Elicit isometric contraction by moving the microscope stage so that the cardiomyocyte moves from the relaxing to the activating solution (pCa=4.5(1)).

NOTE: If the cell is functional, it will immediately contract.

6.9. Upon reaching force plateau, start recording the force data.

NOTE: The tests can be done individually. Depending on software there is the possibility to create a sequence of tests that will correspond to the different Ca^{2+} solutions within a Ca^{2+}-sensitivity protocol (**Figure 1, Supplementary File**).

6.10. Wait ~10 s and then switch the cell immersed in activating solution.

NOTE: It is important to wait 10 s before immersing the cell in relaxing solution. If the cell is moved too early, important data to calculate the redevelopment force of the cell (Ktr value) might be lost.

6.11. Quickly move the stage so that the cardiomyocyte immerses in the relaxing solution.

6.12. Wait until the test stops.

6.13. Repeat steps 6.8 – 6.12 so that the cell is activated twice in activating solution (pCa=4.5(2)).

NOTE: Typically, after the first activation, cardiomyocyte ends can slightly detached from the needle tips, changing the cardiomyocyte length, CSA and/or the sarcomere length. Readjust to the desired sarcomere length and introduce the corrected dimensions in the software.

6.13.1. Continue to step 6.13.2 for Ca^{2+} sensitivity protocol or save the data and detach the cell from needles and clean them with acetone to remove the glue

6.13.2. If needed, end the protocol at this step if basic values of passive and active force of the

cell are the only parameters needed. Adjust the sarcomere length of the cell to 2.2 µm by slightly stretching it again, if necessary.

6.13.3. Replace the activating solution by the next Ca^{2+} solution (55-100 µL here). Repeat steps 6.8 – 6.12.

6.13.4. Repeat exchanging the existing solution by each Ca^{2+} solution and repeat steps 6.8 – 6.12 until all solutions have been tested (5.0, 5.2, 5.4, 5.6, 5.8, 6.0).

6.13.5. Lastly, re-activate the cell with activating solution (pCa4.5(3)). Repeat steps 6.8- 6.12.

7. Incubation with kinases and phosphatases

7.1. After performing the selected baseline protocol, dilute the kinase/phosphatase in Relaxing solution at the recommended concentration.

NOTE: It is recommended to carry out a dose-response curve prior to the experiment.

7.2. Set the temperature of the experimental wells to 20 °C.

7.3. Fill the experimental wells with kinase/phosphatase, relaxing solution and activating solution (55-100 µL).

7.4. Gently move the microscope stage so that the cell becomes immersed in the well containing kinase/phosphatase.

7.5. Incubate the cardiomyocyte with the kinase/phosphatase for at least 30 min or according to the manufacturers' instructions.

7.6. Repeat the selected baseline protocol.

8. Finalizing the experiment

8.1. Unglue the cardiomyocyte from the tips of the force transducer and motor by stretching the cell.

8.2. Carefully remove the glue halo from the needle tips using a cotton swab soaked in acetone.

8.3. Shut down the equipment.

9. Analyzing the data

9.1. Collect all files from each cardiomyocyte tested.

NOTE: Each test will correspond to one file. This means that for each Ca^{2+} solution or sarcomere length, there will be a corresponding file.

9.2. Calculate active and passive forces of a single cardiomyocyte.

9.2.1. Open the file corresponding to first activation (pCa=4.5(1)) using a spreadsheet (**Figure 3A, Appendix A in Supplementary File**).

NOTE: We used a custom made program to perform the analysis. Please see **Appendix A** in the **Supplementary File**.

9.2.2. Average ≈60 values before and average ≈60 values after the 1^{st} slack of the cell (when the cell is immersed in Ca^{2+} solution). These 2 values correspond to a and b, respectively.

9.2.3. Repeat the same analysis for the 2^{nd} slack of the cell (when the cell is immersed in relaxing solution). These 2 values correspond to c and d, respectively.

9.2.4. Calculate the difference between a and b (total force, F_{total}).

9.2.5. Calculate the difference between c and d (passive force, $F_{passive}$).

9.2.6. Calculate active force, $F_{active} = F_{total} - F_{passive}$.

9.2.7. Normalize all force values to CSA (see formula above) to obtain the total tension (T_{total}), passive tension ($T_{passive}$) and active tension (T_{active}).

9.2.8. Repeat step 9.2.1 to 9.2.5 for the 2^{nd} activation (pCa=4.5(2)).

9.2.9. Consider these values as those representing T_{total}, T_{active} and $T_{passive}$ of the cardiomyocyte under analysis.

NOTE: The first activation of the cell with pCa4.5(1) is usually associated with alterations in cell dimensions. For this reason, the 2nd activation with pCa4.5 is more accurate and is the one to be used.

9.2.10. Repeat the steps 9.2.1 to 9.2.5 for each file/pCa tested solutions (5.0, 5.2, 5.4, 5.6, 5.8, 6.0, 4.5(3)).

9.3. Calculate pCa50 and nHill of a single cardiomyocyte.

NOTE: For this analysis, use the non-normalized F_{active} values from the files 4.5(2), 5.0, 5.2, 5.4, 5.6, 5.8, 6.0 and 4.5(3).

9.3.1. Place in a spreadsheet file all non-normalized values of F_{active} for each Ca^{2+} solution tested (4.5(2),5.0, 5.2, 5.4, 5.6, 5.8, 6.0, 4.5(3)).

9.3.2. Calculate the correction factor = $F_{active\ [4.5(2)]}$ - $F_{active\ [4.5(3)]}$ / 7.

9.3.3. Calculate the corrected values of F_{active} for each Ca^{2+} solutions (5.0, 5.2, 5.4, 5.6, 5.8, 6.0) by subtracting F_{active} – correction factor.

9.3.4. Calculate the relative force ($F_{relative}$) for each Ca^{2+} solutions by normalizing each F_{active} values by the corresponding corrected value.

NOTE: The $F_{relative}$ [4.5(3)] should equal 1. Each experimental protocol begins and ends with a control activation at saturating Ca^{2+} concentration (pCa 4.5(2) and 4.5(3)). This allows force normalization and assessment of the rundown of the preparations through the comparison of changes in maximal Ca^{2+} - activated force (F_{max}). If at the end of the experimental protocol, the cardiomyocyte produces less than at least 70% of the maximum force of the first contraction, that cell/measurement should be excluded from the analysis.

9.3.5. Use the $F_{relative}$ and the corresponding pCa values to fit to a sigmoidal curve with the following equation $F(Ca) = Ca^{nHill} / (Ca50^{nHill} + Ca^{nHill})$.

9.3.6. Extrapolate pCa and nHill values from the equation abovementioned.

9.4. Calculate the rate of force redevelopment (ktr) of a single cardiomyocyte.

9.4.1. Perform a fit to the curve that corresponds to the values immediately after the 1st cell slack.

9.4.2. Calculate the slope of the curve and this value will correspond to the rate of force redevelopment.

9.4.3. Repeat step 9.4.1 and 9.4.2 for each Ca^{2+} solution.

NOTE: A poor curve fit will be obtained for the lowest Ca-solutions (R squared≤0.90).

REPRESENTATIVE RESULTS:

Functional permeabilized cardiomyocytes should appear uniform and with a consistent striation pattern throughout the entire experiment. Although a certain degree of deterioration and force decrease is expected after prolonged experiments, the values of active tension should be relatively stable. Cells showing clear signs of striation loss or whose force drops significantly (< 15 $kN \cdot m^{-2}$ or <80% of its initial active force) should be excluded. **Table 6** displays the normal values expected for the most important parameters derived from rodents, pigs and human samples.

The parameters obtained depend mainly on the chosen protocol. **Figure 5** shows representative force traces of 3, out of 8, force recordings needed to carry out a protocol of myofilaments Ca^{2+}-sensitivity. By transferring the cell to a well containing the activating solution, the cardiomyocyte starts to develop force until it reaches a plateau. After a quick slack test (duration of 1 ms), whereby the cardiomyocyte shortens to 80% of its length, we obtain the baseline values of zero force. After the slack test, the cell continues to develop force as it is immersed in the activating solution. Total force (F_{total}) is calculated by subtracting the plateau value from the minimal value. The slope of the last part of this curve gives us the value of the rate of force redevelopment (ktr) (**Figure 6**), which is a measure of the apparent rate of cross-bridge attachment and detachment (f_{app} and g_{aap})[10]. When the ktr Rsquare value is <0.90 the ktr value should be excluded and usually this happens at lower Ca^{2+} concentrations (pCa 5.6, 5.8 and 6.0). After transferring the cell back to a well containing the relaxing solution, the cell relaxes and its force drops. Passive force ($F_{passive}$) is calculated by subtracting the minimal value (obtained after a prolonged cell shortening) to this new value of force. Active force results from the difference between F_{total} and $F_{passive}$.

The maximal active and passive force that characterizes a cardiomyocyte is the one derived from the second cell activation with a saturating Ca^{2+}-solution (pCa = 4.5). The first activation is usually discarded as the sarcomere length often needs to be readjusted.

To carry out a myofilament Ca^{2+}-sensitivity protocol, it is necessary to perform at least 9 activation tests (4.5; 4.5; 5.2; 5.6; 6.0; 5.0; 5.4; 5.8 and 4.5). This sequence is merely exemplifying but should always start with 4.5 (twice) and end with 4.5. The programming of the data-acquisition software for a myofilament Ca^{2+}-sensitivity protocol is depicted in **Figure 1** of the **Supplementary File**.

After calculating active force for all these activation solutions, check if the last activation yielded more than 80% of the initial maximal force (otherwise this cell results should be discarded, as mentioned above). To correct for the decline in F_{max} during the experimental series, the interpolated F_{max} values can be used to normalize the data points. The normalized data can be fit to a sigmoidal curve with the following equation $F(Ca) = Ca^{nHill}/(Ca50^{nHill} + Ca^{nHill})$. The parameter values obtained represent the calcium sensitivity (Ca_{50}, which can be converted into pCa50) and cooperativity (nHill). All force values can be converted to tension values after normalizing to the cross-sectional area. Besides myofilament Ca^{2+}-sensitivity and the length-dependent activation protocols, other tests can be performed. Such is the case of sarcomere length dependencies of T_{active}, $T_{passive}$ (**Figure 7**), and cardiomyocyte residual force. Residual force recordings are calculated from the initial force recovery (pCa 4.5) reached after the length change of the cell (80%) and normalized to each total steady-state force reached before length change[11]. Increase in residual force is usually indicative of cross-bridges with slow detachment kinetics and higher stiffness.

Finally, we should stress that this technique can be carried out in skinned cardiomyocytes extracted mechanically from frozen or freshly collected samples, as well as isolated enzymatically followed by the permeabilization of its membranes. The way the cardiomyocytes are isolated impacts significantly the results derived from this technique. **Figure 8** shows the differences

observed among the three isolation procedures.

FIGURE AND TABLE LEGENDS:

Figure 1: Integrated scheme of the testing apparatus. The testing apparatus includes the microscope, the micromanipulators and the associated computer. The bottom of the figure shows a skinned cardiomyocyte glued between the motor and the force transducer.

Figure 2: Flow chart of the protocol of cell isolation, permeabilization and gluing. The upper left corner image is composed of 4 images showing pieces of the heart sample in the RELAX-ISO solution (**A**) in a Petri dish, (**B**) in a tube used for mechanical homogenization of tissue, (**C**) the homogenizer, (**D**) the tissue immediately after homogenization and (**E**) when it is in a tube for Triton permeabilization.

Figure 3: Determination of length and sarcomere length of a skinned cardiomyocyte. Cell length and width determination at a sarcomere length of ≈2.2 µm.

Figure 4: Length-dependent activation protocol (mimics the Frank-starling mechanism in vitro). Representative force traces and parameters derived from myofilaments' Ca^{2+} sensitivity protocols performed before (**A**, 1.8 µm) and after stretching a cardiomyocyte up to 2.2 µm (**B**).

Figure 5: Myofilaments Ca^{2+}-sensitivity protocol. Representative force traces and derived parameters. For the sake of simplicity, only 3 out of 8 force curves are depicted. Namely a cardiomyocyte activated with the saturating, an intermediate and the lowest Ca^{2+}-containing solution (4.5, 5,6 and 6.0, respectively).

Figure 6: Representative traces from a mice cardiac cell activated at different calcium solutions and the respective ktr fit curve. (**A**) pCa 4.5; (**B**) pCa 5.0; (**C**) pCa 5.2; (**D**) pCa 5.4; (**E**) pCa 5.6; (**F**) pCa 6.0 and E values for total, passive and active tension, ktr value and Rsquare for ktr fit

Figure 7: Protocols of sarcomere length dependencies of $T_{passive}$ (A) and T_{active} (B). Passive tension and active tension were calculated in a single cardiomyocyte at a sarcomere length of 1.8 µm to 2.3 µm.

Figure 8: Representative results for cardiomyocytes mechanically isolated from fresh ("Fresh") and frozen myocardial samples ("Frozen") as well as from collagenase digested heart (modified Laggendorf thecnique) with posterior permeabilization with Triton ("Collag+Triton"). Values of (**A**) Total tension, (**B**) Active Tension and (**C**) Pasisve Tension from cardiomyocytes activated with pCa 4.5 solution at a sarcomere length of ≈2.2 µm. (**D**) Calcium sensitivity curve and the respective values for (**E**) pCa50 and (**F**) nHill. (**G**) Residual Force and (**H**) ktr values calculated at maximum activation solution (pCa 4.5).

Table 1: Instructions for stock solution preparation.

Table 2: Instructions for Relax-ISO solution preparation.

Table 3: Instructions for activating solution preparation.

Table 4: Instructions for relaxing solution preparation.

Table 5: Instructions for pCa solutions preparation.

Table 6: Typical parameters and indices derived from single permeabilized cardiomyocytes from rodents, pigs and humans. Adapted from[12].

Table 7: Troubleshooting table.

DISCUSSION:

In vitro assessment of cardiac function using skinned cardiomyocytes represents an important technique to clarify the modifications occurring at cardiomyocyte level in physiological (e.g., stretch) and pathological context (e.g., ischemia). This methodology has several advantages such as requiring a minimal amount of myocardium to assess function in cardiomyocytes obtained from defrosted samples; using cardiomyocytes from a wide range of species (mice[13], rat[1,14,15], rabbit[16], pig[17], dog[18], guinea pig[19] and human[20]) and different cardiac locations, including the atria, left and right ventricles or a specific region of the infarcted heart. Moreover, this technique allows delivering specific concentrations of Ca^{2+} and energy (ATP) while measuring the function of regulatory and contractile structures in their native configuration.

Despite the simplicity of this technique, there are some critical steps. It is essential to guarantee the quality of each step from the beginning, including sample collection. Myofilament proteins are susceptible to proteases[21]. Thus it is mandatory to store samples in liquid nitrogen immediately after its collection. Fresh samples, which were not previously frozen, will develop significantly higher forces, so it is not advisable to mix measurement done in fresh and frozen samples in the same protocol. The second most critical step is the cardiomyocytes' extraction. During this procedure, it is crucial to maintain the sample on ice most of the time. A protease inhibitor cocktail can be use to reduce the risk of protein degradation during the extraction/permeabilization[22]. Thirdly, samples should be cut in smaller pieces using precise scalpel movements since we noted reduced quality cardiomyocytes when this step was disregarded. Another critical step is washing the cardiomyocytes since it is difficult to have the right balance between washing out Triton (permeabilizes the cell but promotes its ungluing) and not losing too many cells in the supernatant. It is important to first try the extraction and number of washouts for each sample, species or protocol. For instance, in our hands, we noted that ZSF1 obese rat tissue extractions have a "fatty" aspect, which made these cells more slippery during the gluing but not more difficult to measure. The way we circumvent this problem was by performing more experiments to have a reasonable number of cells per animal. Moreover, it is crucial to select a good cell to glue, namely with good striation and reasonable length. If the cardiomyocyte does not have these features, it will mostly detach from the needle tips or develop no/low force. It is also important to use the correct glue for cardiomyocyte attachment, taking into account the time of gluing and its efficacy to glue the cell to the needle. In our hands, the

silicone glue (**Table of Materials**) cures fast (10-15 min) and strong enough. Finally, the last critical step is related with carefully lifting the cardiomyocyte 5 min after gluing the cell (to avoid gluing the cell to the coverslip) and before moving it to the wells (to avoid the cell to be dragged by the microscope stage). **Table 7** summarizes the troubleshooting associated with this technique, its underlying causes and possible solutions to overcome frequent problems.

The major limitation of this method is that it cannot answer all the questions related to the myofilament contractility, such as how fast the myofilaments activate/deactivate. In the in vivo setting, membrane depolarization, intracellular Ca^{2+} increase and its diffusion to myofilaments need to occur in order for the myocytes to contract, whereas in skinned cardiomyocytes Ca^{2+} diffusion to myofilaments occurs immediately when the cell is submerged in the Ca^{2+} solution. This faster rate of Ca^{2+} diffusion will bias myofilaments activation/deactivation analysis[23].

These experiments are influenced by different factors, including the temperature, solution pH, mechanical perturbation (slack-re-stretch vs. slack) and cell attachment procedures (pin tie vs. glue), all of these variables accounting for literature discrepancies in terms of ktr and the sarcomere length-dependent increase in force[4,12].

Future progress of the technique includes performing functional studies in intact rather than permeabilized cardiomyocytes. This technique has the disadvantage of relying on cardiomyocytes freshly isolated (not previously frozen). Another important issue not directly related to this methodology but that may significantly impact it is related to the maximal period of sample frozen storage. Specifically, it is mandatory to establish the degree of myofilament degradation throughout storage time (i.e., for how long can frozen samples be stored in order to assure good quality functional data derived from the extracted cardiomyocytes).

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DISCLOSURES:
The authors have no conflict of interest.

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Figure 1

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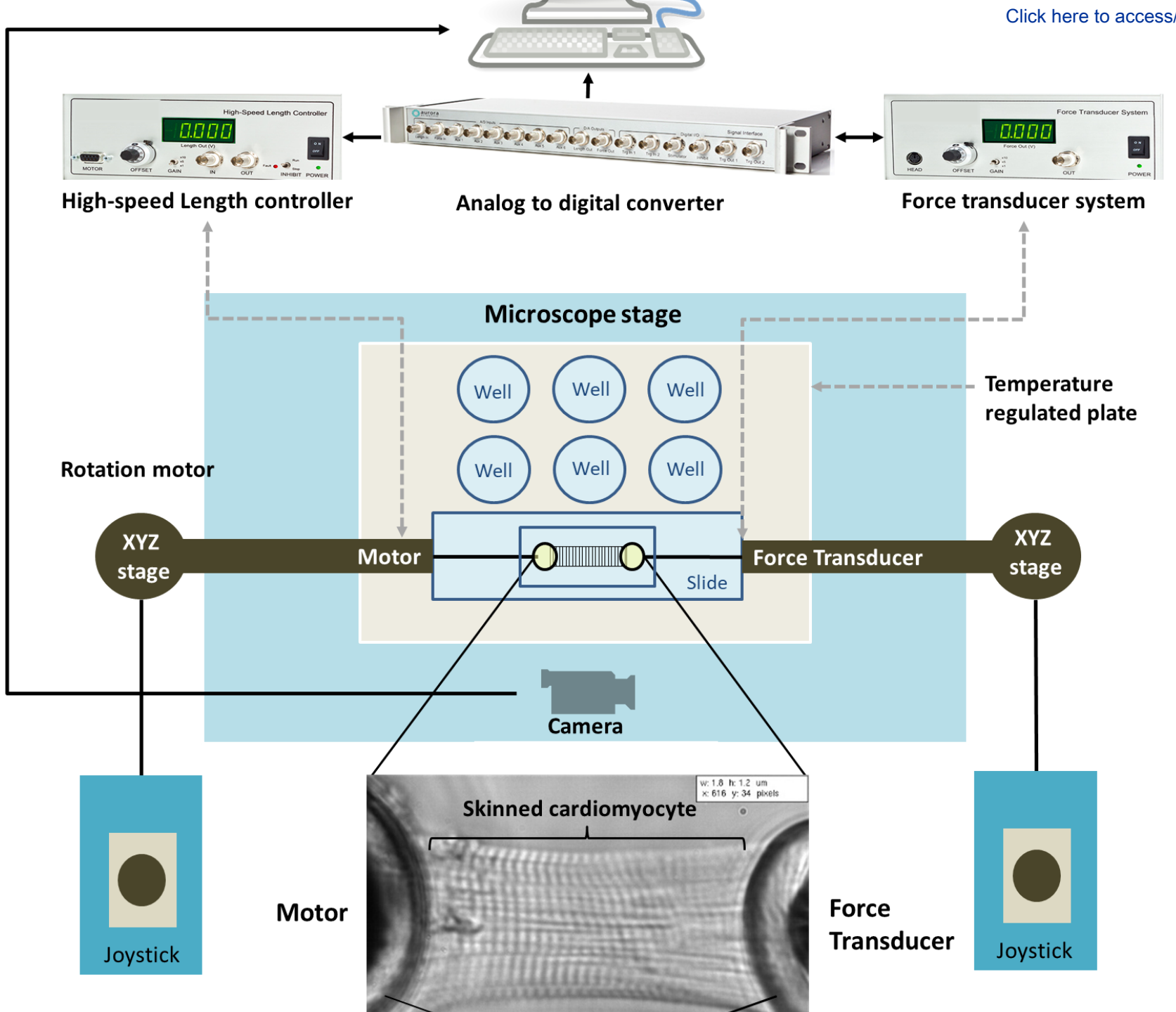
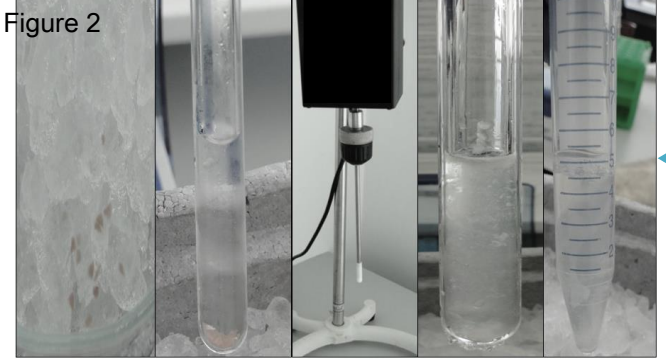
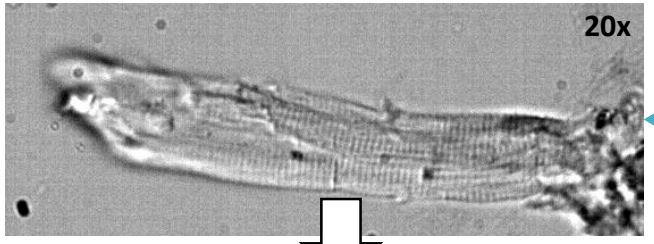
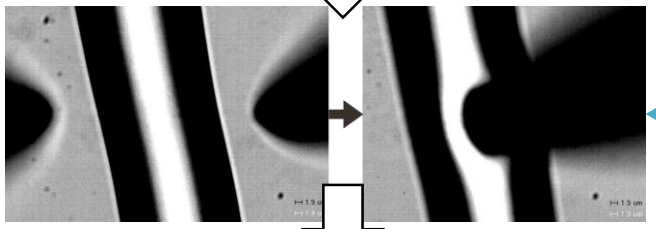
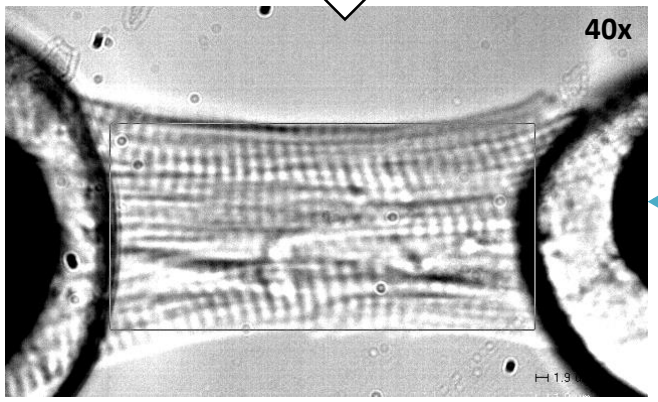


Figure 2









Defrost the biopsy/sample (3-5mg) in RELAX solution

Cut in small pieces and homogenate using the motor and pestle

Incubate at room temprature with triton 0.5% for 5’

Wash 4x the cell with RELAX

1. Fill the tube with RELAX Solution
2. Mix (up and down)
3. Centrifuge (348G, 4ºC for 1’)
4. Discart the supernatant

Select a cell with a good striation

Glue the cell ends to the motor and the transducer

Perform the selected protocol

- Active and passive force
- [Ca^{2+}] sensitivity
- Length-dependente activation
- Stretch

Figure 3

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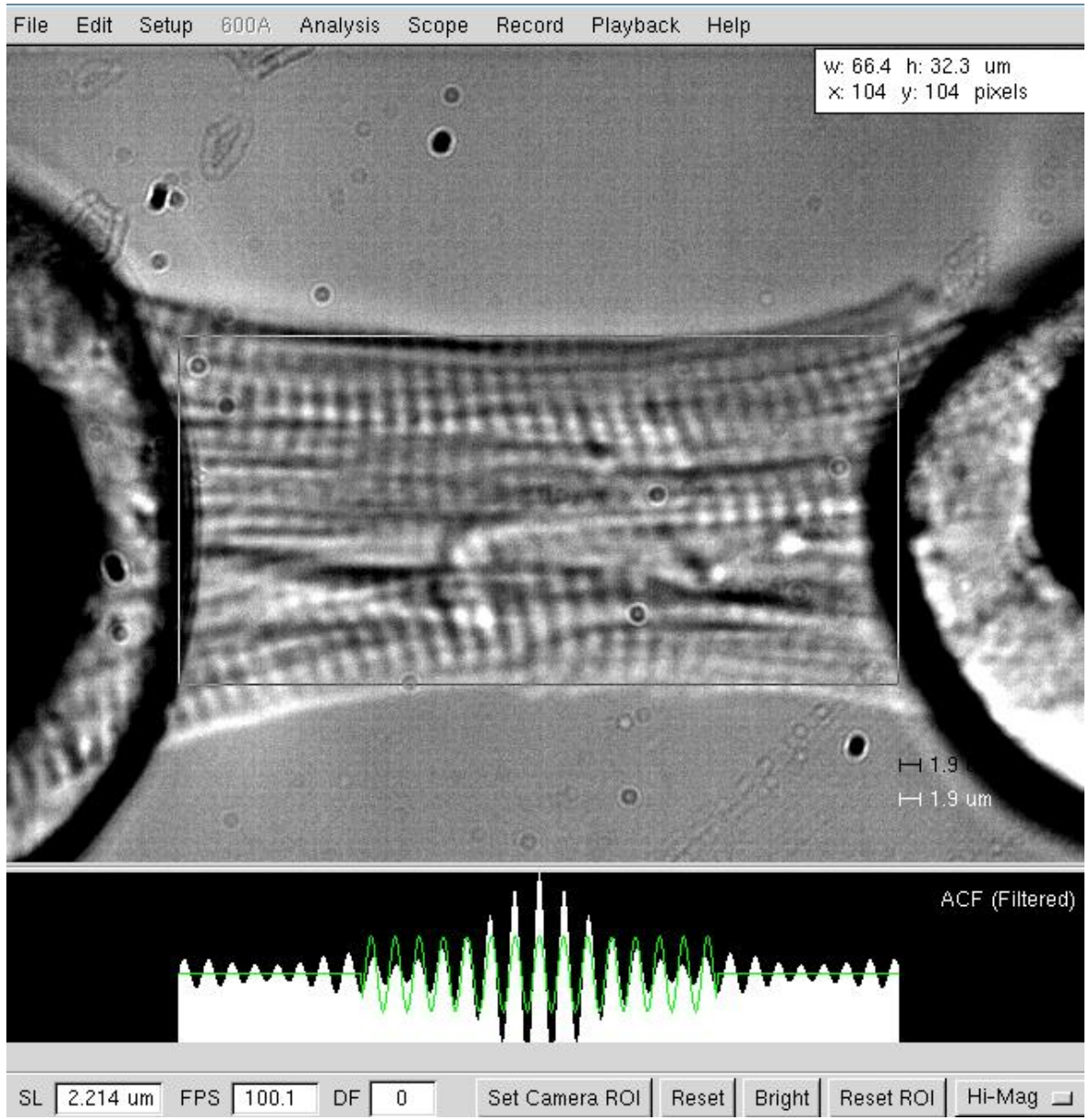


Figure 4

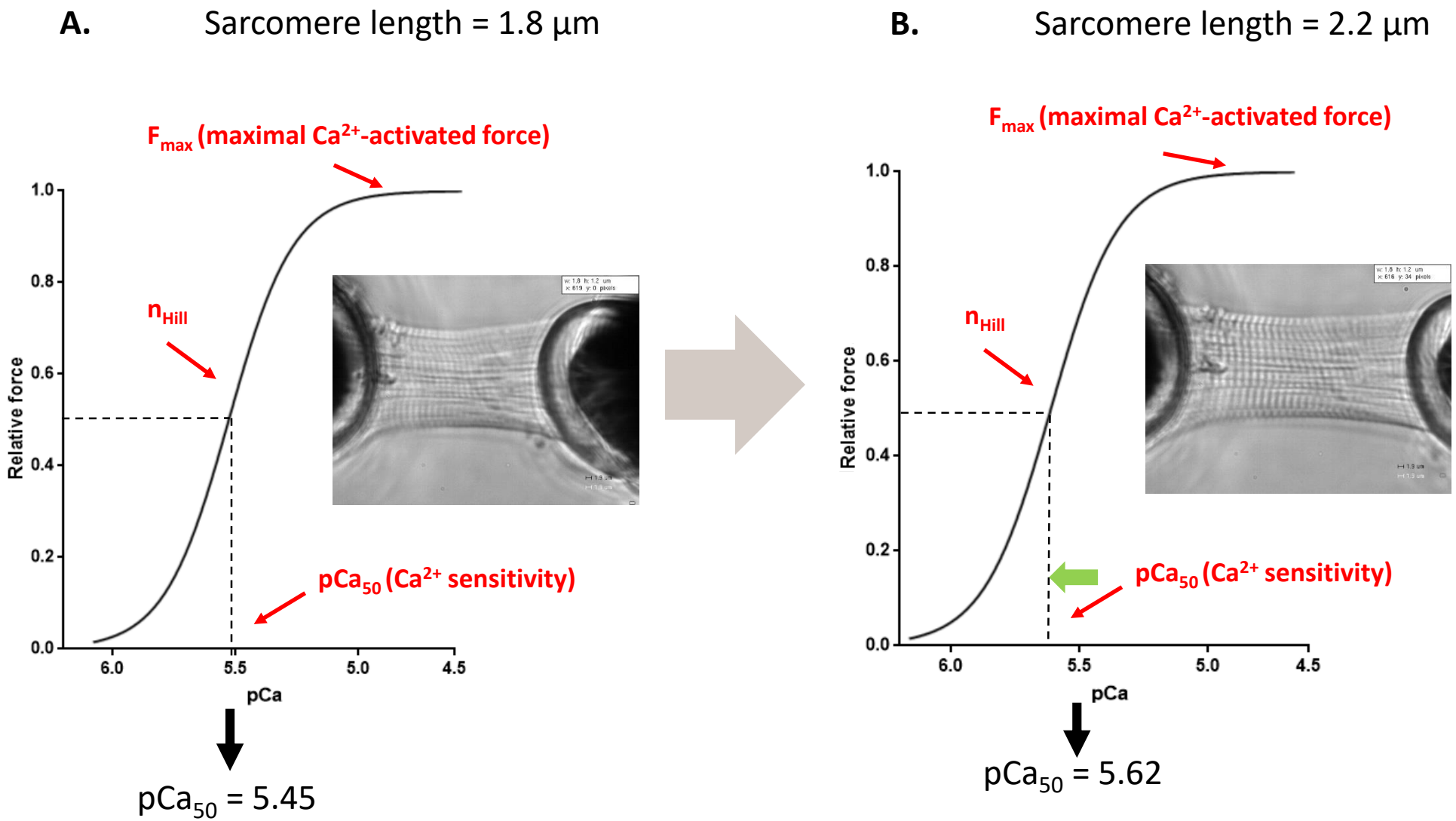
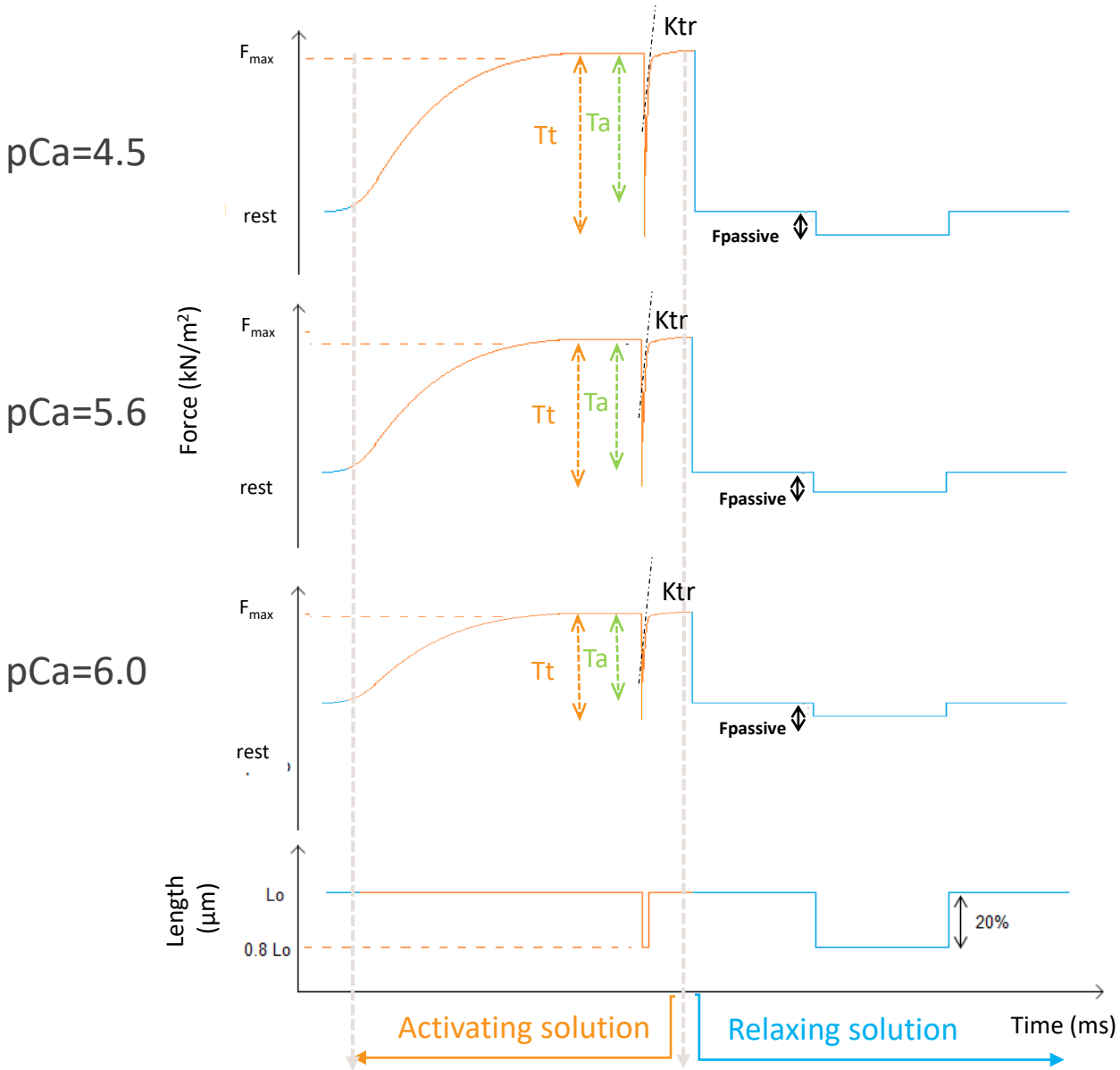


Figure 5



Move the stage so that the cell is transfered to a well containing activating solution

Move the stage so that the cell is transfered to a well containing relaxing solution

$F_{active} = F_{total} - F_{passive}$

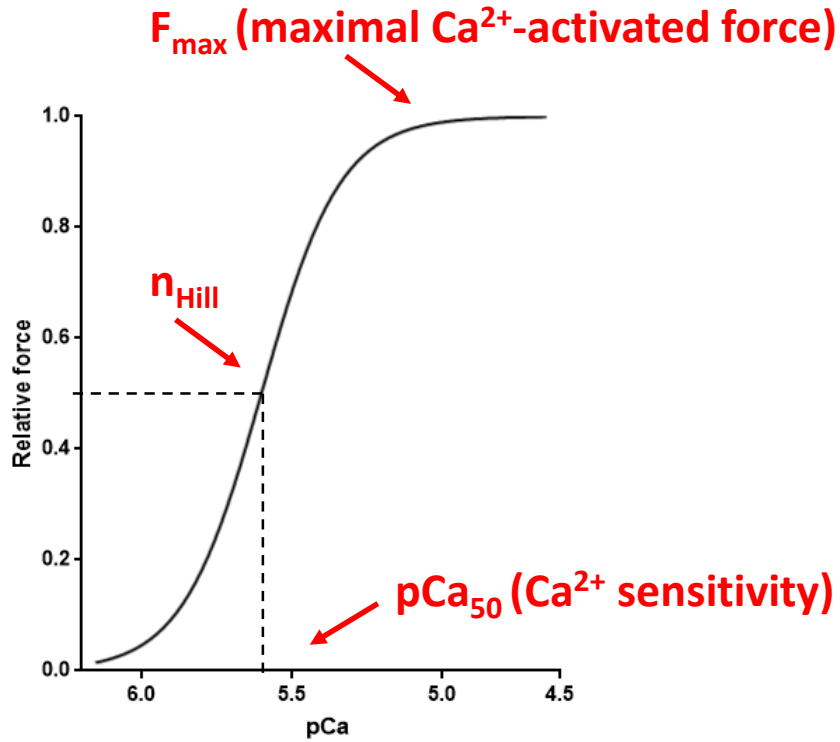
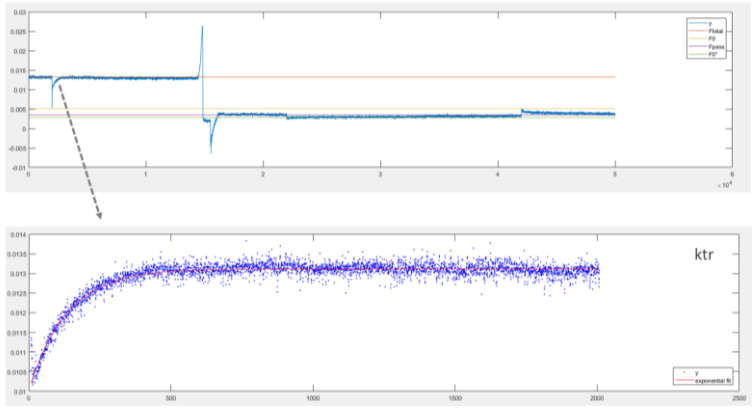
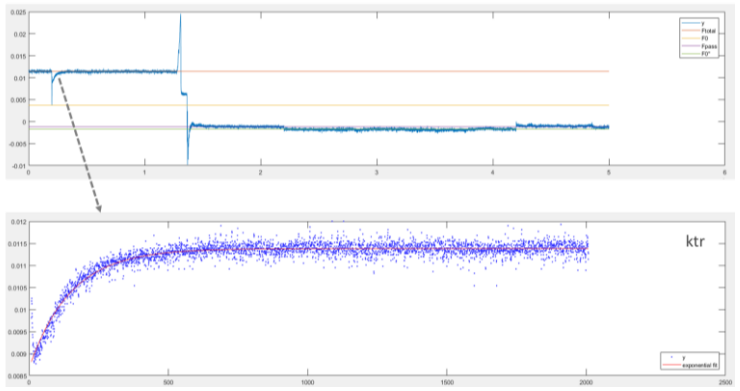


Figure 6

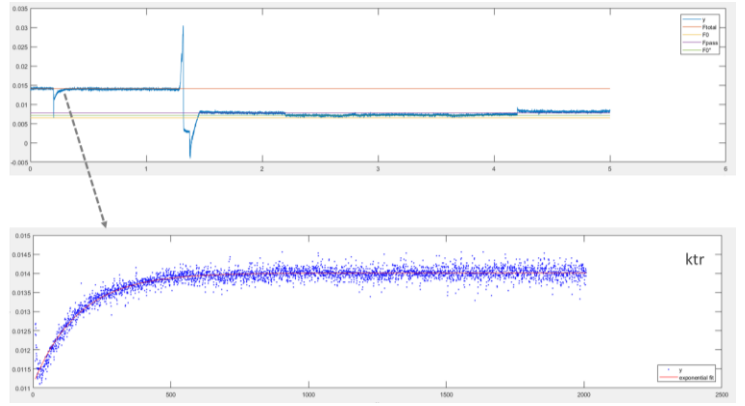
A. pCa 4.5



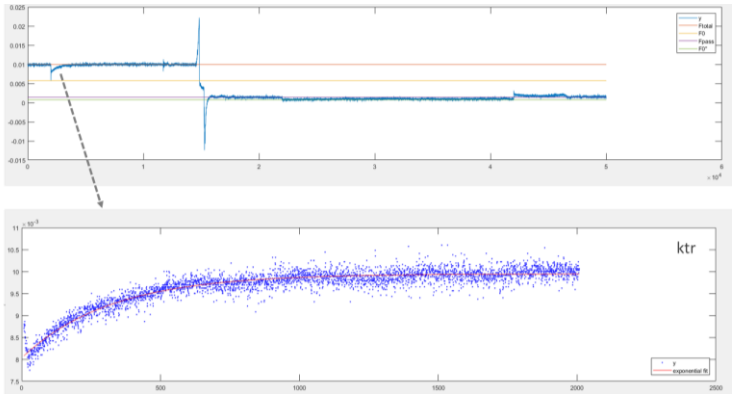
B. pCa 5.0



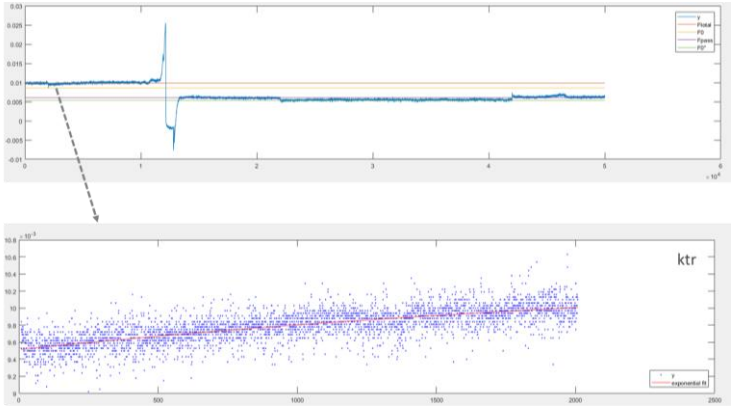
C. pCa 5.2



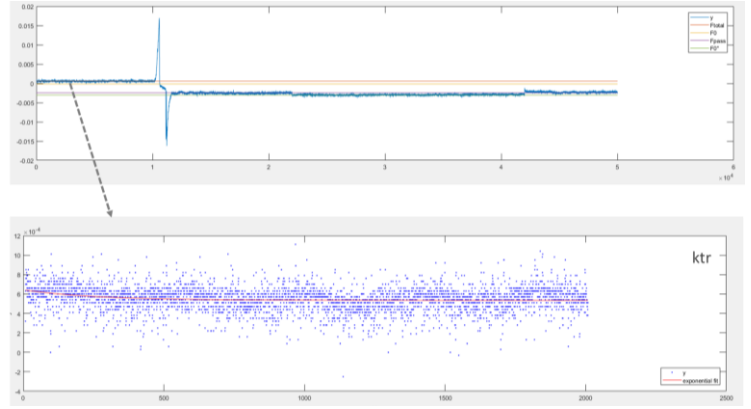
D. pCa 5.4



E. pCa 5.6



F. pCa 6.0



E.

pCa	TtN	TpN	TaN	ktr	Rsquare (ktr)
4.5	37.04	3.17	33.87	7.51	0.92
5	35.06	2.43	32.62	6.74	0.91
5.2	34.72	2.98	31.74	6.09	0.92
5.4	19.26	3.00	16.25	3.13	0.89
5.6	5.74	2.99	2.75	0.35	0.52
6	3.33	2.57	0.76	3.96	0.03

Figure 7

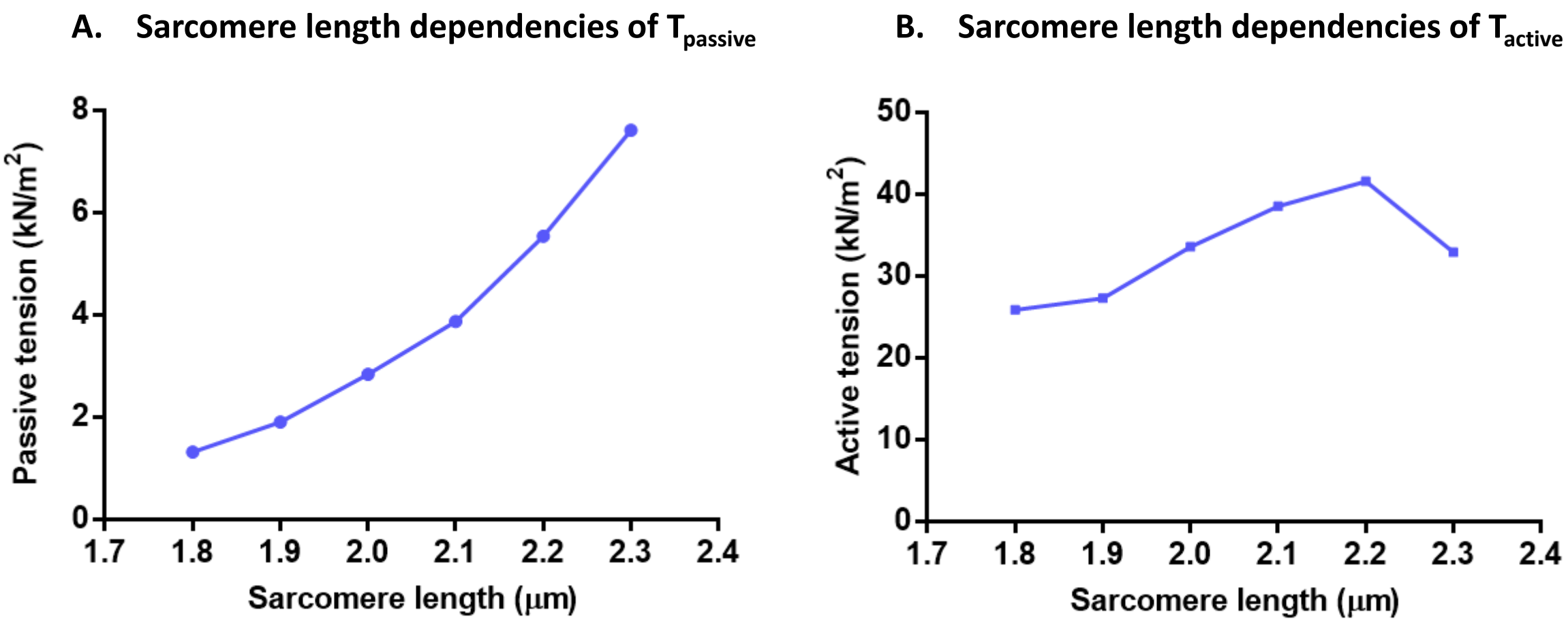


Figure 8

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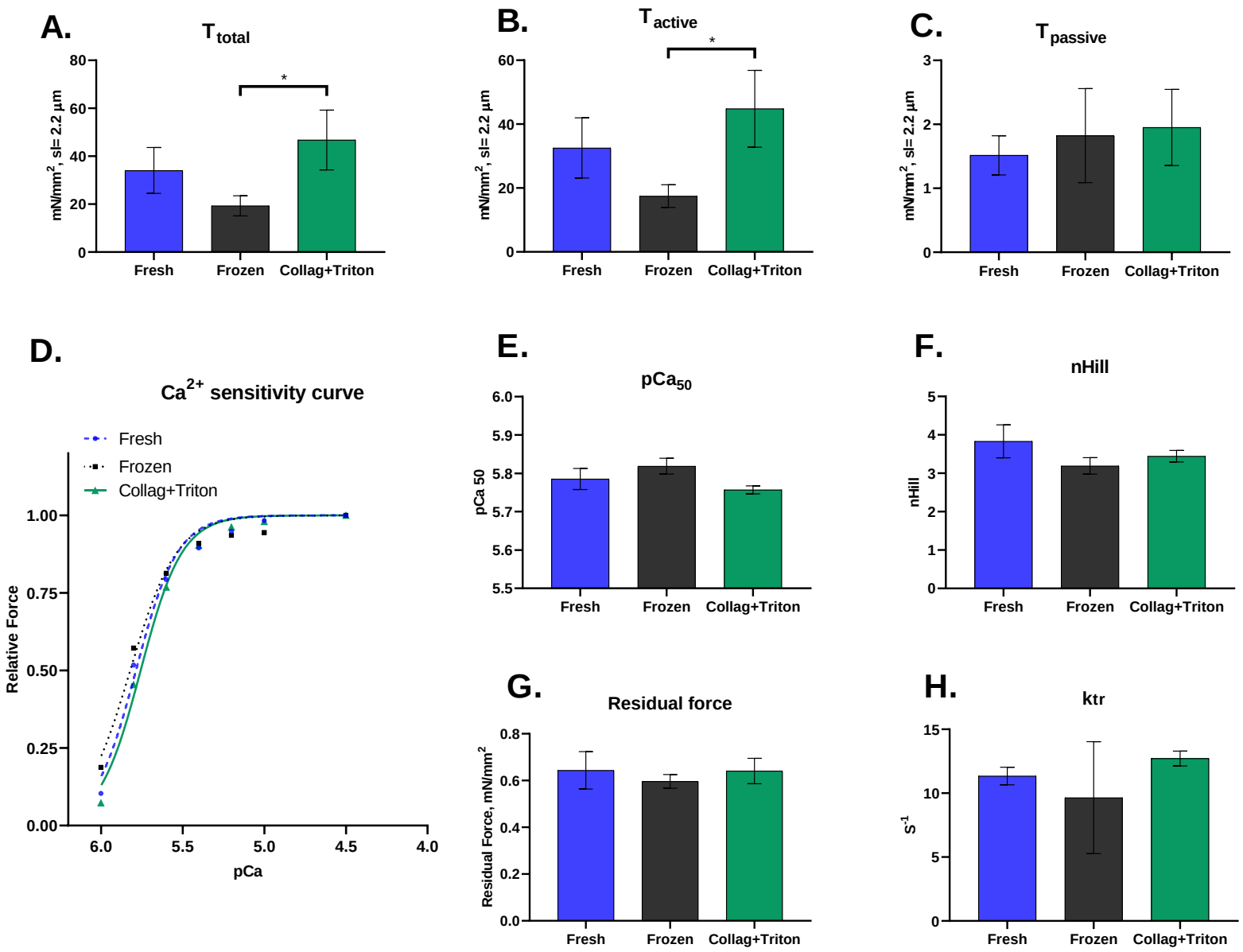

Table 1

Store at	Stock solutions	[M]	Final volume (mL)	Weight/ volume
4°C	Potassium hydroxide (KOH)	1	100	5.611 g
4°C	Potassium hydroxide (KOH)	5	50	14.03 g
4°C	BES	1	50	10.66 g
4°C	Propionic acid	1	100	7.483 mL
4°C	CaEGTA composed of:	0.1	100	
	- $CaCO_3$	0.1		1.001 g
	- EGTA	0.1		3.804

Notes
To adjust pH
To adjust pH
Adjust the pH to 7.0 with 5M or 1M KOH
Mix and heat the solution to 60°C for more than 1 hour. Adjust the pH to 5-6 with 1M KOH.

Table 2

RELAX-ISO (for cardiomyocytes' isolation)	**[mM]**	**Weight**
Na_2ATP	5.95	3.28 g
$MgCl_2.6H_2O$	6.04	1.23 g
Tritiplex (EGTA)	2	0.76 g
KCl	139.6	10.41 g
Imidazole	10	0.68 g

Table 3

Activating solution (for the measurements)	**[mM]**	**Weight / volume**
Na_2ATP	5.97	0.823 g
MgCl 1M	6.28	1.57 mL
Propionic acid	40.64	10.16 mL
BES	100	25 mL
CaEGTA (stock solution previously prepared)	7	17.5 mL
Na_2PCr	14.5	0.925 g

Table 4

Relaxing solution (for the measurements)	**[mM]**	**Weight / volume**
Na_2ATP	5.89	0.325 g
MgCl 1M	6.48	0.65 mL
Propionic acid	40.76	4.08 mL
BES	100	10 mL
EGTA	6.97	0.265 g
Na_2PCr	14.5	0.370 g

Table 5

pCa = -Log [Ca^{2+}]	Relaxing (pCa=9.0) mL	Ativating (pCa=4.5) mL
5	0.86	39.14
5.1	1.2	38.80
5.2	1.54	38.46
5.3	2	38.00
5.4	2.51	37.49
5.5	3.14	36.86
5.6	3.89	36.11
5.7	4.8	35.20
5.8	5.89	34.11
5.9	7.14	32.86
6	8.57	31.43

Table 6

Parameter	Rodent	Pig	Human
Active tension, $kN.m^{-2}$ (at 2.2 µm)	17 – 28	19 – 40	19 – 36
Passive tension, $kN.m^{-2}$ (at 2.2 µm)	3.6 – 5.5	1.9 – 6.8	1.8 – 2.3
pCa50	5.58 – 5.64	5.40 – 5.50	5.43 – 5.82
nHill	2.60 – 2.76	2.95 – 3.36	2.99 – 3.10
ktr, s^{-1}	4.00 – 8.00	1.00 – 3.00	0.90 – 2.00

Table 7 Click here to access/download;Table;Table 7.xlsx

Problem	Possible reason
The cardiomyocyte detaches during maximal activation	sufficient gluing time; The glue is old and has dri
	There is Triton® in the cell suspension solution, which can no longer be removed
The cardiomyocyte has low force under control conditions	The extraction went wrong and delivered low-quality cells
acting but no force is recorded; The cell ha	The force transducer is off
	The force transducer is not well calibrated
	The force transducer needle is loose
The striation pattern is not good enough to determine the sarcomere length	Insufficient light
	The extraction went wrong and delivered low-quality cells
	Needles' tips are not in the same plane
No length and/or force variation during acquisition	The motor or the force transducer are off
	The motor is broken and not producing cell shortening
Too much noise on the acquisition recordings	Too much air flow around the equipment
	Too many vibrations around the equipment

Ca^{2+}-sensitivity curve has strange values and the force values do not increase with $[Ca^{2+}]$.	The mixture of activating and relaxing solution was not done properly (check 3.10 to 3.14 of the methods section, possibly due to insufficient mixing)

Solution
Increase the time of the gluing step
Repeat the extraction procedure with one or two additional Triton® wash out steps
Increase the sample size and do a new extraction. If the problem persists is probably due to improper sample collection - discard this sample
Turn it on
Calibrate the force transducer using a set of known weights (check the manufacturer's instruction manual).
Glue the needle again using crystal bond 509 or jewelers wax.
Increase microscope light or move the cell back to the coverslip and assess sarcomere length again (the wells has lower light intensity)
Increase the sample size and do a new extraction
Using micromanipulators, adjust the needles' tips up or down until finding a focused sarcomeres
Turn them on
Replace it or try to calibrate it using a function generator
Protect the equipment from the direct air flow
A stabilization table is advisable. Even then, it is recommended to remove any equipment that might have a compressor or emit vibrations (freezer, fridges)

Defrost the vials with the same concentration, collect all vial's content in the same beaker, mix with a stirrer and divide them again. Test these solutions again in a new cell. If this does solve the problem, prepare a new batch of Ca^{2+} containing solutions

Name of Material/Equipment	Company	Catalog Number	Comments/Description
Acetone	Sigma	34580	
Adenosine 5’-triphosphate disodium salt hydrate (Na2ATP)	Sigma	A2383	
Calcium carbonate (CaCO3)	Merck	1.02067.0500	
Imidazole	VWR	24720.157	
Magnesium chloride hexahydrate (MgCl2.6H2O)	Merck	1.05833.0250	
Magnesium chloride solution (MgCl2 1M)	Sigma	M1028	
N,N-Bis(2-hydroxyethyl)taurine (BES)	Sigma	B9879	
Phosphocreatine dissodium salt hydrate (Na2PCr)	Sigma	P7936	
Potassium chloride (KCl)	Merck	1.04936.1000	
Potassium hydroxide (KOH)	Merck	8.14353.1000	
Propionic acid (C3H6O2)	Merck	8.00605.0500	
Silicone Squeeze Tube	Marineland	31003	
Tritiplex (EGTA)	Merck	1.08435.0025	
Triton® X-100 10%	Merck	648463	
Tissue homogeneizer (GKH GT Motor Control)	Terre Haute Glas-col		
Length Controller (Model 315C-I)	Aurora Scientific		
Force Transducer (Model 403 A)	Aurora Scientific		
Software ASI 600A	Aurora Scientific		
Sotware VSL (Model 900B)	Aurora Scientific		
Inverted Microscope (IX51)	Olympus		

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CORRESPONDING AUTHOR

Name:	INÊS FALCÃO-PIRES
Department:	SURGERY AND PHYSIOLOGY
Institution:	FACULTY OF MEDICINE, UNIVERSIDADE DO PORTO
Title:	PhD.
Signature:	InesfalcaoPires
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Dear editor:

Please find enclosed the revised version of the manuscript entitled “IN VITRO ASSESSMENT OF CARDIAC FUNCTION USING SKINNED CARDIOMYOCYTES” by Patrícia Goncalves-Rodrigues, João Almeida-Coelho, Alexandre Gonçalves; Flávio Amorim, Adelino F Leite-Moreira, Ger Stienen and Inês Falcão-Pires.

We thank the reviewers for their thorough and detailed analysis of our work, which helped us to improve the manuscript. All the points raised by the reviewers were taken into account, and the manuscript was corrected accordingly ("track changes” in red). Furthermore, we include below a reply where we outline, point by point, each change made as raised in the editor or reviewers’ comments. We sincerely hope that the revised version will comply with their suggestions.

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We asked a native English speaker to revise the entire manuscript, and we hope this version is significantly improved.

2. **Unfortunately, there are a few sections of the manuscript that show significant overlap with previously published work. Though there may be a limited number of ways to describe a technique, please use original language throughout the manuscript. Please check the iThenticateReport attached to this email.**

We have carefully looked to the iThenticateReport attached and revised 2 main sections of the text:

1. *The first, in the introduction, is based on a book chapter that 2 authors have previously written. We restructured the text and included this reference, which, by mistake, was not in the version of the paper previously submitted (Falcão-Pires et al., In vitro Experimental Assessment of Cardiac Function in Cokkinos, Introduction to Translational Cardiovascular Research).*
2. *The second section, a sentence in the introduction and another in the discussion, was based on a JOVE paper, similar to our but in skeletal muscle, which we already cited in the manuscript but we forgot to cite it again in these two sections (Roche et al., JOVE, 2015). Both sections were restructured to use original language.*
3. *The other sections highlighted in iThenticateReport refer to acknowledgments, authors’ affiliations and ethics which appear in many papers of our laboratory. We obviously did not change it.*

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There are no figures from previous publications.

4. Please remove the embedded Table from the manuscript. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xlsx file.

We removed all the table from the manuscript and created .xls files for each of them. We also changed the text accordingly.

5. Please use 12 pt font and single-spaced text throughout the manuscript.

We changed this accordingly.

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We removed all commercial language from the manuscript.

7. Please remove all footnotes and as notes between steps.

Accordingly, we replaced footnotes by Notes next to the corresponding step.

8. There is a 2.75 page limit for filmable content. Please highlight 2.75 pages or less of the Protocol steps (including headings and spacing) in yellow that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

We have highlighted in yellow the filmable content identifying the essential steps of the protocol for the video.

9. Each figure must be accompanied by a title and a description after the Representative Results of the manuscript text.

We are not totally sure what the editor means with this sentence. We changed accordingly to what we understood. Please confirm if the new version is as desired.

10. Please do not abbreviate journal titles for references.

We used JOVE template for EndNote to produce the list of references.

11. Please sort the items in alphabetical order according to the name of material/equipment.

We changed the material table accordingly.

12. Please use h, min, s for time units.

We changed this in the entire manuscript.

13. Please convert centrifuge speeds to centrifugal force (x g) instead of revolutions per minute (rpm).

We changed this throughout the manuscript, although we do not think we should change rpm to g in step 6.6 as we are referring to rotation and not centrifugation, therefore g is not appropriate for this step of the protocol.

Reviewers' comments:

Reviewer #1:

The manuscript submitted by Gonçalves-Rodrigues and colleagues aims to provide the methodological steps required to measure the force and rate of tension redevelopment from skinned single cardiomyocytes. With this respect they provide details on the preparation of high quality, permeabilized cardiomyocytes, the preparation of required solutions, attachment of cardiomyocytes, and protocol for the measuring the mechanical properties of the cardiomyocytes. The premise of the article is excellent, and will provide an excellent resource for those wanting to move into myocardial mechanics. The manuscript is generally well written, but would benefit from additional editing. There are many cases where the tense of plurality changes within a sentence. Specific instances have been pointed out, but is not an exhaustive lit. There are a few additional suggestions to improve the quality and clarity to the reader.

We thank the reviewer for the valuable remarks.

It may be beneficial to the readers to include a section that describes how to calibrate the force transducer to ensure accurate force measurements.

We added a new section (4) where we described this procedure although it can be highly specific depending on the commercial system each lab has (line 178).

It should be stressed to the readers that may not be familiar to the equipment the sensitivity of the force transducer, and the care that should be taken not to break it.

A note was included in the manuscript where this is addressed (line 180)

Abstract: The first sentence is very long and difficult to follow. Please try to break this into at least two sentences.

We agree with the reviewer and the sentence has been rewritten to allow better comprehension.

Table 1: The company name is presumably meant to be Merck?

Yes, the reviewer is correct. We replace “Merk” by “Merck”.

Line 66: Please change "assessing sarcomere length" to "assessment of ... "

We changed this (line 47).

In the stock solutions table, please change "CaEGTA composed by" to "composed of".

We changed this in table 1.

The ionic strength for all experimental solutions should be disclosed.

The ionic strength of the experimental solutions amounted to 180 mM. This information was included in section 3 of the manuscript (line 154).

It would be helpful to describe how the proportions of solutions presented in the mixing table were calculated, so that readers may calculate other pCa solutions if needed. If done

using Fabiato and Fabiato computer program, please disclose this and consider including additional pCa solutions.

The compositions of the solutions were calculated by means of a computer programme similar to that of Fabiato and Fabiato (1). This programme is available on request. Unfortunately, it runs under old Windows operating systems (Windows XP and below). The solutions may also be calculated by means of the MaxChelator programme (2), which yields similar results. The proportions for additional pCa solutions were added to Table 5.

It would be of benefit to the reader to suggest the optimal type of glue used for cardiomyocyte attachment. For example, do the authors use UV sensitive glue?

We added this information in the discussion section (line 643-645). We did not use UV sensitive glue in our experiments but a fast-cure silicon glue.

Equipment is misspelled. Change "assumes" to "assume". Change "detached" to "detach".

We corrected this.

It would benefit readers to describe here how you circumvented the issues of cardiomyocyte attachment with the fatty ZSF1 cardiomyocytes.

Although we anticipated some problems with cardiomyocytes extraction from fatty samples, this ended up not to be a major issue. We had extracted and measured over 60 samples from obese animals (including ZSF1 obese) and we did not detect any major differences from other strains extractions, especially since we recently changed to stronger silicon glue that is able to maintain cells glued even with very high maximal forces. We added a remark in line 639. Changing to this glue, improved significantly the efficiency of skinned myocytes' measurements working day.

Throughout the manuscript, the authors often use the term "myofilamentary", which is rarely if ever used in the field. "Myofilamentary" is easily exchanged with "myofilament" and would be much simpler for readers.

Thank you. We replaced "myofilamentary" to "myofilament" throughout the manuscript.

Figure 5: Would it be possible to show an inset of the ktr for each calcium concentration? This would provide an excellent visual representation of the relationship between calcium and the rate of tension redevelopment.

We added a new figure (Figure 6) to show ktr curves and values for some of the calcium solutions (line 582).

Reviewer #2:

The manuscript describes the steps needed to isolate, attach, and measure mechanical properties of single skinned cardiac myocytes. The preparation is invaluable toward understanding myofilament function, how it translates to in vivo parameters, and the basis for sarcomere based therapies. The description in thorough and favorable for improved reproducibility.

We thank the reviewer for the valuable remarks.

Major Concerns:

The only major concern is data presented in Table 1. The ktr values for rodent preparations are similar to pig and human preparations and much lower (an order of magnitude) than previous reports using skinned mouse/rat cardiac myocytes. The raises issues related to variation between sample preparation (e.g., frozen vs fresh tissue), methodology for mechanical perturbation (slack-re-stretch vs slack), differences in attachment procedures (trough and pin tie in vs glue). These issues should be discussed. In addition, force redevelopment trace(s) (on short time base) should be provided.

After reading the reviewer comment we realized that ktr magnitude values in table 1 were incorrect. We corrected the values in table 6 in the new version of the manuscript.

The differences between frozen vs fresh tissue was addressed in line 627-629 and a new figure was added (Figure 8, line 590-596).

Issues related to methodology for mechanical perturbation and cell attachment procedures were addressed in line 627. This is such a relevant issue that we decided to include a new figure in the manuscript representative of this information (Figure 8).

We decided to include a new figure (figure 6, line 585) with representative traces of a protocol of Ca^{2+} sensitivity (pCa: 4.5, 5.0, 5.2, 5.4, 5.6 and 6.0). These traces are from mice cardiomyocytes. At lower calcium concentration solutions, the ktr fit was not good (Rsquare of fit <0.90) and for that reason, we do not use these values (line 501).

Minor Concerns:

Page 7, line 222. Why is numerator divided by 1000? Perhaps units should be shown.

The numerator is divided by 1000 to have N/m^2 (our software provides force values in mN).

Page 9, line 294. Consider providing force trace in short time frame to show force redevelopment time course. Is residual force (force at the onset of force development) and its variance quantified? What is the impact of residual force? How does residual force affect interpretation of data?

Residual force is beyond the scope of this manuscript. Nevertheless, a new phrase was added in "Results" section were residual force is mentioned as another possible parameter derived from this technique (line 547).

We did notice that there was a mistake in the ktr values presented in table of representative results. We correct this table based in values from our laboratory and other studies (3).

Page 9, line 307. What should data be excluded in active force drops below 70% of initial? Could this exclusion criteria affect interpretation and conclusions related to mechanics and effects of disease, drugs, etc?

Maybe we were not totally clear - this is just a quality-control check and relates to the run-down during the experiment, and not to intrinsic low force development in myocytes from diseased hearts or specific drug-effects. For instance, each experimental protocol to assess Ca^{2+} sensitivity begins and ends with a control activation at saturating Ca^{2+} concentration (pCa of 4.5). By comparing the final to the initial value, its rundown should not be higher than 30% regarding the initial active force (F_{max}). Instead, the final value should be at least 70% of the maximum force of the first contraction. Otherwise, the measurement/cell should be excluded from the analysis, meaning that the myofilaments lost function or that the cell unglued from the transducer or motor. We included this information in the manuscript as a note (line 480-485).

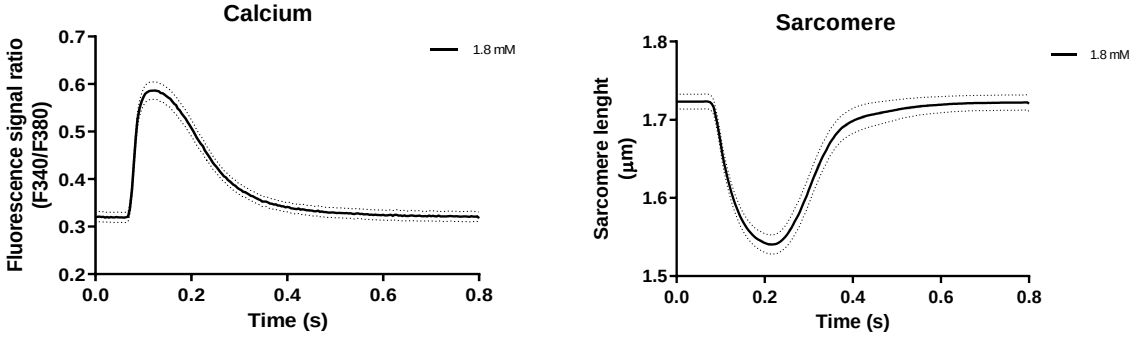
Page 12, line 374. Please clarify what is meant by "the diffusion distances are less than 2um".

We agree with the reviewer and this sentence is confusing and not adding any useful information, so we decided to remove it from the manuscript.

Page 12, lines 396-401. Can estimates be provided that compare calcium activation times in an intact myoyctes (via EC coupling) vs. calcium diffusion in a averaged sized skinned cardiac myocyte preparation?

Intact cardiomyocytes are usually studied at 37ºC and 1.8 mM of Ca2+. Peak Ca2+ under these conditions amounts to approximately 1 µM.To address your question, we performed experiments in intact and skinned cardimyocytes in conditions as similar as possible (20ºC for both preparations; 1.0 µM for skinned versus 1.8 mM of Ca^{2+} for intact).

Myofilaments activation time (10 – 90% of Fmax) in skinned cardiomyocytes at 20ºC with 1.0 µM of Ca2+ ≈ 5.5 sec. However, this activation time is mainly limited by the Ca-EGTA binding kinetics and Ca-EGTA equilibration inside the skinned fibres (4). Ktr at 20ºC provides a more appropriate measure for the activation time because Stehle R et al demonstrated that at a given Ca2+-activated steady-state force, the kinetics of force development by Ca2+ and after a rapid release and restretch are the same (k ACT ≈ k TR) (5). In our experiments 20 oC, a Ktr value of 11.7 s-1 was found. This would correspond to a 10 – 90% rise time of (ln0.9-ln0.1)/ktr, i.e. 0.19 s. As can be seen from the figure below, myofilaments activation time in intact cardiomyocytes at 20ºC with 1.8 mM amounted to Ca2+ ≈ 0.14 sec. This value is in fair agreement with the value derived from the ktr measurements. However, we consider discussion of this topic beyond the scope of the current manuscript and therefore prefer not to include these results.



Please check typos: page 8, line 261 "ecquipment"; page 12, line 371 "assumes"; page 12 line 389, remove "etc"; *We corrected these typos.*

Reviewer #3:

The protocol reviews the techniques involved in assessing contractile properties of single skinned (permeabilized) cardiomyocytes, including stiffness (passive force), maximal tension generation, calcium sensitivity (force-pCa curves), cooperativity, and rate of force redevelopment (ktr). This in vitro assessment of cardiomyocyte contractility can be performed in appropriately preserved frozen tissue samples of minimal size in order to further characterize varying cardiac disease phenotypes and assess the effects of potential therapeutic interventions targeting sarcomeric proteins.

The title and abstract are appropriate and the authors included relevant and appropriate applications of the protocol. Critical steps are appropriately highlighted and the inclusion of the troubleshooting table is helpful.

We thank the reviewer for the valuable remarks.

Major Concerns:

From just reading the manuscript, it is difficult to visualize what kind of data one obtains from each protocol - for example, it is not obvious what kind of data is being generated when you "Start Sequence". Consequently, the data analysis portion (10) is difficult to understand. Perhaps including a sample analysis in the video will be helpful.

We changed section 8 (line 308-391) to became easier to understand to which protocol we are refering. Regarding the section 11 (423-501), this analysis is usually done after the practical experiment is concluded.

The purpose of the software protocols referenced in 7.7 and Figure 1 of the supplementary file should be clarified (i.e. what is the purpose of shortening the cell to 80% of the initial length?).

We added this information in the note immediately after the step 8.7 (line 271). Figure 1 of the supplementary file intended to show the parameters behind a basic test and sequence of tests that facilitate data acquisition. These parameters can be used as a model to input in other software's. But as the name says, it is supplementary, and we can remove it if the reviewer prefers.

Hardware (tissue homogenizer, length controller, force transducer, etc.) and software manufacturer/model numbers should be included.

This information was added to material/equipment table.

Addition of protease inhibitors have been described during cardiomyocyte extraction/permeabilization in order to prevent degradation.

That is correct. However, in our experiments we usually use cells extracted in the same day of the experiments and maintain the solution always on ice, to slow down protein degradation.Neverthless, protease inhibitors can be use to reduce the risk of protein degradation during extraction/permeabilization (6).

Minor Concerns:

The measurement of cardiomyocyte depth using the prism mirror (7.4) is not described in detail, however perhaps this is an aspect that can better be described by video rather than in words.

We do have a mirror in our laboratory but lack a powerful external light source, which is required to visualize the cell. We are currently assuming the cell depth is 70% of cell width. We are now in the process of implement the use of the mirror, and if we have sufficient light intensity at the time of the filming we will definitely describe this procedure in the video. For now we mention the 70 % approximation assumption in the text (line 326).

It is not clear what is being demonstrated in the first picture of Figure 2 (upper left corner).

We detailed what is in the picture in the figure legend (line 560).

Would be helpful to include a labeled picture of the hardware components along with the schematic illustrated in Figure 1.

We believe that additional labelling is not required; all the components were already labelled in figure 1.

The representative data included in the Appendix is helpful, however the use of the custom program (Cellprog) for data analysis is confusing since that is not what most users will have access to.

Currently, there is not any good program to extract data semi-automatically and therefore, we developed one that can help us on this task. As for us, this program is such an important part of the data analysis we found we should include it. Still, we agree with the reviewer and this is the reason why it was included in the appendix.

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6. Woulfe KC, Ferrara C, Pioner JM, Mahaffey JH, Coppini R, Scellini B, et al. A Novel Method of Isolating Myofibrils From Primary Cardiomyocyte Culture Suitable for Myofibril Mechanical Study. Frontiers in cardiovascular medicine. 2019;6:12.