Journal of Visualized Experiments

Isolating Myofibrils from Skeletal Muscle Biopsies and Determining Contractile Function with a nano-Newton resolution Force Transducer

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE61002R2
Full Title:	Isolating Myofibrils from Skeletal Muscle Biopsies and Determining Contractile Function with a nano-Newton resolution Force Transducer
Section/Category:	JoVE Biology
Keywords:	skeletal muscle; Sarcomere Kinetics; Myofibril Mechanics; Contractility; Calcium; Cantilever nano-Newton Force Probe
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Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Amsterdam, Noord-Holland, the Netherlands

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TITLE:

Isolating Myofibrils from Skeletal Muscle Biopsies and Determining Contractile Function with a Nano-Newton Resolution Force Transducer

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KEYWORDS:

skeletal muscle, sarcomere kinetics, myofibril mechanics, contractility, calcium, cantilever nano-Newton force probe

SUMMARY:

Presented here is a protocol to assess the contractile properties of striated muscle myofibrils with nano-Newton resolution. The protocol employs a setup with an interferometry-based, optical force probe. This setup generates data with a high signal-to-noise ratio and enables the assessment of the contractile kinetics of myofibrils.

ABSTRACT:

Striated muscle cells are indispensable for the activity of humans and animals. Single muscle fibers are comprised of myofibrils, which consist of serially linked sarcomeres, the smallest contractile units in muscle. Sarcomeric dysfunction contributes to muscle weakness in patients with mutations in genes encoding for sarcomeric proteins. The study of myofibril mechanics allows for the assessment of actin-myosin interactions without potential confounding effects of damaged, adjacent myofibrils when measuring the contractility of single muscle fibers. Ultrastructural damage and misalignment of myofibrils might contribute to impaired contractility. If structural damage is present in the myofibrils, they likely break during the isolation procedure or during the experiment. Furthermore, studies in myofibrils provide the assessment of actin-myosin interactions in the presence of the geometrical constraints of the sarcomeres. For instance, measurements in myofibrils can elucidate whether myofibrillar dysfunction is the primary effect of a mutation in a sarcomeric protein. In addition, perfusion

with calcium solutions or compounds is almost instant due to the small diameter of the myofibril. This makes myofibrils eminently suitable to measure the rates of activation and relaxation during force production. The protocol described in this paper employs an optical force probe based on the principle of a Fabry-Pérot interferometer capable of measuring forces in the nano-Newton range, coupled to a piezo length motor and a fast-step perfusion system. This setup enables the study of myofibril mechanics with high resolution force measurements.

INTRODUCTION:

Striated muscle cells are indispensable for daily life activities. Limb movement, respiratory function, and the pumping motion of the heart rely on the force generated by muscle cells. Skeletal muscle consists of muscle fascicles containing bundles of single muscle fibers (**Figure 1A**). These muscle fibers are comprised of myofibrils, which are formed by serially linked sarcomeres (**Figure 1B**,**D**). The sarcomeres contain thin and thick filaments. These primarily consist of chains of actin and myosin molecules, respectively (**Figure 1B**). Actin-myosin interactions are responsible for the force-generating capacity of muscle. Patients with mutations in genes encoding for sarcomeric proteins, such as nebulin, actin, and troponin T, suffer from muscle weakness due to contractile dysfunction[1].

The quality of muscle contractility can be studied at various levels of organization, ranging from in vivo whole muscles to actin-myosin interactions in in vitro motility assays. During the past decades, several research groups have developed setups to determine the contractility of individual myofibrils[2–10]. These setups are based on the detection of changes in laser deflection from a cantilever (i.e., optical beam deflection) caused by the contraction of the myofibril (for details, see Labuda et al.[11]). Although determining the contractile function of myofibrils has some limitations (e.g., the dynamics of the excitation-contraction coupling processes that are upstream of the myofibrils are lacking), there are multiple advantages to this approach. These include: 1) the ability to assess actin-myosin interactions in the presence of the geometrical constraints of the sarcomeres; 2) the ability to assess actin-myosin interactions without potential confounding effects of damaged, adjacent myofibrils (when measuring the contractility of single muscle fibers ultrastructural damage and misalignment of myofibrils might contribute to impaired contractility) (**Figure 1D**); 3) the small diameter of myofibrils (~1 µm, **Figure 2A**) and the lack of membranes allow for almost instant calcium diffusion into the sarcomeres. Furthermore, if structural damage is present in the myofibrils, they likely break during their isolation or during the experiment. Hence, assessing myofibril contractility is an elegant method to study the basic mechanisms of muscle contraction and to understand whether disturbed actin-myosin interactions are the primary cause of muscle disease caused by mutations in sarcomeric proteins.

This protocol presents a newly developed setup to determine the contractility of myofibrils incorporating a cantilever force probe with nano-Newton resolution (i.e., Optiforce). This force probe is based on the principle of interferometry. Interferometry enables the use of relatively stiff cantilevers. This makes it possible to measure force with little deflection of the cantilever, approaching isometric contractions of the myofibril. The probe allows for the assessment of low passive and active forces that are produced by a single myofibril isolated from different muscle biopsies, including those from human subjects, with a high signal-to-noise ratio. The optical

cantilever force probe incorporated in this setup is based on a Fabry-Pérot interferometer[12]. The interferometer detects small displacements between an optical fiber and a gold-coated cantilever mounted on a ferrule (**Figure 3**). The gap between the optical fiber and the cantilever is called the Fabry-Pérot cavity. Myofibrils are mounted between the probe and piezo motor using two glue-coated glass mounting fibers. The force produced by the myofibril can be mathematically derived from the interferometer data. Interferometry is based on the superposition or interference of two or more waves (in this setup three light waves). Laser light with a wavelength between 1,528.77–1,563.85 nm is emitted from the interferometer and is sent through the optical fiber. In the probe, the light is reflected 1) at the interface between the optical fiber and the medium (**Figure 3A**); 2) at the interface of the medium and the cantilever (**Figure 3B**); and 3) at the interface between the metal and gold coating of the cantilever (**Figure 3C**). The reflection at interface A and B is dependent on the refractive index (*n*) of the medium in which the probe is submerged. The light, consisting of the three superimposed reflections, returns to a photodiode in the interferometer. The photodiode measures the intensity of the light, which is the result of the interference pattern of the three superimposed reflections. When contractile force is generated by activating or stretching a myofibril, the myofibril pulls on the cantilever. This movement changes the cavity size (*d)* and consequently, the number of wavelengths that fit in the cavity. The light reflected at the cantilever will have a different phase, resulting in a different interference pattern. The photodiode records this change of interference pattern intensity as a change in Volts. Subsequently, myofibril force generation is calculated from this change, taking into account the cantilever stiffness. The force probe is calibrated by the manufacturer by pushing the tip of the mounting needle, attached to the free handing end of the cantilever, against a weighing scale while keeping the bending of the cantilever equal to a multiple of the wavelength of the readout laser[13]. Thus, interferometry is a highly sensitive method to detect small changes in distance, allowing for measurement of forces with nano-Newton resolution. This resolution enables the assessment of myofibrillar force production with a high signal-to-noise ratio. While traditional interferometry limits the range of measurements to the linear part of the interference curve, using a lock-in amplifier and modulation of the laser wavelength overcomes this limitation[14]. This is explained in more detail in the discussion section.

To measure myofibril active tension, a fast-step perfusion system was incorporated to expose the myofibril to calcium solutions (**Figure 4A**). The fast-step perfusion system enables solution changes to occur within 10 ms. Because of their small diameter, calcium diffusion into the myofibrils is nearly instantaneous. Hence, this system is particularly suitable for measuring the rates of actin-myosin binding during activation and release during relaxation. The rate of activation (k_{ACT}) and relaxation (k_{REL}) can be determined from the activation-relaxation curves. Also, by exposing the myofibrils to calcium solutions of increasing concentration, the force-calcium relationship and calcium sensitivity can be determined.

Furthermore, a piezo length motor enables fast stretching and shortening of the myofibril. This offers the possibility to study the viscoelastic properties (i.e., passive tension) of the myofibril, as well as performing a rapid shortening and restretch of the myofibril to determine the rate of tension redevelopment (k_{TR}). The parameters retrieved from both active and passive tension experiments can be altered by gene mutations in a sarcomeric protein.

This custom-built setup was used to measure the active and passive contractile characteristics of myofibrils isolated from healthy human, patient, and mouse skeletal muscle.

PROTOCOL:

The protocol for obtaining human biopsies was approved by the institutional review board at VU University Medical Center (#2014/396) and written informed consent was obtained from the subjects. The protocol for obtaining animal muscle biopsies was approved by the local animal ethics committee at VU University (AVD114002016501)

1. Preparation and myofibril isolation

NOTE: Use previously described methods to glycerinate biopsies, prepare the different calcium concentration (pCa) solutions[7,16,17], and isolate myofibrils[2,18].

1.1. Thaw the relaxing (pCa 9.0, Rx) and activating (pCa 4.5, Act) solutions as well as the inhibitors (1 M E64, 1 M DTT, 1 M leupeptin, 1 M PMSF), which are stored at -80 ˚C.

1.2. Take a glycerinated piece of striated muscle biopsy of approximately 1 mm^3 and place it in a small Petri dish with 1:1 Rx/glycerol (v/v) solution and place the Petri dish on a cold plate at 4 ˚C.

1.3. Dissect the piece of muscle using dissection microscope and forceps, separating single muscle fibers without isolating them from the piece of muscle.

NOTE: Remove as much fatty and connective tissue as possible to prevent the contamination of the myofibril suspension.

1.4. Transfer the piece of dissected tissue to a 5 mL tube with 1.5 mL of relaxing solution with inhibitors (1 µL/mL E-64, 1 µL/mL leupeptin, 1 µL/mL DTT, and 125 µL/mL PMSF). Allow the tissue to temper at approximately 4 ˚C for 1 h.

1.5. During incubation, boot up both PC's, turn on the devices, and open the associated software (see **Table of Materials**).

1.6. Submerge the force probe in ultrapure water in a Petri dish and calibrate the probe.

1.6.1. Press the '**Start Wizard**' on the interferometer and follow the onscreen instructions. After pressing **Calibrate**, tap on the microscope stage.

NOTE: Tapping on the microscope stage will cause the cantilever to deflect and pass through fringes. This enables calibration of the probe.

1.6.2. Leave the probe submerged in the ultrapure water in the Petri dish after calibration.

1.7. Initialize the piezo motor position. To do so, follow one of the steps detailed below.

1.7.1. When the piezo motor will be used for k_{TR} tension, set the length to 0 µm.
Signal generator settings can be found in **Table 1**, **Figure 5C**.

1.7.2. When the piezo motor will be used for passive tension, set the length to 50 µm.
Signal generator settings can be found in **Table 1**.

NOTE: The difference between steps is the initial position of the piezo length motor. To stretch the myofibril, the piezo motor needs to pull to increase the distance between both mounting needles and to lengthen the myofibril. To slacken the myofibril, the piezo motor needs to push to decrease the distance between both mounting needles and to shorten the myofibril.

1.8. Prepare a microscope slide. Pipette 150 µL of polyhydroxyethylmethacrylate (poly-HEMA) solution (5% poly-HEMA in 95% ethanol, w/v) on a microscope slide and spread it across the slide so it is all covered.

NOTE: If a myofibril suspension is pipetted on an uncoated microscope slide, the myofibrils that sink to the bottom will stick to the microscope slide and it will not be possible to glue them.

1.9. Fill syringes with pCa solutions (see **Figure 4A**) and prime the perfusion system.

NOTE: In these steps all tubes are prefilled with the appropriate solution to make sure all air bubbles are removed from the tubing.

1.9.1. Fill the inflow tubing of the flow chamber (**Figure 3**) inflow with Rx.

1.9.2. When used, flush the manifold with ultrapure water to remove air. To do so, connect the syringe with ultrapure water to the outlet and flush in it in the reverse direction. Block the unused ports of the manifold.

1.9.3. Enable each pCa syringe to fill their respective tubes with pCa solution. Then, connect them to the manifold and the Θ-glass.

1.9.4. Open valves 1 and 6 with the data acquisition panel software (see **Table of Materials**) by checking the button '**1+6**' (**Figure 6B**) to fill the Θ-glass with the relaxing (pCa 9.0) and activating (4.5) solutions and close valves when the Θ-glass is filled (**Figure 6A**).

2. Mounting a myofibril

2.1. Coat a microscope slide with poly-HEMA to prevent myofibrils from sticking to the glass.

2.2. Prepare the homogenizer (see **Table of Materials**) for tissue homogenization. Clean the internal rotor rod with clean tissue paper, assemble the homogenizer, and spin 1x for 15 s in alcohol and thrice for 15 s each in ultrapure water. Prerinse the homogenizer in relaxing solution 1 x for 15 s on ice.

2.3. Place the homogenizer rod in the tube containing the muscle tissue as described in step 1.4 and, while keeping the tube on ice, spin the rotor for 15 s on speed 5 to tear the muscle tissue and obtain a myofibril suspension.

2.4. Pipette ~50 µL of the myofibril suspension and ~250 µL of the relaxing solution on the microscope slide coated with poly-HEMA in the tissue bath. This will form a liquid drop. Cover the bath with a lid to protect from dust and wait 5–10 min to allow the myofibrils to sink to the bottom.

NOTE: The ratio between the suspension and the relaxing solution is dependent on the quality of the isolation, therefore, adjust accordingly. For example, if the myofibril yield is low and few suitable myofibrils are present in the suspension, add more myofibril suspension and dilute with less relaxing solution (e.g., 75 µL of myofibril suspension and 225 µL of relaxing solution). Heart and skeletal muscle tissue is easy to recognize due to its striation pattern. Using a 10x or 40x objective, this pattern is also visible in a single myofibril. In case other tissue is present in the suspension, myofibrils can be selected visually. One can skip the 5–10 min wait. However, this increases the difficulty of gluing a myofibril.

2.5. Coat mounting needles with glue (shellac + ethanol; 120 mg shellac in 2 mL of 70% ethanol). To do so, heat the glue at 65 ˚C for 30–60 s and pipette ~6 µL on a new uncoated glass slide. Dip the tip of each mounting needle in the glue and repeat until a layer of glue is visible. Move the probe and piezo up vertically with the micromanipulators to make room to place the tissue bath on the microscope stage. Remove the glass slide containing the glue.

2.6. Mounting the myofibrils

2.6.1. Place the tissue bath with the microscope slide coated with poly-HEMA containing the myofibril suspension on the microscope stage. Use the stage to find a suitable myofibril with the 40x objective. If necessary, move and rotate the tissue bath to move the myofibril to a mountable position.

NOTE: Look for myofibrils with a visible striation pattern that are approximately 30 µm long. As described in detail in steps 3.1 and 3.2.1 it is possible to check length and sarcomere length prior to gluing the myofibril. Do not glue torn myofibrils, because these are likely to break during contraction.

2.6.2. Slide the flow chamber into place directly above the liquid drop containing the myofibrils in the tissue bath (pipetted onto the slide in step 2.4) and lower it. Stop before it hits the liquid

drop.

2.6.3. Lower the piezo mounting needle and press it on the bottom tip of the myofibril. Lift it slightly to check if the myofibril is attached to the needle.

2.6.4. Lower the flow chamber far enough for the mounting needle of the probe to reach the bottom without the probe touching the flow chamber.

2.6.5. Press the mounting needle of the probe on the top tip of the myofibril. Lift it slightly to check if the myofibril is attached to the needle.

2.6.6. Lift the myofibril from the bottom of the bath as far as possible without losing the ability to focus without the objective touching the bottom of the glass.

3. Initializing experiment

3.1. Use the micromanipulators, camera, and system controller software (**Figure 7A**, see **Table of Materials**) to measure sarcomere length. Move the piezo and/or force probe to set the initial sarcomere length of the myofibril to 2.5 µm.

NOTE: A sarcomere length of 2.5 µm ensures optimal overlap between myosin heads and actin.

3.2. Using the vessel function of the system controller software measure the myofibril length and width (**Figure 7B**,**C**).

NOTE: When rotating the camera, it may tilt horizontally and/or vertically. To check the alignment of the camera, a spirit level can be used to verify that the camera is rotated and not tilted.

3.2.1. Position the myofibril in the center of the video image using the microscope stage.

3.2.2. Draw a square from one side of the myofibril to the other. For the length, make sure to include the dark edge of the glue droplets (**Figure 2A**) in the square because the image processing is based on contrast.

3.2.3. Start recording the data in the system controller software (see **Table of Materials**) by pressing '**Start**' and after 5 s pause the system controller software data recording by pressing the '**Pause**' button. The length is now recorded in the data.

3.2.4. For the width first rotate the camera 90˚ (see **Table of Materials**) and then use the contrast of the edge of the myofibril itself.

3.2.5. Start recording the data in the system controller software (see **Table of Materials**) by pressing '**Start**' and after 5 s pause the system controller software data recording by pressing

the '**Pause'** button. The width is now recorded in the data.

3.3. If active tension of the myofibril needs to be determined, the perfusion setup needs to be used. If so, continue to step 3.4. If only passive tension will be determined, skip steps 3.4–4.1.3.7 and continue at step 4.2.

3.4. Position and initialize the perfusion setup.

NOTE: This is only necessary for generation of active force. Continue to step 4.2 when performing passive tension experiments.

3.4.1. Set the fast-step motor position at 4 V (**Figure 5B**).

3.4.2. Slide the perfusion stand on the table to align the left bottom corner of the stand with the tape on the table.

NOTE: Be careful not to hit the force probe or the piezo motor.

3.4.3. Use the manipulator to roughly position the Θ-glass by eye.

3.4.4. Look through the eyepiece and carefully move the Θ-glass towards the myofibril using the manipulator.

3.4.5. Align the top channel of the Θ-glass with the myofibril using the manipulator and check the position by performing a fast-step (signal generator settings can be found in **Table 1**) with the system controller software (**Figure 2B–C**, see **Table of Materials**).

NOTE: Make sure that the bottom channel will be aligned with the myofibril during the activation phase of the fast-step (**Figure 2B–C**).

3.5. Turn on the background flow of Rx (**Figure 4A**) to create a laminar background flow in the flow chamber.

NOTE: The background flow is necessary to prevent turbulent flow as a result of the pCa solution flow from the Θ-glass.

3.5.1. Turn on inflow of flow chamber with a Luer valve lever.

3.5.1.1. Send the following parameters to the outflow pump to start draining the flow chamber and prevent overflowing of the flow chamber (**Figure 9**): Valve = Bath valve (2); Microstep mode = Micro; Plunger target = 48,000; Plunger speed = 38–40 (arbitrary).

NOTE: Make sure the fluid level is stable at all times. The myofibril should not run dry and neither should the cantilever. It is better to have a little overflow than too little flow.

3.6. To set the temperature to a desired value with the thermoelectric temperature controller (**Figure 8**, see **Table of Materials**), enter the desired temperature and press '**Start**'. Wait until the desired temperature is reached by checking the graph in the thermoelectric temperature controller software and continue.

NOTE: When performing experiments at room temperature, the thermoelectric temperature controller does not have to be used.

4. Experimental protocol(s)

4.1. Decide which active force protocols need to be performed.

NOTE: Depending on the data necessary for the study, multiple types of active force experiments can be performed: step 4.1.1, measurement of the maximum force at saturating [Ca^{2+}]; step 4.1.2, obtaining a Force-pCa curve to determine calcium sensitivity in addition to step 4.1.1; step 4.1.3, determining the rate of tension redevelopment by doing a shortening-restretch protocol in addition to step 4.1.1 or 4.1.2.

4.1.1. Measure maximal active force.

4.1.1.1. Start recording the data in the system controller software (see **Table of Materials**) by pressing '**Start**'.

4.1.1.2. Open valves 1 and 6 with the data acquisition panel (see **Table of Materials**) software by checking the button '**1+6**' to start Θ-glass flow of the relaxing solution and activating solution through the Θ-glass (**Figure 6A**).

4.1.1.3. Reset the range of the interferometer so that the baseline force is 0 V by selecting and pressing '**Reset Range**' on the interferometer (see **Table of Materials**).

4.1.1.4. When the force trace is stable, perform the Θ-glass fast-step (step size = 100 µm). Signal generator settings can be found in **Table 1** (**Figure 5C**). An activation-relaxation trace similar to **Figure 4D** will be recorded and visible in the system controller software.

4.1.1.5. Pause the system controller software data recording by pressing the '**Pause**' button.

4.1.1.6. If no more activations are to be performed, close valves 1 and 6 to stop Θ-glass flow by unchecking the button '**1+6**' (**Figure 6B**), stop the syringe pump (**Figure 9**, see **Table of Materials**) by pressing '**Terminate**', and stop the background flow by closing the Luer valve.

4.1.2. Force-pCa curve

NOTE: This is similar to step 4.1.1 to obtain the maximal active force, but with multiple activations using different pCa solutions.

4.1.2.1. Start recording the data in the system controller software by pressing '**Start'**.

4.1.2.2. Open valves 1 and 2 with the data acquisition panel software to start the flow of relaxing solution and pCa 6.2 through the Θ-glass.

4.1.2.3. Reset range of the interferometer so that the baseline force is 0 V by selecting and pressing '**Reset Range**' on the interferometer.

4.1.2.4. When the force trace is stable, perform the Θ-glass fast-step (step size = 100 µm). Signal generator settings can be found in **Table 1**.

4.1.2.5. Pause the system controller software by pressing the '**Pause**' button.

4.1.2.6. Repeat steps 4.1.2.1–4.1.2.4 for valves 1 and 3 (pCa 5.8), valves 1 and 4 (pCa 5.6), valves 1 and 5 (pCa 5.4), and valves 1 and 6 (pCa 4.5).

4.1.2.7. If no more activations are to be performed, close valves 1 and 6 to stop Θ-glass flow by unchecking the button '**1+6**' (**Figure 6A**), stop the syringe pump (**Figure 9**) by pressing '**Terminate**', and stop the background flow by closing the Luer valve.

4.1.3. Measure rate of tension redevelopment (k_{TR}).

NOTE: This is similar to step 4.1.1 for maximal active force but with some changes and added steps.

4.1.3.1. Calculate the piezo movement necessary to slacken the myofibril 15% and enter this value in the signal generator (**Figure 5D**, **Table 1**).

4.1.3.2. Start recording the data in the system controller software by pressing '**Start**'.

4.1.3.3. Open valves 1 and 6 with the data acquisition panel (**Figure 6A**) software to start flow of the relaxing solution and pCa 4.5 through the Θ-glass .

4.1.3.4. Reset range of the interferometer so that the baseline force is 0 V by selecting and pressing '**Reset Range**' on the interferometer.

4.1.3.5. When the force trace is stable, perform the Θ-glass fast-step (step size = 100 µm). Signal generator settings can be found in **Table 1**.

4.1.3.6. When the force plateau is reached, perform the shortening-restretch with the piezo. Signal generator settings can be found in (**Figure 5D**, **Table 1**). An activation-relaxation trace similar to **Figure 4E** will be recorded and visible in the system controller software.

NOTE: A custom protocol can be made to automate the steps above.

4.1.3.7. Pause the system controller software by pressing the '**Pause**' button.

4.1.3.8. If no more activations are to be performed, close valves 1 and 6 to stop Θ-glass flow by unchecking the button '**1+6**' (**Figure 6B**), stop the syringe pump (**Figure 9**) by pressing '**Terminate**', and stop the background flow by closing the Luer valve.

4.2. Perform passive force measurements.

4.2.1. Perform a continuous stretch.

4.2.1.1. Calculate the piezo movement necessary to stretch the myofibril and enter this value in the signal generator (**Table 1**).

NOTE: These are example settings. Calculate the amount of stretch and time of stretch relative to the sarcomere length. These settings are necessary to ensure that the speed of stretch per sarcomere remains equal across myofibrils.

4.2.1.2. Start recording the data in the system controller software by pressing '**Start**'.

4.2.1.3. Reset range of the interferometer so that the baseline force is 0 V by selecting and pressing '**Reset Range**' on the interferometer.

4.2.1.4. Perform continuous stretch with the signal generator in the system controller software to operate the piezo. Example signal generator settings can be found in **Table 1**.

4.2.1.5. Shorten the myofibril to slack length with the piezo after the stretch is finished (**Table 1**).

4.2.2. Perform a stepwise stretch.

4.2.2.1. Start recording the data in the system controller software by pressing '**Start**'.

4.2.2.2. Reset range of the interferometer so that the baseline force is 0 V by selecting and pressing '**Reset Range**' on the interferometer.

4.2.2.3. Perform a stepwise stretch with the signal generator in the system controller software to operate the piezo. Example signal generator settings can be found in **Table 1** (**Figure 5E**).

4.2.3. Shorten the myofibril to slack length with the piezo after the stretch is finished. Example signal generator settings can be found in **Table 1**.

4.3. Pause the system controller software by pressing the '**Pause**' button.

4.4. Stop the recording of data by pressing the '**Stop**' button in the system controller software.

4.5. Save the data by pressing '**File**' and '**Save Data**' in the system controller software.

5. Cleaning

5.1. Remove the measured myofibril and prepare for the next myofibril.

5.1.1. To do so, carefully tear off the myofibril while looking through the ocular with the 40x objective.

5.1.2. Move up the force probe and piezo. Move up the Θ-glass all the way up, to the right and to the back. Then move up and slide away the flow chamber. Remove the tissue bath.

5.1.3. To clean the mounting needle, bring it into focus using the 10x and the ocular. Dip the brush in ethanol and carefully brush off and remove the glue from the needle.

NOTE: Keep in mind that it might take some time before the glue comes off.

5.1.4. Rinse the flow chamber and tissue bath with ultrapure water.

5.1.5. Place the probe in small a Petri dish filled with ultrapure water. Ensure that the probe is completely submersed.

5.2. When the experiments are finished, clean the setup as above and perform the following additional steps.

5.2.1. Empty the tubing from the flow bath. Send the parameters to the outflow syringe pump (see **Table of Materials**, **Figure 9**). Valve = Bath valve (2); Microstep mode = Normal; Plunger target = 0; Plunger speed = 30.

NOTE: Terminate the command when the tubing is empty.

5.2.2. Initialize the pump several times (**Figure 9B**).

5.2.3. Drain the syringes. To do so, close all the Luer valves, open all the valves, remove the tubing from the needle of the syringe, hold the tube of specific pCa under the needle, and open the Luer valve. Use the pressure plugs to speed up the process.

5.2.4. Reattach the tubing to the needle of the syringe. Fill syringes with ~5 mL of ultrapure water. Place a cup underneath the Θ-glass. Open all the valves and open the pressure valve to flush the system.

5.2.5. Shut down the system. Turn off the PC, interferometer, and piezo controller power block.

6. Data analysis

6.1. Export data traces from the system controller software (see **Table of Materials**) to a spreadsheet software program or clipboard by opening the data file and selecting the desired segment. The traces shown will be exported (e.g., raw force, sarcomere length, and piezo position).

6.2. Perform analysis with the software of choice (e.g., MATLAB).

REPRESENTATIVE RESULTS:

Data traces were recorded and opened with the system controller software (see **Table of Materials**). Complete traces or selected segments were exported to the clipboard or text file for further analysis with a desired software. Valves to control flow of the different solutions were switched with custom software or manually. A custom MATLAB script was used to analyze the rates of activation, tension redevelopment, and relaxation. The maximum active force and the peak and the plateau force of the passive force experiments were taken directly from the system controller software force trace. After mounting a myofibril (**Figure 2**), the desired protocol was selected.

Maximum active force and calcium-sensitivity of the force in myofibrils isolated from mouse and human skeletal muscle biopsies

In **Figure 4A** the experimental setup used for the active force experiments is depicted schematically. Force traces of an active force experiment with a myofibril isolated from healthy human quadriceps muscle are shown. The myofibril was activated 5 times with solutions with varying pCa (pCa 6.2, 5.8, 5.6, 5.4, 4.5; data shown in **Figure 4B**). The average maximum force of all myofibrils in this experiment was ~123 mN/mm^2. A force-pCa curve was constructed from the plateau forces reached during each activation in each of the five calcium solutions. The results are shown in **Figure 4C**. From this curve the pCa at 50% of maximum force production (pCa_{50}) was calculated. In this myofibril, the pCa_{50} was 5.75.

Additionally, one or multiple compounds could be added to the perfused solution to measure its effect on the force produced by the myofibril. In **Figure 4D**, the effect of N-benzyl-p-toluene sulphonamide (BTS), a fast twitch muscle (type II) myosin heavy chain II (MHCII) inhibitor, is illustrated.[19] A myofibril was activated first with a pCa 5.6 solution, and subsequently with a pCa 5.6 + BTS solution. During the second activation less force was produced, indicating that this was a myofibril that contained MHCII. There are mutations in proteins that are present exclusively in specific muscle types, and thus only affect myofibrils from that specific muscle type. In that case, 'typing' the myofibrils is important to discern the mutation effect on the

various muscle types. Also, this example illustrates the possibility for testing the efficacy of therapeutic compounds in myofibrils.

Figure 4E shows an active force trace of a single myofibril isolated from mouse skeletal soleus muscle tissue. The myofibril was mounted in the setup and perfused with relaxing solution (pCa 9.0), followed by perfusion with activating solution (pCa 4.5, ~0.032 mM calcium). We simultaneously recorded the force and sarcomere length. This was a near isometric contraction, as the cantilever deflection was ~0.5 μm, which was approximately 1% of the myofibril's slack length (~50 μm). In **Figure 4E** a rapid shortening-restretch protocol was performed during active contraction to assess the rate of tension redevelopment (k_{TR}, yellow dashed line). The k_{TR} is a measure of cross-bridge cycling kinetics. Also, the activation and relaxation curves were fitted to determine the rate of activation (k_{ACT}, red dashed line) and relaxation (k_{REL}, green dashed line), respectively. **Figure 4** shows a more detailed view of the relaxation phase highlighted in **Figure 4F**. Two phases became apparent: 1) an initial slow phase of relaxation (dominated by cross-bridge detachment) and 2) a fast phase of relaxation (dominated by cross-bridge detachment and calcium-dissociation)[20].

Passive force in myofibrils isolated from a human skeletal muscle biopsy

Figure 10 shows a trace of a passive force experiment with a myofibril isolated from healthy human diaphragm muscle tissue. The first protocol involved one or multiple passive stretches to determine the viscoelastic properties of the sarcomeres. **Figure 10** shows a force trace of a continuous stretch of a myofibril (stretch from sarcomere length 2.2–3.0 μm). During the stretch, myofibrils displayed both viscous and elastic characteristics. This is evident from the curve shown in **Figure 10A**. The sharp peak represents both characteristics, whereas the plateau force is a measure of elasticity. Viscosity resists strain linearly. Thus, the force dropped after the strain was removed. **Figure 10B** highlights the stretch itself and illustrates the high signal-to-noise ratio. Note that force traces are unfiltered.

FIGURE LEGENDS:

Figure 1: Schematic depiction and electron microscopy images of a skeletal muscle and its morphology. (**A**) Shows the structure of skeletal muscle and (**B**) shows the structure of the sarcomere, the smallest contractile unit. These schematic images are adapted from Servier Medical Art. (**C**) Shows an image of a single muscle fiber and (**D**) shows an electron microscopy image of a muscle fiber revealing myofibrillar damage as well as preserved myofibrillar ultrastructure.

Figure 2: Images showing a mounted myofibril, **Θ-glass alignment, and piezo mounting needle.** (**A**) A myofibril mounted at slack length between the glass fiber needles coated with shellac as seen through a 40x objective. (**B**) Images of the position of the Θ-glass relative to the myofibril (highlighted with the white ovals) as seen through a 10x objective. (Top) Aligned to the top channel (relaxing solution, pCa 9.0); (Bottom) Aligned to the bottom channel (activating solution, pCa 4.5) to perfuse the myofibril with calcium and induce contraction. (**C**) Schematic depictions of the position of the Θ-glass relative to the myofibril. (Top) Aligned with the top channel (relaxing solution, pCa 9.0); (Bottom) Aligned with the bottom channel (activating solution, pCa 4.5) to

perfuse the myofibril with calcium and induce contraction. (**D**) Mounting needle attached to the carbon rod of the piezo holder.

Figure 3: Schematic representation of the setup and end part of the tissue flow chamber. In dark blue the tissue flow chamber made out of aluminum, and in white the cavity in which the force probe and Θ-glass are shown in position; (Center) Myofibril attached between two glass fiber mounting needles attached to the force probe and piezo length motor. The Θ-glass is aligned with the myofibril. The Θ-glass can move up and down to expose the myofibril to the calcium solution. (Right) Close-up of the cantilever force probe. Indicated are the cavity size (or Fabry-Pérot cavity, *d*); reflection interfaces A, B, and C; and an example of a light wave emitted by the laser (red). The cantilever is mounted on the shoulder of the ferrule. The fiber that carries the laser from the interferometer exits the ferrule at the tip of the cantilever. A glass mounting fiber is fixed on the cantilever using wax. (Top left) The interferometer analyzes the interferometer signal transmitted to the system controller software.

Figure 4: Experimental setup and data from active tension experiments. (**A**) Schematic representation of the perfusion setup and solutions used. Note that the first and last tubes (light blue) contain calcium-free solution (i.e., relaxing solution). (**B**) Example force traces of an active tension experiment with a myofibril isolated from human skeletal muscle tissue showing five activations from relaxing solution (pCa 9.0) to multiple activation solutions (pCa 6.2 – 4.5). (**C**) A force-calcium curve; force levels at the plateaus in panel (**B**) were normalized and plotted against their respective calcium levels. (**D**) Example force trace of a type II (fast twitch) myofibril isolated from human skeletal muscle activated with pCa 5.6 solution (blue) and subsequently with pCa 5.6 + BTS (a type II specific cross-bridge inhibitor, red). (**E**) Example data trace of an active tension experiment with myofibrils isolated from mouse soleus skeletal muscle tissue with a rapid shortening-restretch protocol during activation to determine the rate of tension redevelopment (k_{TR}, yellow dashed line). Also, the activation and relaxation curve were fitted to determine the rate of activation (k_{ACT}, red dashed line) and relaxation (k_{REL}, green dashed line), respectively. (**F**) A zoom of the relaxation phase (Top left), highlighted in (**E**). The fast-step motor signal (Bottom left) indicated the time point at which the solution changed from an activation solution (pCa 4.5) to a relaxing solution (pCa 9.0). The relaxation phase consisted of a linear, slow phase (Top right) and an exponential, fast phase (Bottom right).

Figure 5: Example setting for the signal generator in the system control software (see **Table of Materials**). 1) Indicates the button to execute commands entered in the signal generator. (**A**) Setting up the piezo length motor. (**B**) Setting up the fast-step motor. (**C**) Performing a fast-step to activate a myofibril for a duration of 5 s. (**D**) Performing a rapid shortening-restretch of a myofibril to determine the k_{TR}. (**E**) Performing a stepwise stretch of a myofibril to determine the viscoelastic properties.

Figure 6: Valve controller software as used on the PC. (**A**) The button used to open valves 1 (Rx) and 6 (Act). (**B**) The state of the buttons when all valves are closed.

Figure 7: Measuring sarcomere length, myofibril length, and myofibril width with the system controller software. A ruler is used as an example. (**A**) Measuring the sarcomere length: the purple box is placed around the myofibril and the sarcomere length is shown in (1). (**B**) Measuring the length: The cyan box is placed from beginning to end of the myofibril. (**C**) Measuring width: After rotating the camera 90°, the cyan box is placed from one side of the myofibril to the other.

Figure 8: Thermoelectric temperature controller software. (**A**) Establish connection with the thermoelectric temperature controller. (**B**) Expand temperature settings. (**C**) Set desired temperature, in this case: 15 °C. (**D**) Turn on thermoelectric temperature controller and send voltage to Peltier thermoelectric cooler module.

Figure 9: Settings for the syringe outflow pump. (**A**) Open connection to the pump by pressing (1). (**B**) Start the pump with predefined settings by pressing (2). (**C**) Start outflow pumping by setting the '**Valve Commands**' to '**Bath Valve**' (2) and entering the '**Command Set Parameters**' as shown. Execute the command by pressing (3). Commands can be terminated by pressing (4).

Figure 10: Example data trace of a passive tension experiment with myofibrils isolated from human skeletal muscle tissue. (**A**) Recording of the force (Upper) and sarcomere length (Lower) during a stretch and release protocol. (**B**) Zoom of (**A**) showing the force (Upper) and sarcomere length during the stretch phase of the myofibril.

Figure 11: Experimental setup and data from cardiomyocyte calcium preactivation experiments. (**A**) Schematic representation of the perfusion setup. Note that the last tube (light blue) contains calcium-free solution (relaxing solution). (**B**) Superimposed curves of activation of a cardiomyocyte without (light blue) and with (dark blue) calcium preactivation, with calcium concentrations of 1 nM and 80 nM, respectively. (**C**) Comparison of calcium preactivation in wild type (WT) and heterozygous RBM20 (HET) cardiomyocytes isolated from rat left ventricle. This figure has been modified from Najafi et al.[21].

Table 1: Table describing the various signal generator settings used in the system controller software to operate the piezo length motor and fast-step motor.

DISCUSSION:

Described is a protocol to assess the contractile function of myofibrils isolated from human or animal skeletal muscle tissues. The force resolution of this setup has been described before by Chavan et al.[12]. In short, it is determined by the random fluctuations of the length of the Fabry-Pérot cavity formed between the detection fiber and the cantilever, which produce the dominant part of the noise at the output of the readout (expressed in V) that, multiplied by the deflection sensitivity (expressed in m/V) and by the spring constant of the cantilever (expressed in N/m), provides the force noise. For our setup, the root mean square (rms) noise in air at the output of the readout, sampled at a 1,000 data points/s (sample/s), is approximately 2 mV. For a typical myofibril measurement, a ferrule-top probe is used with a spring constant of ~0.7 N/m (deflection sensitivity ~300 nm/V). This rms value corresponds to a cantilever deflection

resolution of 0.6 nm, which translates to a force sensitivity of ~0.37 nN. The force probe is calibrated by pushing the tip of the mounting needle against a weighing scale while keeping the bending of the cantilever equal to a multiple of the wavelength of the readout laser[13]. This method of calibration entails both the cantilever and mounting needle stiffness as well as possible variations in torque of the cantilever and mounting needle due to speed and magnitude of myofibril contraction. Currently, a setup for assessing myofibril contractility is available, which is based on the detection of a laser deflected from the cantilever, i.e., optical beam deflection (1,700 A; ~1 nN force resolution). This system was developed by Labuda et al. using an optical periscope to guide a laser light towards and away from the cantilever in constraining configurations[11]. In this system, a myofibril is mounted between the atomic force cantilever and a rigid glass needle. An advantage of the system described here is the higher force sensitivity and signal-to-noise ratio. Furthermore, in this setup, relatively stiff cantilevers can be used, which results in small cantilever deflection when myofibrillar force is applied. This is important, as it allows for force measurements at nearly constant sarcomere length. Finally, compared to the system described by Labuda et al., the system here utilizes similar or identical methods to control the temperature, to induce length changes on the myofibril, and to change the perfusion solutions using a Θ-glass and fast-step motor. The advantage of the system described by Labuda et al. is that a change of solution composition (between cantilever and optical periscope) does not affect the signal output. In the system described here, the solution composition between cantilever and optical fiber must remain constant. The solution to this limitation is described in more detail below.

Optimization

The optical force probe in combination with the fast-step perfusion system led to complications. The difference in optical properties between low and high concentration Ca^{2+} solutions interferes with the force measurements. To prevent backflow of the high calcium solution, a custom flow chamber was engineered (**Figure 3**). A constant background flow of calcium-free solution is induced from right to left to keep the solution constant between the top of the optical fiber and the cantilever (**Figure 3D**).

To control the temperature, a Peltier element with liquid cooling is mounted on the flow chamber. This flow chamber is thermally uncoupled from the microscope by mounting it on a plastic adapter. With the Peltier element, controlled by a TEC system, it is possible to control the temperature of the solution over time with 0.1 °C precision. Temperature is monitored by a temperature sensor mounted on the flow chamber. Temperature stability is important due to the nature of the force transducer. The cantilever consists of a gold-coated glass strip, effectively making it a thermometer. Thus, the cantilever bends with temperature changes.

The setup uses a fast-step perfusion system (see **Table of Materials**) to control the movement of the Θ-glass. This system allows for perfusion switches within 10 ms. Combining the method of temperature control and solution switching makes this system particularly suitable to measure the kinetics of sarcomere contractility (i.e., rates of force development, tension redevelopment, and relaxation) in myofibrils.

Initially, the downside of using interferometry was the small usable range due to the necessity to use the linear part of the interference curve (λ/8, with λ being the wavelength of the laser). However, recent innovations eliminated this need by combining wavelength modulation with a lock-in amplifier. Therefore, the system is not limited to a single linear part of the interference curve. This enables the measurement of infinite deflection of the cantilever[14]. Thus, the range of cantilever deflection readout of this system is greatly enlarged compared to traditional interferometry. Additionally, the force probes described are easy to replace and there are many cantilevers available, with stiffnesses ranging from 0.5 N/m to >20 N/m. Therefore, it is possible to quickly change between cantilevers and select the stiffness most suitable for the experiment conducted.

Challenges

The current system is a prototype based on a cardiomyocyte measuring system (see **Table of Materials**). Several components can be improved to provide a better user experience and data of higher quality. First, due to add-ons to the system, vibration and resonance can be an issue that will add noise to the signal. Also, the Θ-glass holder and fast-step motor attachment method could be improved to make it less prone to vibration.

Second, it is desirable to replace the fast-step motor with a piezo length actuator to increase the speed of solution switching and to obtain a more consistent motion.

Third, the calcium solutions we previously used to activate single striated muscle fibers included propionic acid, but these solutions absorb near-infrared light, interfering with the force measurements. Calcium chloride was used to eliminate the need for propionic acid, which greatly reduced this effect. This issue is inherent to a system based on interferometry and not present when utilizing optical beam deflection.

Fourth, a custom flow bath was engineered to create a laminar flow, to match the flow of the Θ-glass. This prevents backflow due to turbulence of the calcium-rich solution. Therefore, the solution between the tip of the optical fiber and the cantilever remains constant. The coverslip with the myofibrils can move freely under the flow chamber and therefore, selection of suitable myofibrils is not confined to the small area of the flow chamber.

Reproducibility and variability

There are several elements of the system and protocol that are important for the degree of reproducibility and variability of the data obtained.

First, the quality of the measurements strongly depends on the quality of the myofibril isolation. Identical protocols yield different qualities and quantities of myofibrils from different biopsies. In some cases, biopsies barely yield usable myofibrils or none at all. Common consensus is that damaged myofibrils will break during contraction and thus are not accounted for in the results.

Second, there is uncertainty in the determination of the cross-sectional area of the myofibril. Due to technical constraints, it is possible to measure the width of the myofibril in only one plane.

Therefore, to calculate the cross-sectional area we assume that the width and depth are equal. When force is normalized to a cross-sectional area to calculate maximal active tension, one should be aware of this assumption.

Mounting of myofibrils due to myofibril mounting angle, position, and the integrity of the glue. Although mounting angle and position can largely be controlled visually, small variations between myofibrils might be present. Glue integrity has not been investigated extensively. However, glue integrity can be verified by monitoring the sarcomere length in the myofibril before and after activation. When more sarcomeres are between the glue after a protocol, this suggests that slippage of the myofibril in the glue has occurred. Consequently, this myofibril should be excluded from the dataset.

Other applications of the setup: Calcium preactivation in cardiomyocytes isolated from rat left ventricle

In addition to assessing the contractile function of myofibrils, the system can also be used to measure cardiomyocyte mechanics. For example, **Figure 11** illustrates the use of membrane-permeabilized single cardiomyocytes isolated from rat left ventricle[21]. Contrary to the experiments described above, relaxing solution was changed and activating solution was kept constant. Each cardiomyocyte underwent five sets of activations, exposing it to a 2 µM free calcium solution for 1 s. The 1 s time constraint is chosen to mimic the time-limited nature of cardiac contractions, where the exposure to low calcium concentration solutions mimics the diastolic phase and the exposure to high calcium concentration solutions mimics the systolic phase of cardiac muscle contraction (**Figure 11A**). For each of the five sets diastolic calcium was varied (1, 80, 160, 250, and 400 nM calcium), while systolic calcium remained constant (**Figure 11A**). A set consisted of two sets of three activation-relaxation cycles at 1.8 µm versus 2.0 µm and 2.0 µm versus 2.2 µm for different experimental groups. Peak force was measured at 1 s from the switch of the pipette and averaged for the set of three activation-relaxation cycles. The high signal-to-noise ratio and the high dynamic range of this force transducer allowed us to measure both the small changes in diastolic force and the much larger systolic forces (**Figure 11B**). Increasing diastolic calcium resulted in a higher force at 2 µM calcium relative to the first activation (**Figure 11B**). WT rat cardiomyocytes were compared with heterozygous (HET) RMB20 rat cardiomyocytes. Due to alternative splicing, HET rats have a more compliant titin protein as compared to the WT rats. The effect was exaggerated in HET cardiomyocytes at 80 and 160 µM calcium (**Figure 11C**).

ACKNOWLEDGEMENTS:

This project was funded by AFM-Telethon and A Foundation Building Strength for Nemaline Myopathies. The authors wish to acknowledge the creator of the products mentioned in this article, IONOptix Inc.

DISCLOSURES:

Michiel Helmes is shareholder and co-owner of IONOptix Inc.

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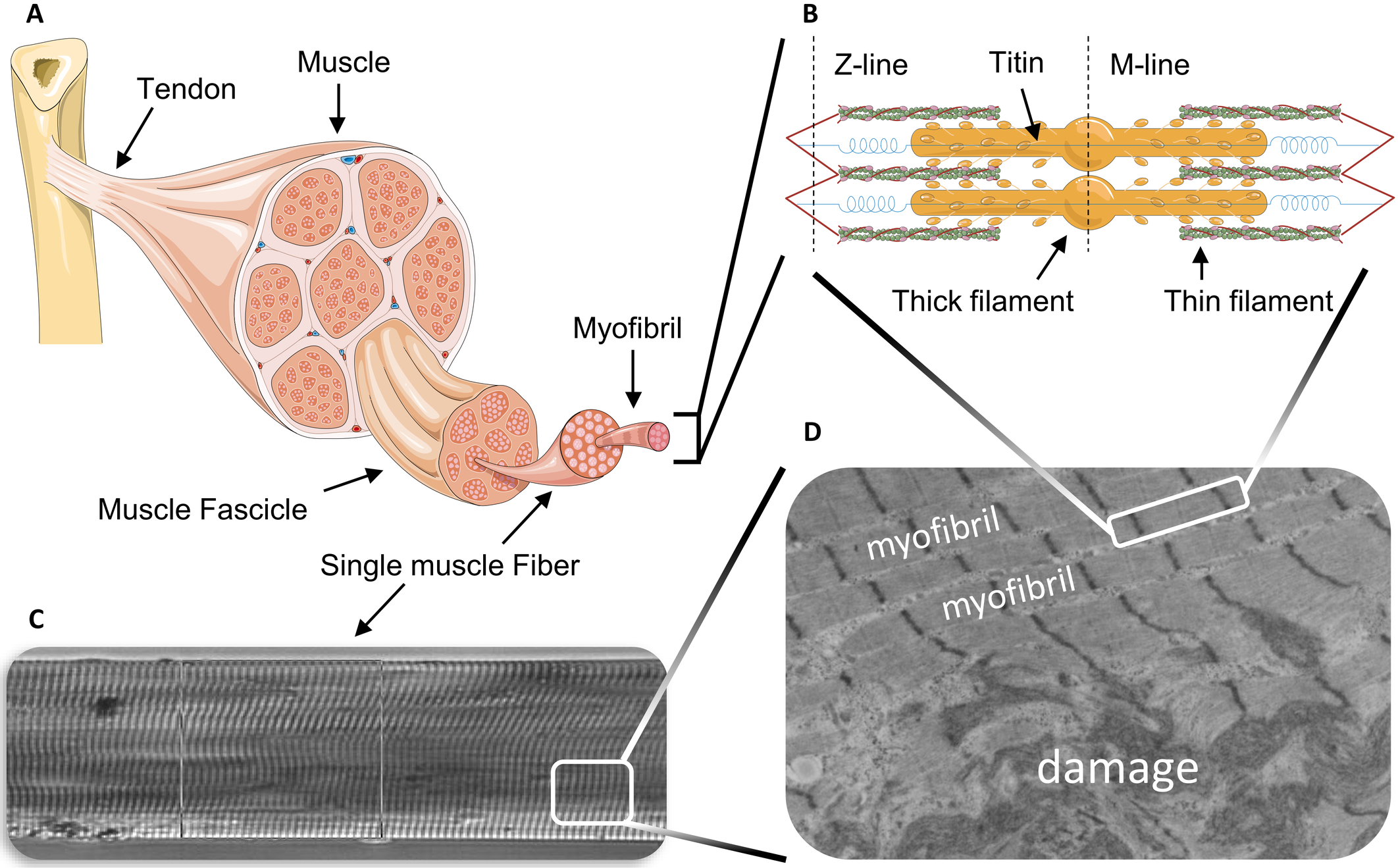

Figure 2

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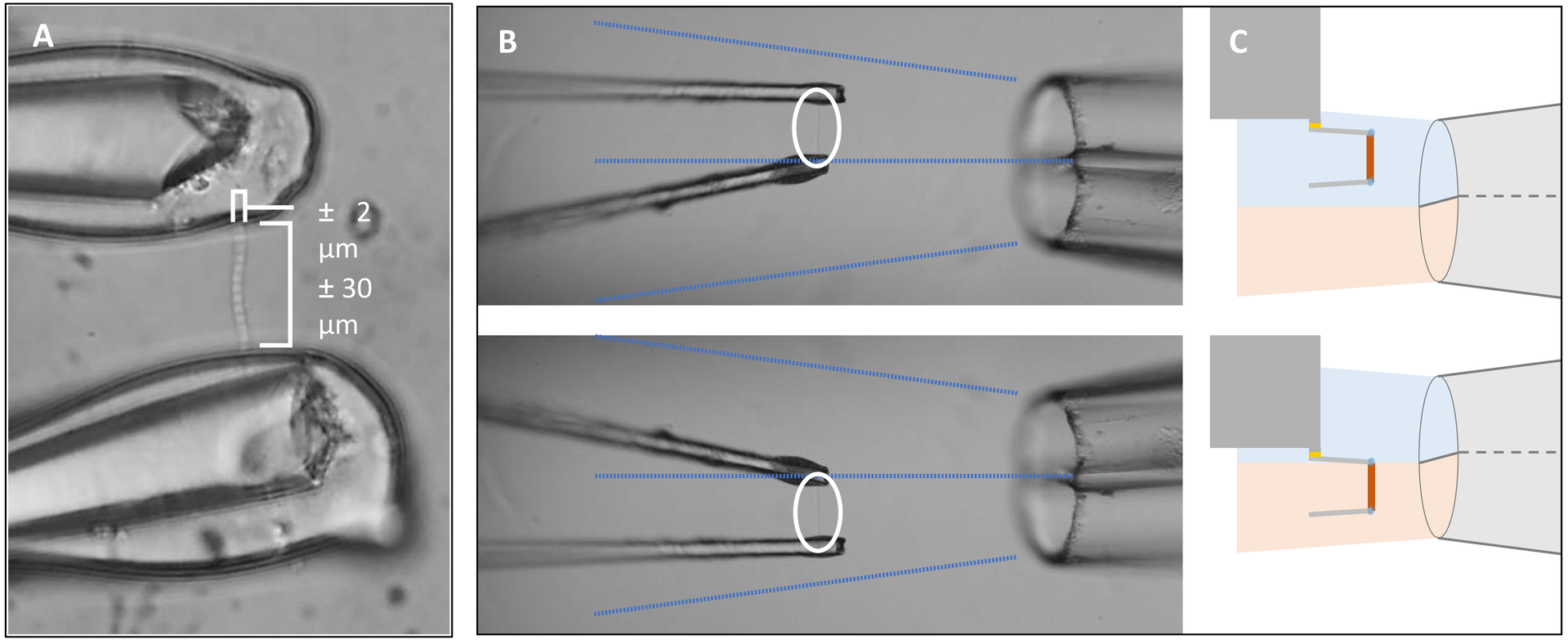

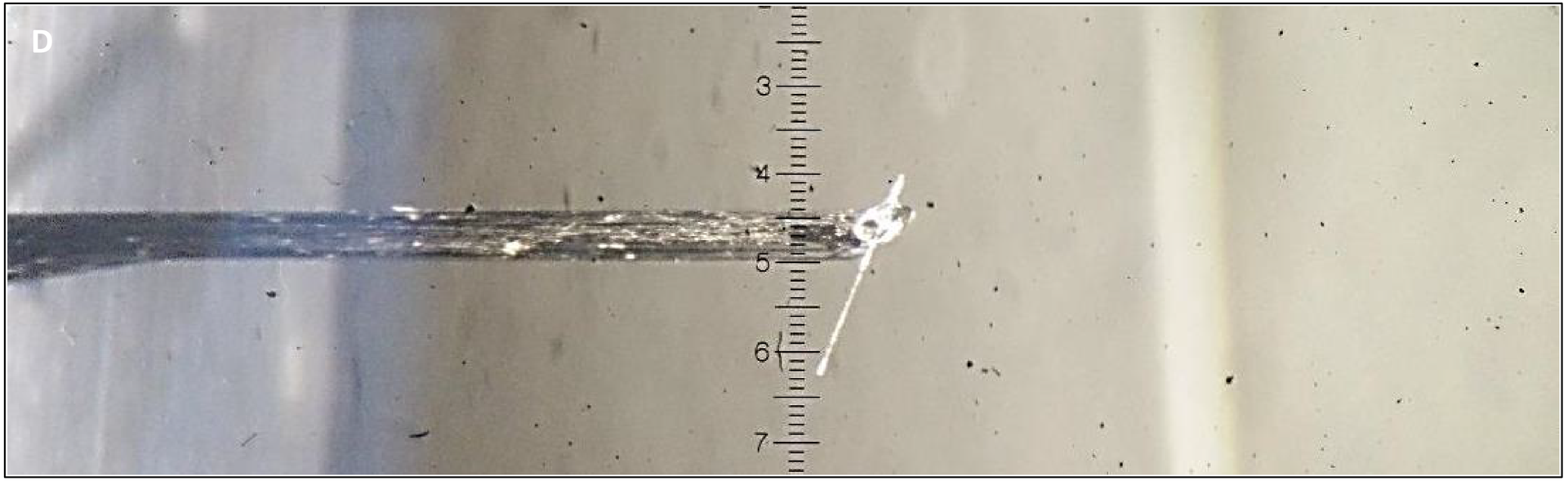

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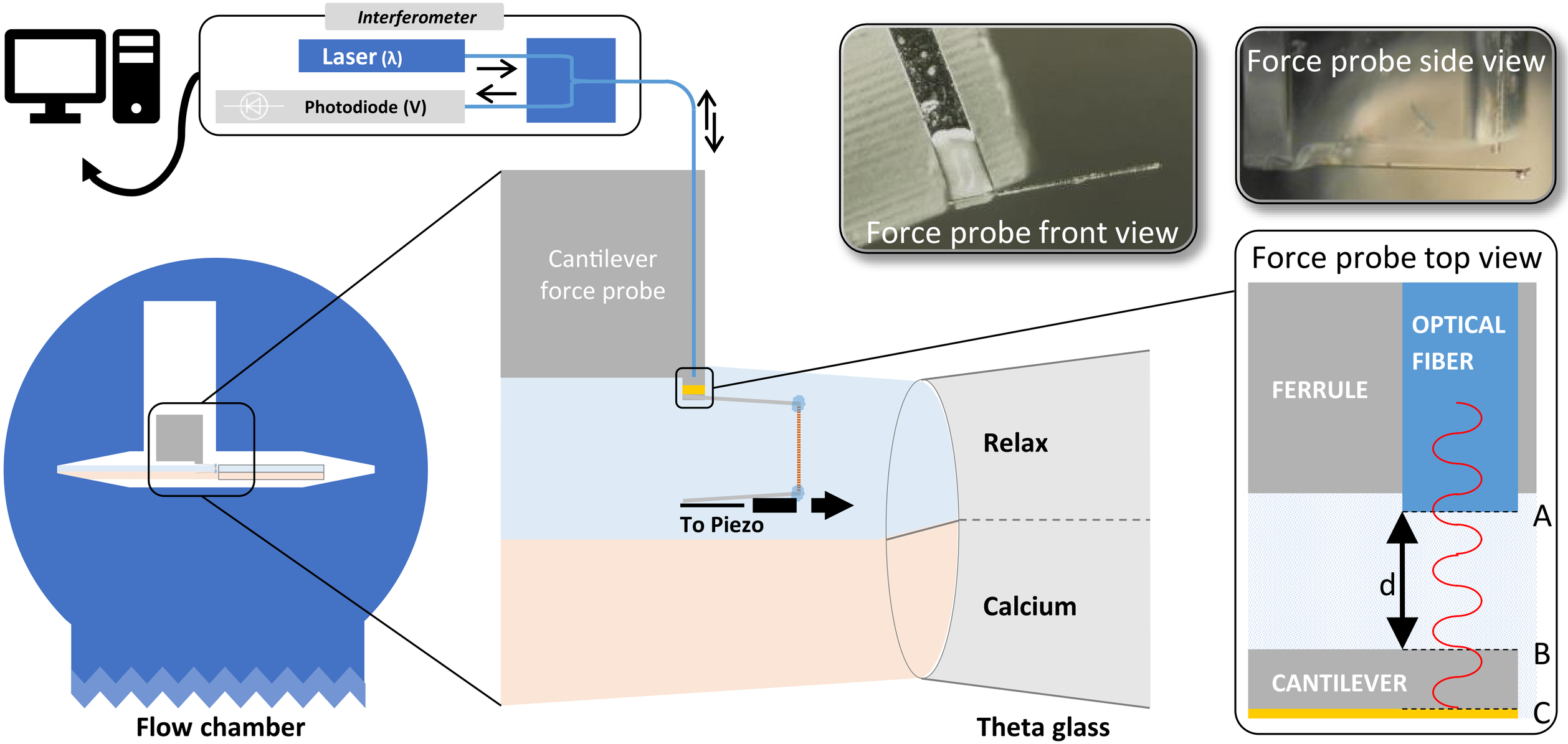

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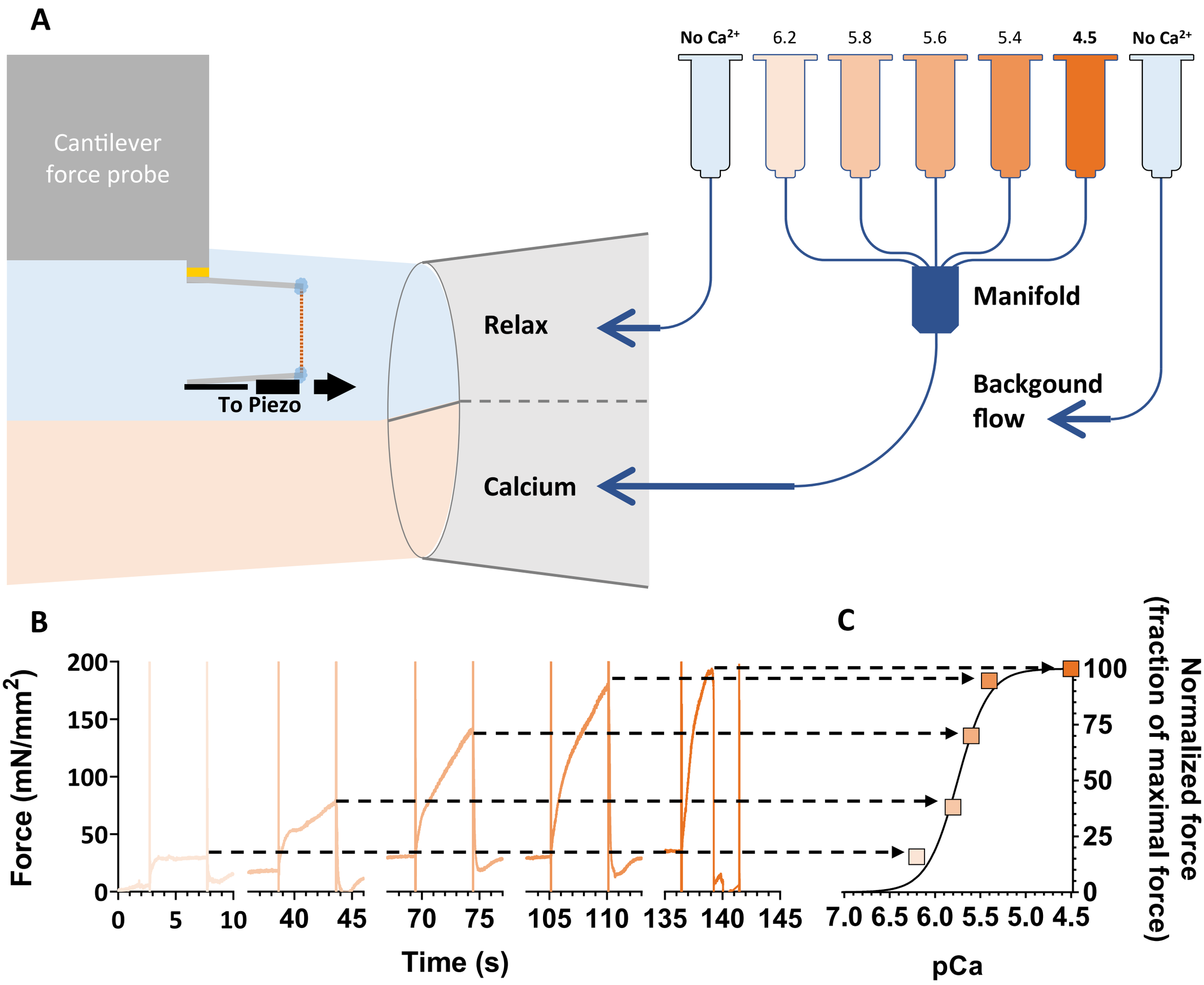

Figure 5

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A

Piezo signal generator Manual Control -4.99V

Source: Manual
Shape: Fixed
Initial: 0um

Fixed | Pulse | Sawtooth | Triangle | Trapezoid | Sine | ?

Initial

Piezo movement (um) 0

1

Stop Signal Generator

B

Fast-Step signal generator Manual Control 3.00V

Source: Manual
Shape: Fixed
Initial: 3Volts

Fixed | Pulse | Sawtooth | Triangle | Trapezoid | Sine | ?

Initial

Potential (Volts) 4

Stop Signal Generator

C

Fast-Step signal generator Manual Control 3.00V

Source: Manual
Shape: Fixed
Initial: 3Volts

Fixed | Pulse | Sawtooth | Triangle | Trapezoid | Sine | ?

	Initial		Level	Delay	Level	Delay
Potential (Volts)	4	1 x	3		4	
Duration (s)	1			5		1

Stop Signal Generator

D

Piezo signal generator Manual Control -4.99V

Source: Manual
Shape: Fixed
Initial: 0um

Fixed | Pulse | Sawtooth | Triangle | Trapezoid | Sine | ?

	Initial		Ramp	Delay	Ramp	Delay
Piezo movement (um)	0	1 x	15		0	
Duration (s)	0.5		0.01	0.01	0.01	1

Stop Signal Generator

E

Piezo signal generator Manual Control -4.99V

Source: Manual
Shape: Fixed
Initial: 0um

Fixed | Pulse | Sawtooth | Triangle | Trapezoid | Sine | ?

	Initial		Ramp	Delay	Ramp	Delay
Piezo movement (um)	51.6	10 x	51.6-5		51.6-5	
Duration (s)	2		0.5	10	0	0

Stop Signal Generator

Figure 6

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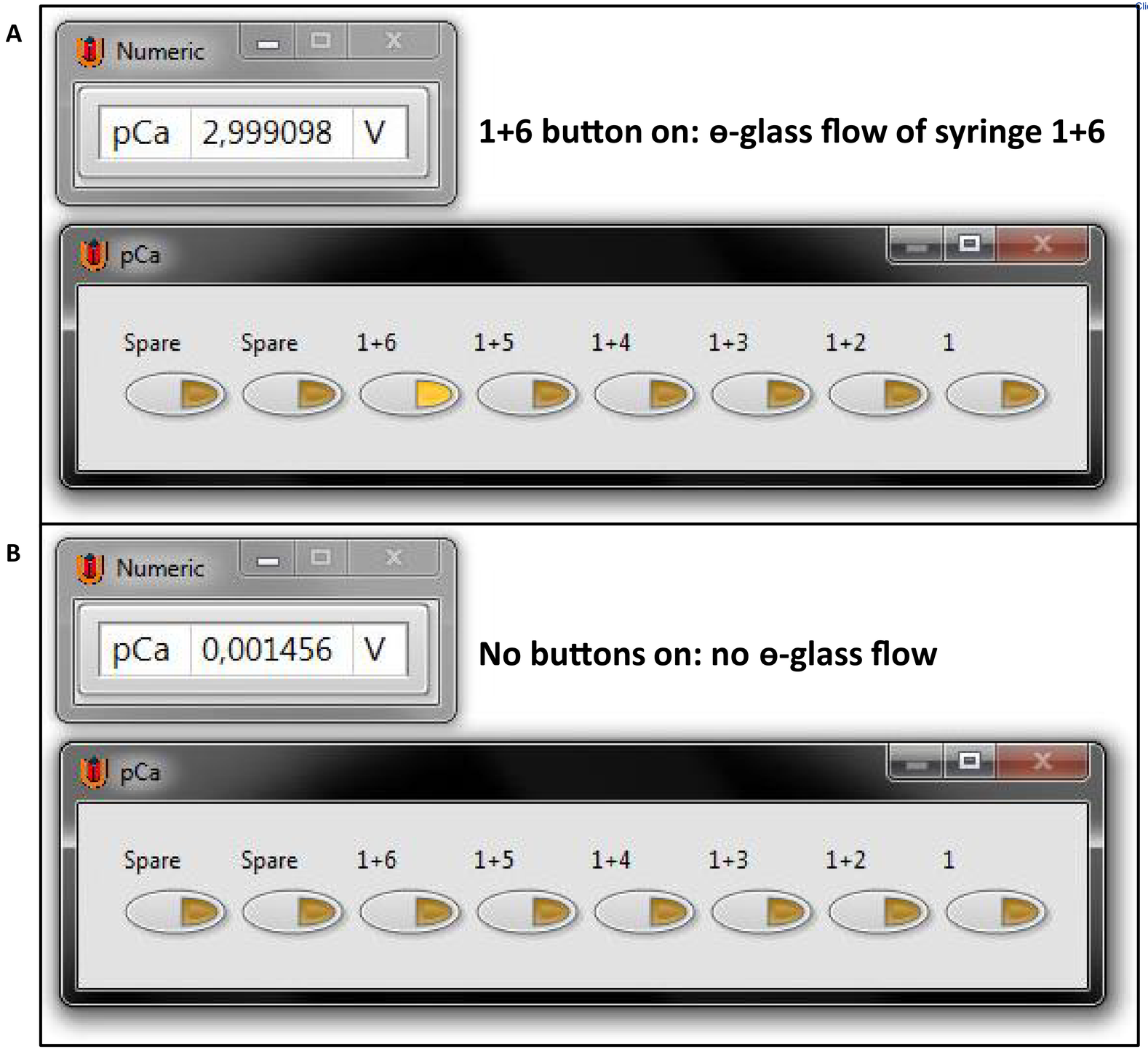

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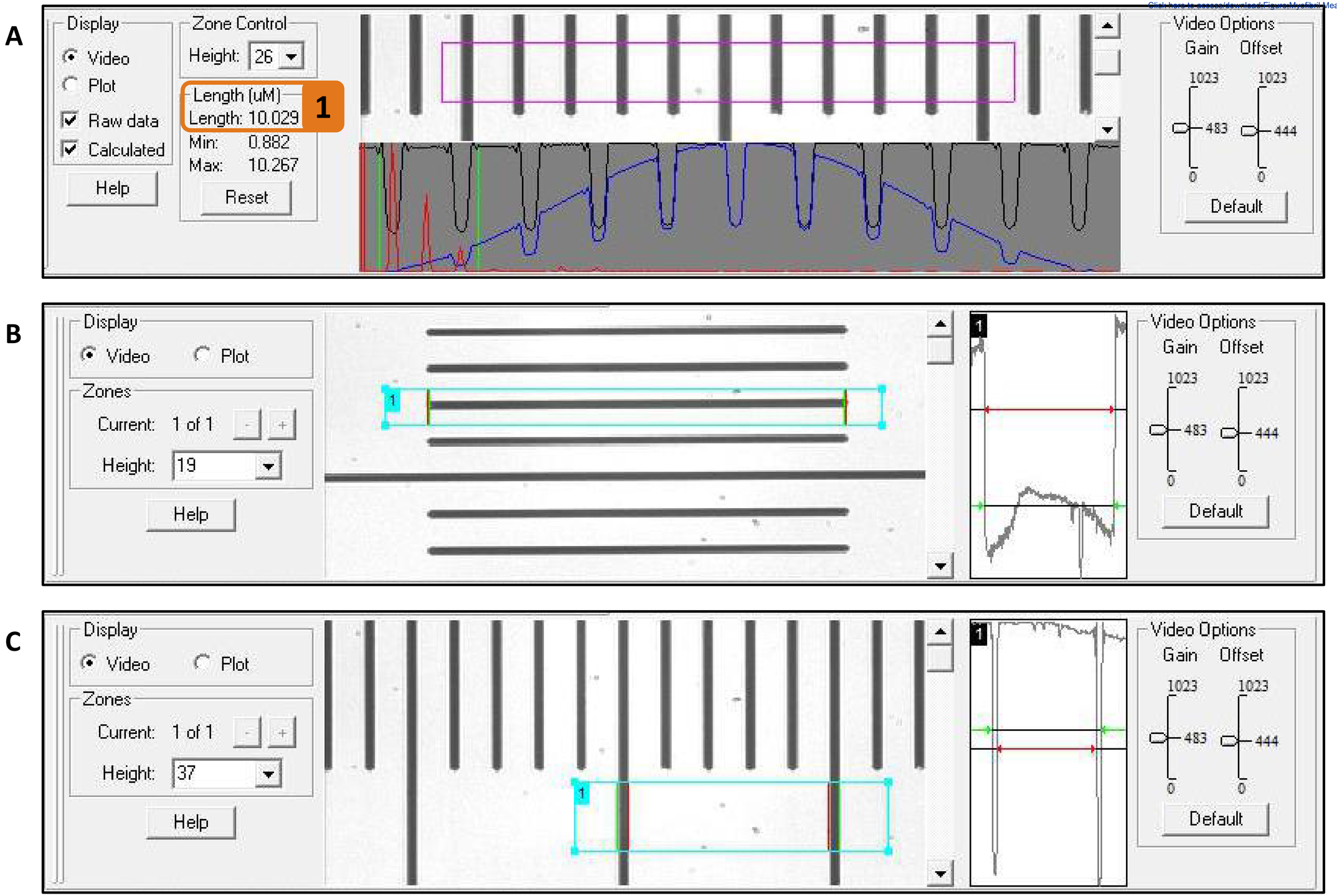

Figure 8

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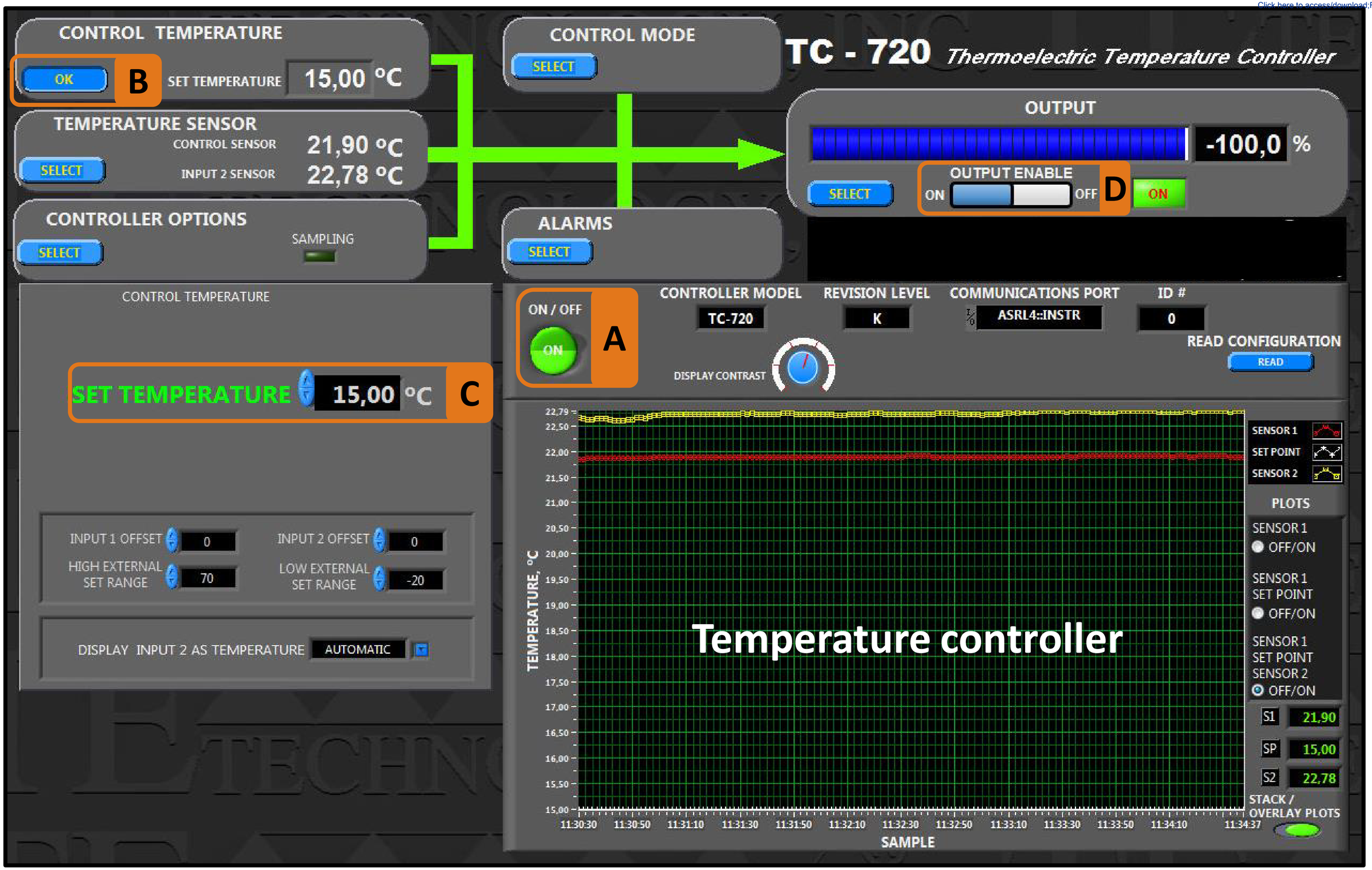

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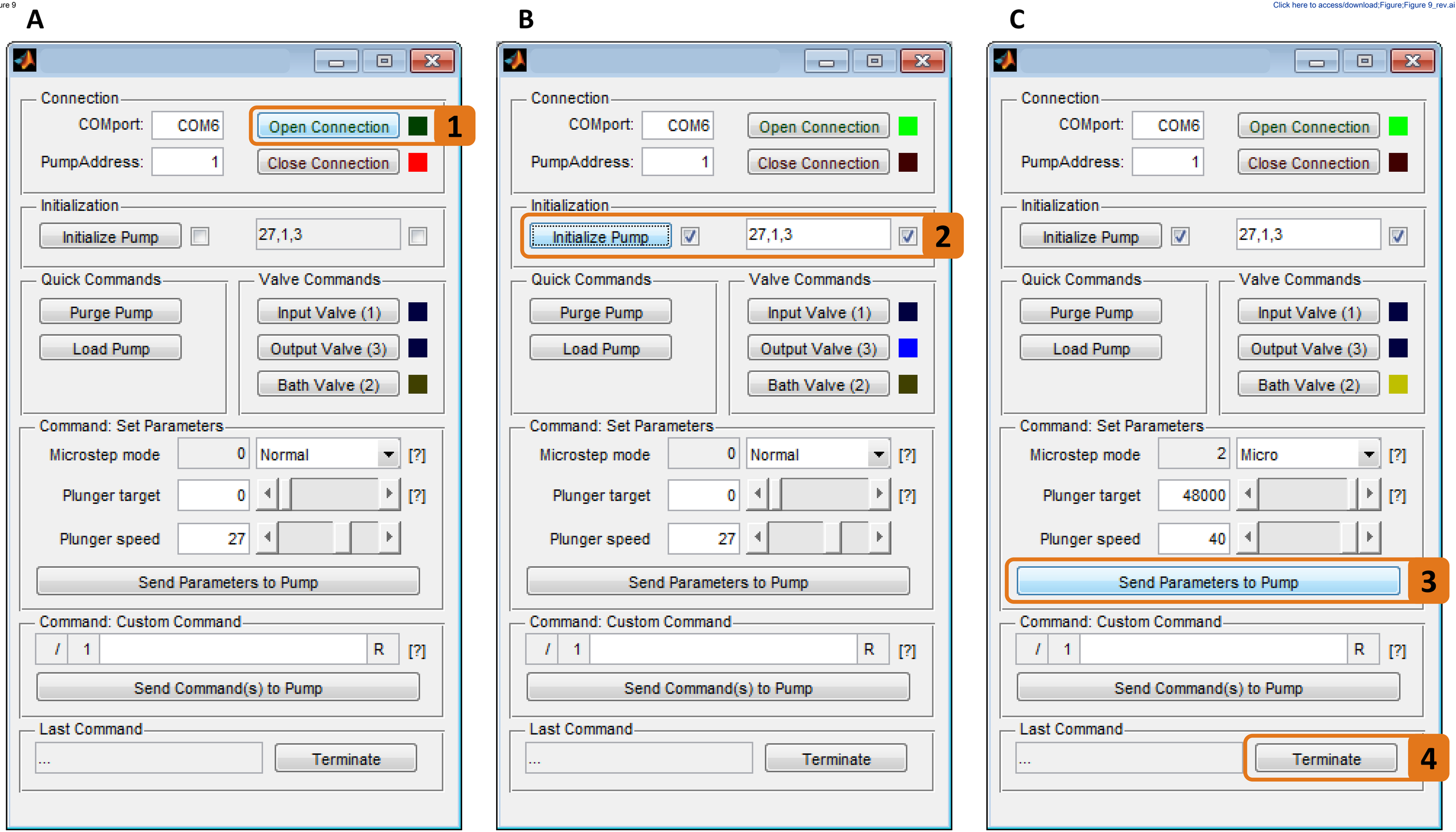

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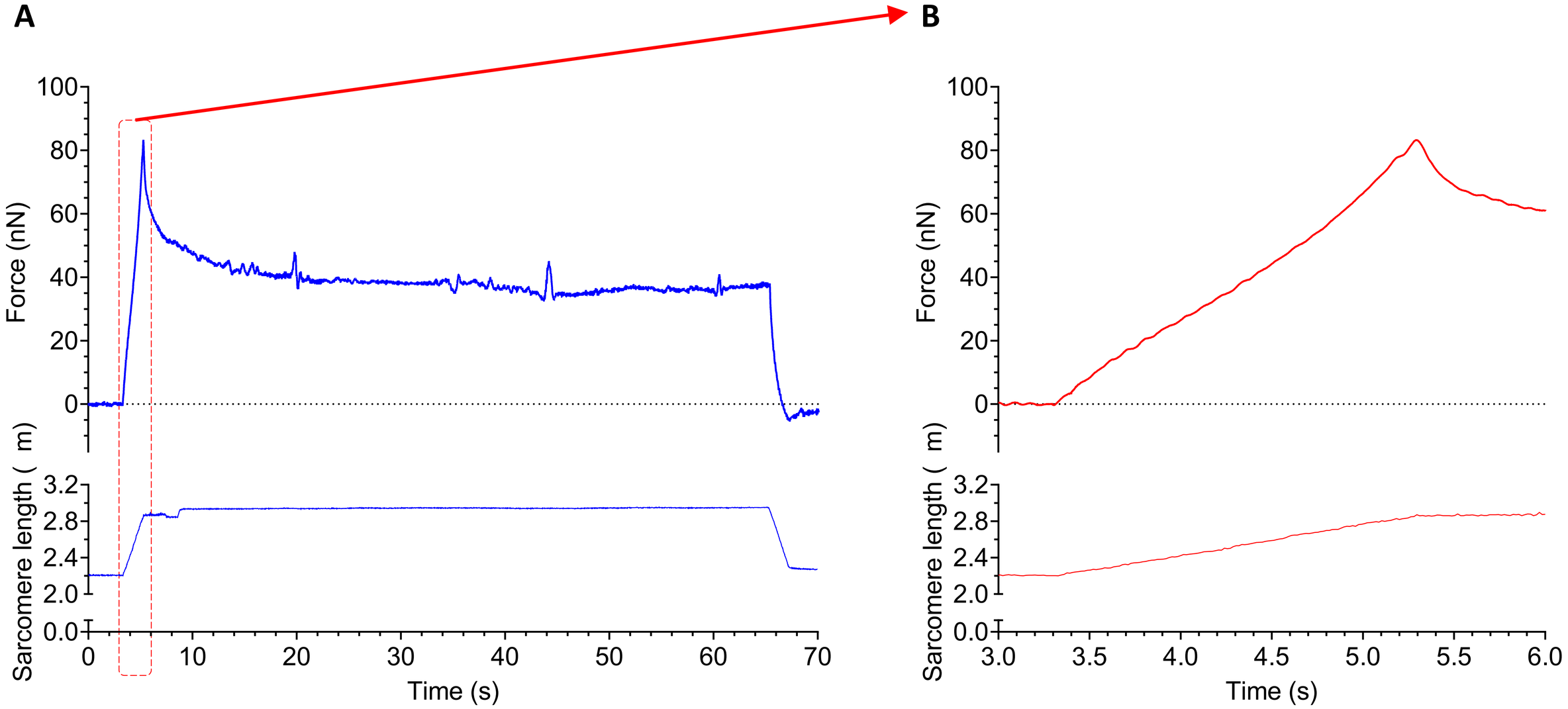

Figure 11

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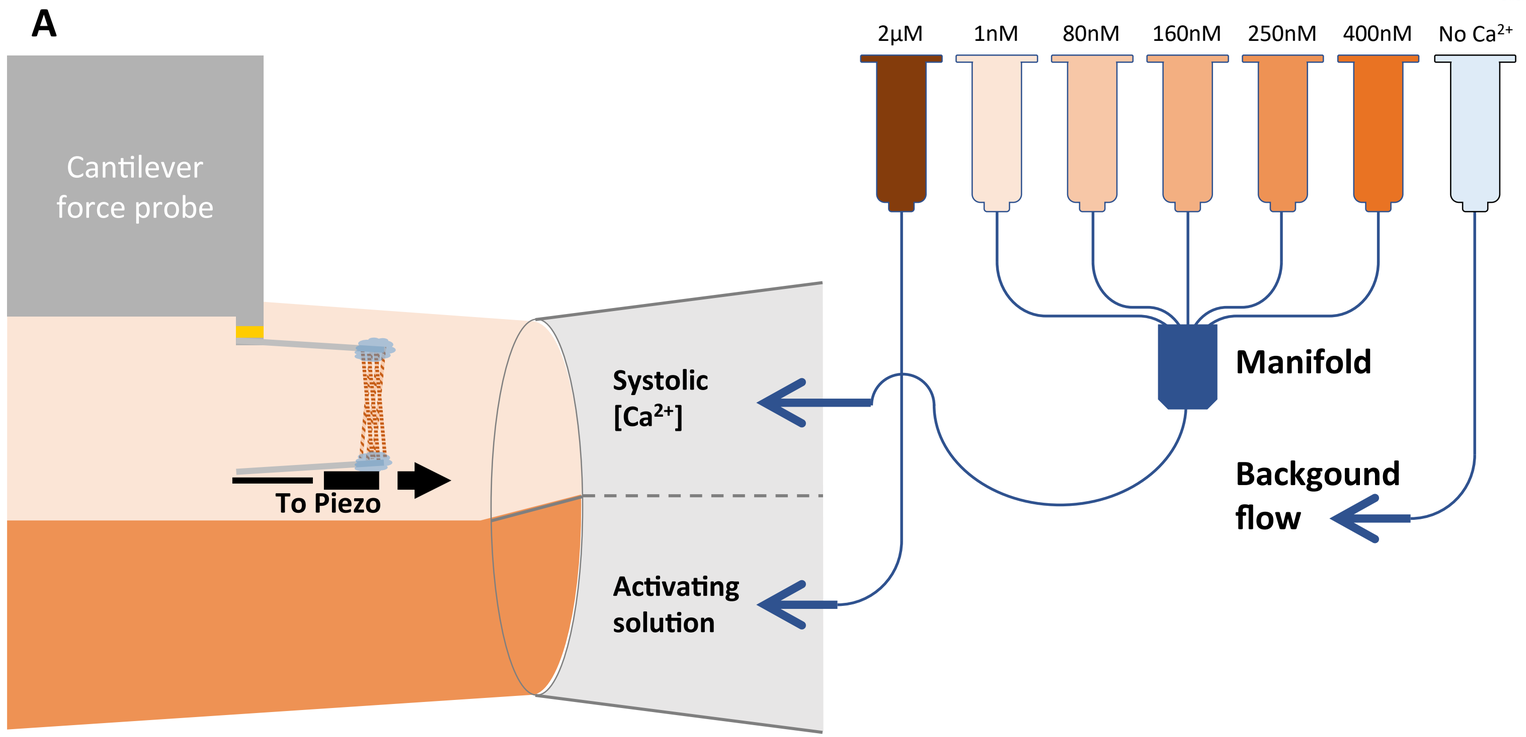

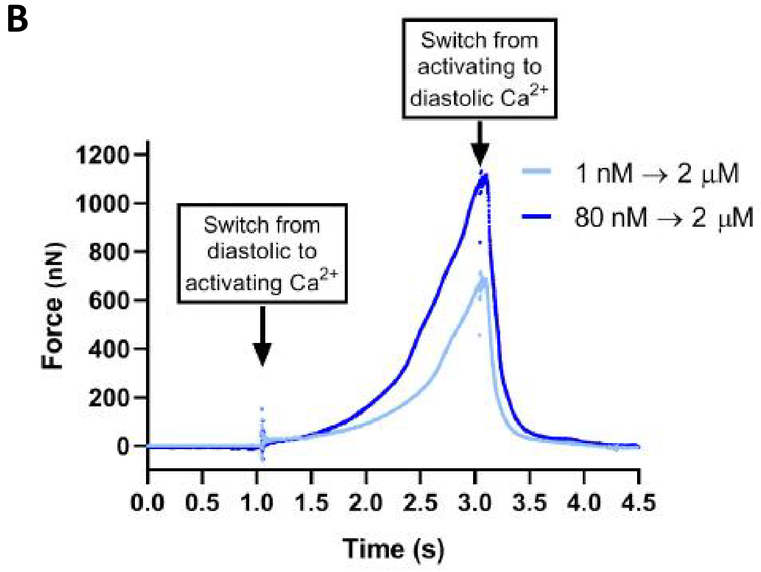

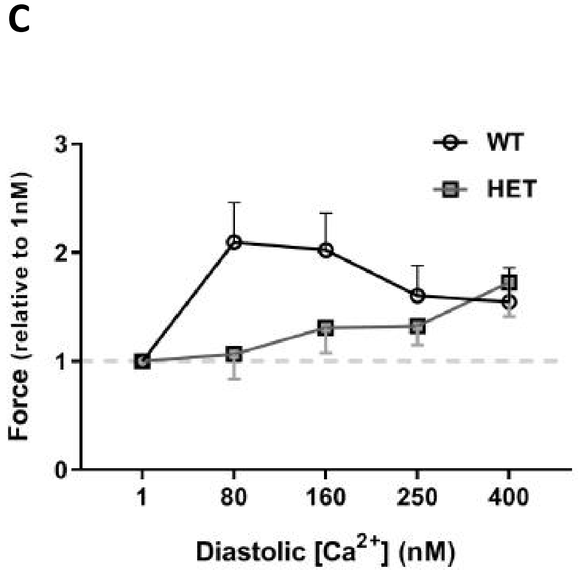

Table 1

Click here to access/download;Table;Myofibril Measurement System - Table 1.xlsx

Step	Device	Description	Shape	Initial	
1.7.1.	Piezo	Initialisation passive tension	Fixed	51.6 µm	
1.7.2.	Piezo	Initialisation active tension	Fixed	0 µm	

Step	Device	Description	Shape	Initial	Delay
3.4.4. / 4.1.1.4. / 4.1.2.4.	Fast-step	Testing ɵ-glass position / Activation of myofibril	Pulse	4 V	1 s
4.1.3.4.	Fast-step	Activation of myofibril (include k_{TR})	Pulse	4 V	1 s

Step	Device	Description	Shape	Initial	Delay
4.1.3.1. / 4.1.3.6.	Piezo	Shortening-Restretch for k_{TR}	Trapezoid	0 µm	0.5 s
4.2.1.1. / 4.2.1.4.	Piezo	Continues stretch	Trapezoid	51.6 µm	2 s
4.2.1.4.	Piezo	Return myofibril to slack length	Trapezoid	1.6 µm	2 s
4.2.2.3.	Piezo	Stepwise stretch	Trapezoid	51.6 µm	2 s
4.2.3.	Piezo	Return myofibril to slack length	Trapezoid	1.6 µm	2 s

Reps	Level	Delay	Level	Delay
1 x	3 V	5 s	4 V	1 s
1 x	3 V	10s	4 V	1 s

Reps	Ramp Level	Ramp Duration	Delay	Ramp Level	Ramp Duration	Delay
1 x	0 + 0.15 * L_0 = __ µm	0.01 s	0.01 s	0 µm	0.01 s	1 s
1 x	51.6 - 0.30 * L_0 = __ µm	2 s	0 s	51.6 - 0.30 * L_0 = __ µm	0 s	1 s
1 x	51.6 µm	5 s	0 s	51.6 µm	0 s	1 s
10 x	51.6 - 5 µm	0.5 s	10 s	51.6 - 5 µm	0 s	0 s
1 x	51.6 µm	5 s	0 s	51.6 µm	0 s	0 s

Table of materials

Click here to access/download;Table of Materials;Myofibril Measurement System - Table of Materials.xlsx

Name of Material/Equipment	Company	Catalog Number	Comments/Description
50 ml XLP Glass Syringe	Bio Spec Products, Inc.	985370-XL	To isolate myofibrils
AC Power Adapter for EXT/EX2 Systems	Custom coded		Matlab
Cavro pump control software	Custom fabricated		Includes Labview program to control over serial connection; To control valves
Cavro® XLP 6000 Pump	Custom fabricated		To cool the Peltier module
Computer Liquid Cooling System, Rev1.3	Custom fabricated		
Data acquisition panel	Custom fabricated		Aluminum tissue chamber
Excel	Custom fabricated		To control the valves; Includes PC software to control over USB
Fast step perfusion system	IonOptix		System controller software: data recording software with advanced signal generator for piezo and fast-step
Inverted microscope	IonOptix	MCS100	To record sarcomere length
IonWizard	IonOptix		Includes: Optiforce (interferometer), Micromanipulators, Signal interface, Piezo motor and controller. Based on the MyoStretcher
Liquid cooling block for Peltier module	IonOptix		Force probe
Microscope light	Koolance	ADT-EX004S	
Microscope slides 40x40mm #0	Koolance	EX2-755	To cool the Peltier module
Myocam-S	Microsoft		Data registration
Myostretcher system	Olympus	IX71	
Optical force transducer (0.7-1.0 N/m)	Olympus	TH4-200	
PC systems	Sigma-Aldrich	529265	Poly(2-hydroxyethyl methacrylate); Coating for microscope slides to prevent sticking of tissue
Peltier Thermoelectric Cooler Module	Sigma-Aldrich	78471	Crystals to dissolve in ethanol resulting in glue
Poly-HEMA	TE Technology, Inc.	TE-63-1.0-1.3	To cool the tissue flow chamber
Shellac wax-free	TE Technology, Inc.	TC-720	Includes PC software to control over USB
Thermoelectric temperature controller	Tecan Trading AG	20736652	
Theta glass	Tecan Trading AG	20739263	Syringe pump to induce backgroundflow together with fast-step perfusion system; Outflow from tissue flow chamber
Theta glass holder	Thermo scientific	2441081	
Tissue flow chamber	Warner Instruments (Harvard Bioscience, Inc.)	Discontinued	Alternative: SF-77CST/VCS-77CSP
Tissue-Tearor + Probe	Warner Instruments (Harvard Bioscience, Inc.)	TG150-4	To perfuse the tissue
UniFRY valve controller			1 PC for IonWizard and 1 PC for other software

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TITLE:

Isolating Myofibrils from Skeletal Muscle Biopsies and Determining Contractile Function with a Nano-Newton Resolution Force Transducer

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KEYWORDS:

Skeletal Muscle, Sarcomere Kinetics, Myofibril Mechanics, Contractility, Calcium, Cantilever nano-Newton Force Probe

SUMMARY:

Here, we present a protocol to assess the contractile properties of striated muscle myofibrils with nano-Newton resolution. We employ a setup with an interferometry-based, optical force probe. This setup generates data with a high signal to noise ratio and enables the assessment of the contractile kinetics of myofibrils.

ABSTRACT:

Striated muscle cells are indispensable for the daily life activity of humans and animals. They are comprised of myofibrils which consist of serially linked sarcomeres, the smallest contractile units in muscle. Sarcomeric dysfunction contributes to muscle weakness in patients with mutations in genes encoding for sarcomeric proteins. Myofibril mechanics allows for the assessment of actin-myosin interactions without potential confounding effects of damaged, adjacent myofibrils – when measuring the contractility of single muscle fibers (a collection of interconnected myofibrils) ultrastructural damage and misalignment of myofibrils might contribute to impaired contractility. If structural damage is present in the myofibrils, they likely break during the isolation procedure or during the experiment. Furthermore, studies in myofibrils provide the assessment of actin-myosin interactions in the presence of the geometrical constraints of the sarcomeres. For instance, measurements in myofibrils can elucidate whether myofibrillar dysfunction is the primary effect of a mutation in a sarcomeric protein. In addition, perfusion

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with calcium solutions or compounds is near instant due to the small diameter of the myofibril. This makes myofibrils eminently suitable to measure the rates of activation and relaxation during force production. The protocol described in this paper employs an optical force probe based on the principle of a Fabry-Pérot interferometer capable of measuring forces in the nano-Newton range. Coupled to a piezo length motor and a fast-step perfusion system, this setup enables the study of myofibril mechanics with high resolution force measurements.

INTRODUCTION:

Striated muscle cells are indispensable for daily life activities. Limb movement, respiratory function and the pumping motion of the heart rely on the force generated by muscle cells. Skeletal muscle consists of muscle fascicles containing bundles of single muscle fibers (**Figure 1A**). These muscle fibers are comprised of myofibrils, which are formed by serially linked sarcomeres (**Figure 1B, D**). The sarcomeres contain thin and thick filaments. These primarily consist of chains of actin and myosin molecules, respectively (**Figure 1B**). Actin-myosin interactions are responsible for the force generating capacity of muscle. Patients with mutations in genes encoding for sarcomeric proteins, such as nebulin, actin, and troponin T, suffer from muscle weakness, due to the contractile dysfunction[1].

The quality of muscle contractility can be studied at various levels of organization, ranging from in vivo whole muscles to actin-myosin interactions in in-vitro motility assays. During the past decades, several research groups have developed setups to determine the contractility of individual myofibrils[2–10]. These setups are based on the detection of changes in laser deflection from a cantilever, i.e., optical beam deflection, caused by the contraction of the myofibril (for details, see Labuda et al.[11]). Although determining the contractile function of myofibrils has some limitations (e.g., the dynamics of the excitation-contraction coupling processes which are upstream of the myofibrils are lacking), there are multiple advantages provided by this approach. These include (1) the ability to assess actin-myosin interactions in the presence of the geometrical constraints of the sarcomeres; (2) the ability to assess actin-myosin interactions without potential confounding effects of damaged, adjacent myofibrils – when measuring the contractility of single muscle fibers (a collection of interconnected myofibrils) ultrastructural damage and misalignment of myofibrils might contribute to impaired contractility (**Figure 1D**); (3) the small diameter of myofibrils (~1 µm, **Figure 2A**) and the lack of membranes allows for the near instant calcium diffusion into the sarcomeres. Furthermore, if structural damage is present in the myofibrils, they likely break during the isolation or during the experiment. Hence, assessing myofibril contractility is an elegant method to study the basic mechanisms of muscle contraction and to understand whether disturbed actin-myosin interactions are the primary cause of muscle disease caused by mutations in sarcomeric proteins.

Recently, we have developed a setup to determine the contractility of myofibrils, in which we incorporated a cantilever force probe i.e., Optiforce with nano-Newton resolution. This force probe is based on the principle of interferometry. Interferometry enables the use of relatively stiff cantilevers. This makes it possible to measure force with little deflection of the cantilever, approaching isometric contractions of the myofibril. The probe allows for the assessment of low passive and active forces, with high signal-to-noise ratio, that are produced by a single myofibril

isolated from different muscle biopsies, including those from human subjects. The optical cantilever force probe, incorporated in our setup, is based on a Fabry-Pérot interferometer[12]. The interferometer detects small displacements between an optical fiber and a gold coated cantilever mounted on a ferrule (**Figure 3**). The gap between the optical fiber and the cantilever is called the Fabry-Pérot cavity. Myofibrils are mounted between the probe and piezo using two glue coated glass mounting fibers. The force production of the myofibril can be mathematically derived from the interferometer data. Interferometry is based on the superposition - or interference - of two or more waves (in our setup three light waves). Laser light with a wavelength between 1528.77 nm and 1563.85 nm is emitted from the interferometer and is sent through the optical fiber. In the probe, the light is reflected (1) at the interface between the optical fiber and the medium (**Figure 3A**), (2) at the interface of the medium and the cantilever (**Figure 3B**) and (3) at the interface between the metal and gold coating of the cantilever (**Figure 3C**). The reflection at interface A and B are dependent on the refractive index, *n* of the medium in which the probe is submerged. The light, consisting of the three superimposed reflections, returns to a photodiode in the interferometer. The photodiode measures the intensity of the light, which is the result of the interference pattern of the three superimposed reflections. When contractile force is generated by activating or stretching a myofibril, the myofibril pulls on the cantilever. This movement changes the cavity size (*d)* and consequently, the number of wavelengths that fit in the cavity. The light reflected at the cantilever will have a different phase, resulting in a different interference pattern. The photodiode records this change of interference pattern intensity as a change in Volts. Subsequently, myofibril force generation is calculated from the change in Volts, taking in to account the cantilever stiffness. The force probe is calibrated by the manufacturer by pushing the tip of the mounting needle, attached to the free handing end of the cantilever, against a weighing scale while keeping the bending of the cantilever equal to a multiple of the wavelength of the readout laser[13]. Thus, interferometry is a highly sensitive method to detect small changes in distance, allowing for measurement of forces with nano-Newton resolution. This resolution enables the assessment of myofibrillar force production with a high signal-to-noise ratio. While traditional interferometry limits the range of measurements to the linear part of the interference curve, using a lock-in amplifier and modulation of the laser wavelength overcomes this limitation[14]. This is explained in more detail in the discussion section in this paper.

To measure myofibril active tension, we incorporated a fast-step perfusion system to expose the myofibril to solutions with calcium (**Figure 4A**). The fast-step perfusion system enables solution changes to occur within 10 ms. Because of their small diameter, calcium diffusion into the myofibrils is near instantaneous. Hence, this system is particularly suitable to measure the rates of actin-myosin binding during activation and release during relaxation. The rate of activation (k_{ACT}) and relaxation (K_{REL}) can be determined from the activation-relaxation curves. Also, by exposing the myofibrils to calcium solution of increasing concentration, the force-calcium relationship and subsequently, calcium sensitivity can be determined.

Furthermore, a piezo length motor enables fast stretching and shortening of the myofibril. This offers the possibility to study the viscoelastic properties (passive tension) of the myofibril, as well as performing a rapid shortening and re-stretch of the myofibril to determine the rate of tension

redevelopment (k_{TR}). The parameters retrieved from both active and passive tension experiments can be altered by gene mutations in a sarcomeric protein.

In this protocol, we describe the use of our custom-built setup to measure the active and passive contractile characteristics of myofibrils isolated from healthy human, patient and mouse skeletal muscle.

PROTOCOL:

The protocol for obtaining human biopsies was approved by the institutional review board at VU University Medical Center (#2014/396) and written informed consent was obtained from the subjects. The protocol for obtaining animal muscle biopsies was approved by the local animal ethics committee at VU University (AVD114002016501)

1. Preparation and Myofibril isolation

NOTE: Use previously described methods to glycerinate biopsies, prepare the calcium (pCa) solutions[7,16,17] and to isolate myofibrils[2,18].

1.1. Thaw the pCa solutions – relaxing (pCa 9.0, Rx) and activating (pCa 4.5, Act) solutions – and inhibitors – 1 M E64, 1 M DTT, 1 M leupeptin, 1 M PMSF – which are ~~(~~stored at -80 ˚C~~)~~.

1.2. Take a glycerinated piece of striated muscle biopsy of approximately 1 mm^3 and place it in a small Petri dish with 1:1 Rx/glycerol (vol/vol) solution and place the Petri dish on a cold plate at 4 ˚C.

1.3. Dissect the piece of muscle using dissection microscope and forceps, separating single muscle fibers without isolating them from the piece of muscle.

NOTE: Remove as much fatty and connective tissue as possible. This will prevent the contamination of the myofibril suspension.

1.4. Transfer the piece of dissected tissue to a 5 mL tube with 1.5 mL of relaxing solution with inhibitors (1uL/mL E-64, 1uL/mL Leupeptin, 1uL/mL DTT and 1,25uL/mL PMSF). Allow the tissue to temper at approximately 4 ˚C for 1 h.

1.5. During incubation, boot up both PC's, turn on devices and open the associated software (see **Table of Materials**).

1.6. Submerge the force probe in ultrapure water in a Petri dish and calibrate the probe.

1.6.1. Press the '**Start wizard**' on the interferometer and follow the onscreen instructions. After pressing **calibrate**, tap on the microscope stage.

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Please remove the redundancy from the protocol and make it crisp in a stepwise manner.

Please remove all commercial terms from the manuscript.

Commented [A3]: Compositions of the solution, what kind of inhibitors are used?

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Commented [A5]: Is this relaxing solution containing glycerol?
Volume used?

Commented [A6]: What kind of inhibitors are used?

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NOTE: Tapping on the microscope stage will cause the cantilever to deflect and pass through fringes. This enables calibration of the probe.

1.6.2. Leave the probe submerged in the ultrapure water in the Petri dish after calibration.

1.7. Initialize the piezo motor position. To do so, follow one of the steps details below.

1.7.1. When the piezo motor will be used for k_{TR} tension, set the length to 0 µm. Signal generator settings can be found in (**Table 1,** **Figure 5C**).

1.7.2. When the piezo motor will be used for passive tension, set the length to 50 µm. Signal generator settings can be found in (**Table 1**).

NOTE: The difference between steps is the initial position of the piezo length motor. To stretch the myofibril, the piezo motor needs to pull to increase the distance between both mounting needles and lengthen the myofibril. To slacken the myofibril, the piezo motor needs to push to decrease the distance between both mounting needles and shorten the myofibril.

1.8. Prepare microscope slide: Pipette 150 µL poly-HEMA solution (5% poly-HEMA in 95% ethanol ratio, weight per volume) on a microscope slide and spread it across the slide so that all is covered.

NOTE: If a myofibril suspension is pipetted on an uncoated microscope slide, myofibrils which sink to the bottom will stick to the microscope slide and it will not be possible to glue them.

1.9. Fill syringes with pCa solutions (see **Figure 4A)** and prime the perfusion system.

NOTE: In these steps all tubes will be pre-filled with the appropriate solution to make sure all air bubbles will be removed from the tubing.

1.9.1. Fill the inflow tubing of the flow chamber (**Figure 3**) inflow with Rx;

1.9.2. When used, flush the manifold with ultrapure water to remove air (connect syringe with ultrapure water to the outlet and flush in it in the reverse direction. Block the ports of the manifold that are unused).

1.9.3. Enable flow for each pCa syringe to fill their respective tubes with pCa solution and following, connect them to the manifold and the Θ-glass.

1.9.4. Open valve 1 and 6 with the data acquisition panel software (see **Table of Materials**) by checking the button '1+6' (**Figure 6B**) to fill the Θ-glass with pCa 9.0 and 4.5 solution and close valves when the Θ-glass is filled (**Figure 6A**).

Commented [A9]: Submerged in?

Commented [A10]: Reworded, please check.

Commented [MvdL11R10]: We appreciate the effort the reviewer put in, but we believe the original sentence better describes the NOTE. The piezo motor is not pushed or pulled, the piezo motor is the device that exerts the motion on the mounting needle and thus, on the myofibril. We have reworded the sentences to clarify the NOTE.

Commented [MvdL12]: This step (1.8) was removed from the manuscript by the editor. However, our opinion is this step is quite essential for the protocol. In our experience, when not coating the microscope slide, myofibrils will stick to the bottom and it will not be possible to glue them.
We have reworded the step and added a NOTE to provide clarity.

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Commented [A14]: What are the respective solutions?

2. Mounting a myofibril

2.1. Coat a microscope slide with polyhydroxyethylmethacrylate (poly-HEMA) to prevent myofibrils from sticking to the glass.

2.2. Prepare the homogenizer (see **Table of Materials**) for tissue homogenization. Clean internal rotor rod with a clean tissue paper, assemble the homogenizer and spin once for 15 s in alcohol and thrice for 15 s each in ultrapure water. Pre-rinse the homogenizer in relaxing solution 1 x 15 s on ice.

2.3. Place homogenizer rod in the tube containing the muscle tissue (as described in step 1.4.)and, while keeping the tube on ice, spin the rotor for 15 s on speed 5 to tear the muscle tissue and obtain a myofibril suspension.

2.4. Pipette ~50 µL from myofibril suspension and ~250 µL relaxing solution on the microscope slide coated with Poly-HEMA in the tissue bath. This will form a liquid drop. Cover the bath with a lid to protect from dust and wait 5-10 minutes to allow the myofibrils to sink to the bottom.

NOTE: The ratio between the suspension and the relaxing solution is dependent on the quality of the isolation, adjust accordingly. For example, if the myofibril yield is low and only few suitable myofibrils are present in the suspension, take more myofibril suspension and dilute with less relaxing solution (for example: 75 µL myofibril suspension and 225 µL relaxing solution).
Heart and skeletal muscle tissue is easy to recognize due to its striation pattern. Using a 10x or 40x objective, this pattern is also visible in a single myofibril. Thus, in the case other tissue is present in the suspension one can select myofibrils visually.
One can opt to skip waiting 5-10 mins. However, this increases difficulty to glue a myofibril.

2.5. Coat mounting needles with glue (Shellac + Ethanol; 120 mg shellac in 2 mL of 70% EtOH). To do so, heat the glue at 65 ˚C for 30-60 s and pipette ~6 µL on a new uncoated glass slide. Dip the tip of each mounting needle in the glue and repeat until a layer of glue is visible. Move the probe and piezo up vertically with the micromanipulators to make room to place the tissue bath on the microscope stage. Remove the glass slide containing the glue.

2.6. Mounting of myofibril

2.6.1. Place the tissue bath – with the microscope slide coated with Poly-HEMA containing the myofibril suspension – on the microscope stage. Use the stage to find a suitable myofibril with 40x objective (if necessary, move and rotate the tissue bath to move the myofibril to a mountable position).

NOTE: Look for myofibrils with a visible striation pattern and that are approximately 30 µm long. It is possible to check length and sarcomere length prior to gluing the myofibril (as

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Commented [A18]: So, the tissue is not sticking to the slide and the slide is kept in the tissue bath? Please explain why this is needed.

Commented [MvdL19R18]: We regret that we do not fully understand the editors' question. The microscope slide in the tissue bath is coated with Poly-HEMA to prevent myofibrils from sticking to the microscope slide (as described in step 1.8.).

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Commented [A21]: Are there any criteria for adjustment?

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Commented [A25]: Is this the same glass slide as above?

Commented [A26]: This part is unclear. Where do you mount the myofibril? What is the use of glass slide both in step 2.4 2.5?

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described in detail in step 3.1. and 3.2.1.). Refrain of gluing myofibrils which are torn as these are likely to break during contraction.

2.6.2. Slide flow chamber into place directly above the liquid drop (as pipetted onto the slide in step 2.4.) containing the myofibrils in the tissue bath and lower it. Stop before it hits the liquid drop.

2.6.3. Lower the piezo mounting needle and press it on the bottom tip of the myofibril. Lift it slightly to check if the myofibril is attached to the needle.

2.6.4. Lower the flow chamber far enough for the mounting needle of the probe to reach the bottom without the probe touching the flow chamber.

2.6.5. Press the mounting needle of the probe on the top tip of the myofibril. Lift it slightly to check if the myofibril attached to the needle.

2.6.6. Lift the myofibril from the bottom of the bath as far as possible without losing the ability to focus without the objective touching the bottom of the glass.

3. Initializing experiment

3.1. Using the micromanipulators, camera and system controller software (**Figure 7**A, see **Table of Materials**) to measure sarcomere length. Move the piezo and/or force probe to set the initial sarcomere length of the myofibril to 2.5 µm.

NOTE: A sarcomere length of 2.5 µm ensures optimal overlap between myosin heads and actin.

3.2. Measure myofibril dimensions: Using the vessel function of the system controller software measure length and width (**Figure 7B, C**; demonstrated in the **Video**):

NOTE: When rotating the camera, it may tilt horizontally and/or vertically. To check the alignment of the camera, a spirit level can be used to verify that the camera is rotated, and not tilted (as demonstrated in the **Video**).

3.2.1. Position the myofibril in the center of the video image using the microscope stage.

3.2.2. Draw a square from one side of the myofibril to the other side of the myofibril. For the length, make sure you include the dark edge of the glue droplets (**Figure 2A**) in the square since the image processing is based on contrast.

3.2.3. Start recording the data in the system controller software (see **Table of Materials**) by pressing 'Start' and after 5 seconds pause the system controller software data recording by pressing the 'pause' button. The length is now recorded in the data.

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3.2.4. For the width you first rotate the camera (see **Table of Materials**) 90 ˚ and then use the contrast of the edge of the myofibril itself.

3.2.5. Start recording the data in the system controller software (see **Table of Materials**) by pressing 'Start' and after 5 seconds pause the system controller software data recording by pressing the 'pause' button. The width is now recorded in the data.

3.3. If active tension of the myofibril needs to be determined, the perfusion setup needs to be used. If so, continue to step 3.2. If only passive tension will be determined, skip step 3.2.-4.1.3.7. and continue at step 4.2.

3.4. Position and initialize the perfusion setup

NOTE: this is only necessary for generation of active force. Continue to **step 4.2.** when performing passive tension experiments.

3.4.1. Initialize the fast-step motor position at 4 V (**Figure 5B**).

3.4.2. Slide the perfusion stand on the table to align the left bottom corner of the stand with the tape on the table.

NOTE: Be careful not to hit the force probe or the piezo motor!

3.4.3. Use the manipulator to roughly position the Θ-glass by eye;

3.4.4. Look through the eyepiece and carefully move the Θ-glass towards the myofibril using the manipulator;

3.4.5. Align the top channel of the theta glass with the myofibril using the manipulator and check the position by performing a fast-step (signal generator settings can be found in **Table 1**) with the system controller software (**Figure 2B-C,** see **Table of Materials**).

Note: Make sure that the bottom channel will be aligned with the myofibril during the activation phase of the fast-step (**Figure 2B-C**).

3.5. Turn on background flow of Rx (**Figure 4A**) to create a laminar background flow in the flow chamber:

NOTE: The background flow is necessary to prevent turbulent flow as a result from the pCa solution flow from the Θ-glass.

3.5.1. Turn on inflow of flow chamber with Luer valve lever;

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3.5.1.1. Send parameters to outflow pump to start drain the flow chamber and prevent overflowing of the flow chamber (**Figure 9**):

Valve: Bath valve (2); Microstep mode: Micro; Plunger target: 48000; Plunger speed: 38-40 (arbitrary).

NOTE: Make sure fluid level is stable at all times! The myofibril should not run dry and neither should the cantilever! Better have little overflow instead of to little flow.

3.6. Set temperature to desired value with the thermoelectric temperature controller (**Figure 8**, see **Table of Materials**):
Enter desired temperature and press 'Start'. Wait until the desired temperature is reached by checking the graph in the thermoelectric temperature controller software and continue.

NOTE: When performing experiments at room temperature, the thermoelectric temperature controller does not have to be used.

4. Experiment protocol(s)

4.1. Decide which active force protocol(s) you want to perform.

Note: Depending on the data necessary for the study, multiple types of active force experiments can be performed: (4.1.1) Maximum force at saturating [Ca^{2+}], (4.1.2.) Force-pCa curve to determine calcium sensitivity in addition to 4.1.1., (4.1.3.) Determine the rate of tension redevelopment by doing a shortening-restretch protocol in addition to 4.1.1. or 4.1.2.

4.1.1. Measure maximal active force.

4.1.1.1. Start recording the data in the system controller software (see **Table of Materials**) by pressing 'Start'.

4.1.1.2. Open valve 1 and 6 with the data acquisition panel (see **Table of Materials**) software by checking the button '1+6' to start ϴ-glass flow of relaxing solution and activating solution through the theta glass (**Figure 6A**).

4.1.1.3. Reset range of the interferometer so that the baseline force will be at 0 V, by selecting and pressing 'Reset range' on the interferometer (see **Table of Materials**).

4.1.1.4. When the force trace is stable, perform the theta glass fast-step (step size: 100 µm). Signal generator settings can be found in **Table 1** (**Figure 5C**). An activation–relaxation trace similar to Figure 4D will be recorded and visible in the system controller software (see **Table of Materials**).

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4.1.1.5. Pause the system controller software (see **Table of Materials**) data recording by pressing the 'pause' button.

4.1.1.6. If no more activations are to be performed, close valve 1 and 6 to stop Θ-glass flow by unchecking the button '1+6' (Figure 6B), stop the syringe pump (**Figure 9**, see **Table of Materials**) by pressing 'Terminate' and stop the background flow by closing the Luer valve.

4.1.2. Force-pCa

NOTE: this is similar to 4.1.1 Maximal active force, but with multiple activations using different pCa solutions.

4.1.2.1. Start recording the data in the system controller software (see **Table of Materials**) by pressing 'Start'.

4.1.2.2. Open valve 1 and 2 with the data acquisition panel (see **Table of Materials**) software to start flow of relaxing solution and pCa 6.2 through the theta glass.

4.1.2.3. Reset range of the interferometer so that the baseline force will be at 0 V, by selecting and pressing 'Reset range' on the interferometer (see **Table of Materials**).

4.1.2.4. When the force trace is stable, perform the theta glass fast-step (step size: 100 µm). Signal generator settings can be found in (**Table 1**).

4.1.2.5. Pause the system controller software (see **Table of Materials**) by pressing the 'pause' button.

4.1.2.6. Repeat steps 4.1.2.1 to 4.1.2.4 for Valve 1 and 3 (pCa 5.8), Valve 1 and 4 (pCa 5.6), Valve 1 and 5 (pCa 5.4), and Valve 1 and 6 (pCa 4.5).

4.1.2.7. If no more activations are to be performed, close valve 1 and 6 to stop Θ-glass flow by unchecking the button '1+6' (**Figure 6A**), stop the syringe pump (**Figure 9**, see **Table of Materials**) by pressing 'Terminate' and stop the background flow by closing the Luer valve.

4.1.3. Measure rate of tension redevelopment (K_{TR}).

NOTE: Similar to step 4.1.1 Maximal active force but with changed and added steps

4.1.3.1. Calculate the piezo movement necessary to slacken the myofibril 15 % and enter this value in the signal generator (**Figure 5D**, **Table 1**).

4.1.3.2. Start recording the data in the system controller software (see **Table of Materials**) by pressing 'Start'.

4.1.3.3. Open valve 1 and 6 with the data acquisition panel (**Figure 6A**, see **Table of Materials**) software to start flow of relaxing solution and pCa 4.5 through the theta glass.

4.1.3.4. Reset range of the interferometer so that the baseline force will be at 0 V, by selecting and pressing 'Reset range' on the interferometer (see **Table of Materials**).

4.1.3.5. When the force trace is stable, perform the theta glass fast-step (step size: 100 µm). Signal generator settings can be found in **Table 1**.

4.1.3.6. When the force plateau is reached, perform the shortening-restretch with the piezo. Signal generator settings can be found in (**Figure 5D**, **Table 1**). An activation–relaxation trace~~curve~~ similar to **Figure 4E** will be recorded and visible in the system controller software (see **Table of Materials**).

NOTE: a custom protocol can be made to automate the steps above.

4.1.3.7. Pause the system controller software (see **Table of Materials**) by pressing the 'pause' button.

4.1.3.8. If no more activations are to be performed, close valve 1 and 6 to stop Θ-glass flow by unchecking the button '1+6' (**Figure 6B**), stop the syringe pump (**Figure 9**, see **Table of Materials**) by pressing 'Terminate' and stop the background flow by closing the Luer valve.

4.2. Perform passive force measurements.

4.2.1. Perform a continues stretch.

4.2.1.1. Calculate the piezo movement necessary to stretch the myofibril and enter this value in the signal generator (**Table 1**).

NOTE: These are example settings. Calculate the amount of stretch and time of stretch relative to the sarcomere length. These settings are necessary to ensure the speed of stretch per sarcomere remains equal across myofibrils.

4.2.1.2. Start recording the data in the system controller software (see **Table of Materials**) by pressing 'Start'.

4.2.1.3. Reset range of the interferometer so that the baseline force will be at 0 V, by selecting and pressing 'Reset range' on the interferometer (see **Table of Materials**).

4.2.1.4. Perform continues stretch with the signal generator in the system controller software (see **Table of Materials**) to operate the piezo. Example signal generator settings can be found in (**Table 1**).

4.2.1.5. Shorten the myofibril to slack length with the piezo after the stretch is finished (**Table 1).**

4.2.2. Perform a stepwise stretch.

4.2.2.1. Start recording the data in the system controller software (see **Table of Materials**) by pressing 'Start'.

4.2.2.2. Reset range of the interferometer so that the baseline force will be at 0 V, by selecting and pressing 'Reset range' on the interferometer (see **Table of Materials**).

4.2.2.3. Perform a stepwise stretch with the signal generator in the system controller software (see **Table of Materials**) to operate the piezo. Example signal generator settings can be found in **Table 1** (**Figure 5**E).

4.2.3. Shorten the myofibril to slack length with the piezo after the stretch is finished. Example signal generator settings can be found in **Table 1**.

4.3. Pause the system controller software by pressing the 'pause' button (see **Table of Materials**).

4.4. Stop the recording of data by pressing the 'Stop' button in the system controller software (see **Table of Materials**).

4.5. Save the data by pressing 'File' and 'Save data' in the system controller software (see **Table of Materials**).

5. Cleaning

5.1. Remove the measured myofibril and prepare for the next myofibril:

5.1.1. Carefully tear off the myofibril while looking through the ocular with the 40x objective;

5.1.2. Move up the force probe and piezo;

5.1.3. Move up the Θ-glass and move it all the way up, to the right and to the back;

5.1.4. Move up and slide away the flow chamber;

5.1.5. Remove tissue bath;

5.1.6. Clean mounting needles and remove the glue;

5.1.6.1. Using the 10x and the ocular, bring the mounting needle in focus;

5.1.6.2. Dip the brush in EtOH and carefully brush of the glue from the needle.

CAUTION: have patience! It might take some time before the glue comes off.

5.1.7. Rinse flow chamber and tissue bath with milli-Q;

5.1.8. Place probe submersed in small petri dish filled with milli-Q.

5.1.9. When done for the day clean the setup as above and:

5.1.9.1. Empty the tubing from the flow bath. Send parameters to outflow syringe pump (see **Table of Materials**, **Figure 9**). Valve: Bath valve (2); Microstep mode: Normal; Plunger target: 0; Plunger speed: 30.

NOTE: Terminate the command when you see the tubing is empty.

5.1.10. Initialize the pump several times (**Figure 9B**)

5.1.11. Drain the syringes:

NOTE: Use the pressure plugs to speed up the process.

5.1.11.1. Close all the luer valves;

5.1.11.2. Open all the valves;

5.1.11.3. Remove to tubing from the needle of the syringe;

5.1.11.4. Hold tube of specific pCa under the needle and open the luer valve.

5.1.12. Flush the system:

5.1.12.1. Re-attach the tubing to the needle of the syringe;

5.1.12.2. Fill syringes with ~5mL of milli-Q;

5.1.12.3. Place cup underneath the Θ-glass;

5.1.12.4. Open all the valves and open the pressure valve.

5.1.13. Shutdown the system; Turn of all the PC, interferometer, piezo controller power block.

6. Data analysis

6.1. Data traces can be exported from the system controller software (see **Table of Materials**) to excel file or clipboard by opening the data file and selecting the desired segment. The traces shown will be exported, e.g. raw force, sarcomere length and piezo position.

6.2. Data can be analyzed with the software of choice (e.g., Matlab)

REPRESENTATIVE RESULTS:

Data traces are recorded and opened with the system controller software (see **Table of Materials**). Complete traces or selected segments can be exported to the clipboard or text file for further analysis with desired software. Valves to control flow of the different solutions are switched with custom software or manually. A custom Matlab script is used to analyze rates of activation, tension redevelopment and relaxation. Maximum active force and the peak and the plateau force of the passive force experiments are taken directly from the system controller software force trace. After mounting a myofibril (**Figure 2**), the desired protocol can be selected.

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Maximum active force and calcium–sensitivity of force in myofibrils isolated from mouse and human skeletal muscle biopsies

In **Figure 4A** the experimental setup used for the active force experiments is depicted schematically. Force traces of an active force experiment with a myofibril isolated from (healthy) human quadriceps muscle. The myofibril was activated five times with solutions with varying pCa (pCa 6.2 – 5.8 – 5.6 – 5.4 – 4.5; data shown in **Figure 4B**). The average maximum force of all myofibrils in this experiment was ~123 mN/mm^2. A force-pCa curve was constructed from the plateau forces reached during each activation in each of the five calcium solutions. The results are shown in **Figure 4C**. From this curve the pCa at 50% of maximum force production (pCa_{50}) can be calculated. In this myofibril, the pCa_{50} was 5.75.

Additionally, one or multiple compounds can be added to the perfused solution to measure its effect on the force produced by the myofibril. In **Figure 4D**, the effect of N-benzyl-p-toluene sulphonamide (BTS), a fast twitch muscle (type II) myosin heavy chain II (MHCII) inhibitor, is illustrated.[19] A myofbril is activated first with a pCa 5.6 solution, and subsequently with a pCa 5.6 + BTS solution. During the second activation less force is produced, indicating this is a myofibril which contains MHCII. Certain mutations are in proteins that are present exclusively in specific muscle types, and thus only affect myofibrils from that specific muscle type. Therefore, 'typing' the myofibrils is important to discern the mutation effect on the various muscle types. Also, this example illustrates the possibility for testing the efficacy of therapeutic compounds in myofibrils.

Figure 4E shows an active force trace of a single myofibril isolated from mouse skeletal soleus muscle tissue. The myofibril was mounted in the setup and perfused with relaxing solution (pCa 9.0), followed by perfusion with activating solution (pCa 4.5, ~0.032 mM calcium). We simultaneously recorded the force and sarcomere length. Note that this is a near isometric contraction as cantilever deflection is ~0.5 µm, which is approximately 1% of the myofibril's

slack length (~50 µm). In **Figure 4E** a rapid shortening-restretch protocol was performed during active contraction to assess the rate of tension redevelopment (k_{TR}, yellow dashed line). K_{TR} is a measure of cross-bridge cycling kinetics. Also, the activation and relaxation curves were fitted to determine the rate of activation (k_{ACT}, red dashed line) and relaxation (k_{REL}, green dashed line), respectively. **Figure 4** shows a more detailed view of the relaxation phase highlighted in **Figure 4F**. Two phases become apparent: 1) an initial slow phase of relaxation (dominated by cross-bridge detachment) and 2) a fast phase of relaxation (dominated by cross-bridge detachment and calcium-dissociation).[20]

Passive force in myofibrils isolated from a human skeletal muscle biopsy

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Figure 10 shows a trace of a passive force experiment with a myofibril isolated from healthy human diaphragm muscle tissue. The first protocol involves one or multiple passive stretches to determine the viscoelastic properties of the sarcomeres. **Figure 10** shows a force trace of a continuous stretch of a myofibril (stretch from sarcomere length 2.2 to 3.0 µm). During stretch, myofibrils display both viscous and elastic characteristics. This is evident from the curve shown in **Figure 10A**. The sharp peak represents both characteristics, whereas the plateau force is a measure of elasticity. Viscosity resists strain linearly. Thus, the force drops after the strain is removed. **Figure 10B** highlights the stretch itself and illustrates the high signal to noise ratio (note that force traces are unfiltered).

DISCUSSION

Here, we describe a protocol to assess the contractile function of myofibrils isolated from human or animal skeletal muscle tissues. The force resolution of our setup has been described before by Chavan et al. (2012).[12] In short, it is determined by the random fluctuations of the length of the Fabry-Perot cavity formed between the detection fiber and the cantilever, which produce the dominant part of the noise at the output of the readout (expressed in V) that, multiplied by the deflection sensitivity (expressed in m/V) and by the spring constant of the cantilever (expressed in N/m), provides the force noise. For our setup, the root mean square (rms) noise in air at the output of the readout, sampled at a 1000 data points/s (sample/s), is approximately 2 mV. For a typical myofibril measurement, a ferrule-top probe is used with a spring constant of ~0.7 N/m (deflection sensitivity ~300 nm/V), this rms value corresponds to a cantilever deflection resolution of 0.6 nm, which translates in a force sensitivity of ~0.37 nN. The force probe is calibrated by pushing the tip of the mounting needle against a weighing scale while keeping the bending of the cantilever equal to a multiple of the wavelength of the readout laser.[13] This method of calibration entails both the cantilever and mounting needle stiffness as well as possible variations in torque of the cantilever and mounting needle due to speed and magnitude of myofibril contraction. Currently, a setup for assessing myofibril contractility is available, which is based on the detection of a laser deflected from the cantilever, i.e. optical beam deflection (1700A; force resolution ~1 nN resolution). This system was developed by Labuda et al. (2011) using an optical periscope to guide a laser light towards and away from the cantilever in constraining configurations.[11] In this system, a myofibril is mounted between the atomic force cantilever and a rigid glass needle. An advantage of the system described here is the higher force sensitivity and signal to noise ratio. Furthermore, in the setup described here, relatively stiff cantilevers can be used, which results in small cantilever deflection when myofibrillar force is

applied. This is important, as it allows for force measurements at nearly constant sarcomere length. Finally, compared to the system described by Labuda et al. (2011), the system described here utilizes similar or identical methods to control for temperature, to induce length changes on the myofibril, and to change the perfusion solutions (using a theta glass and fast step motor). The advantage of the system described by Labuda et al. (2011) is that a change of solution composition (between cantilever and optical periscope) does not affect the signal output. In the system described here, the solution composition between cantilever and optical fiber must remain constant. The solution to this limitation is described in more detail below.

Optimization

The optical force probe in combination with the fast-step perfusion system led to complications. The difference in optical properties between low and high [Ca^{2+}] solutions interferes with the force measurements. To prevent backflow of the high calcium solution a custom flow chamber was engineered (**Figure 3**). A constant background flow of calcium-free solution was induced from right to left to keep the solution constant between the top of the optical fiber and the cantilever (**Figure 3d**).

To control the temperature, a Peltier element with liquid cooling was mounted on the flow chamber. This flow chamber is thermally uncoupled from the microscope by mounting it on a plastic adapter. With the Peltier element, controlled by a TEC system, it is possible to control the temperature of the solution over time with 0.1°C precision. Temperature is monitored by a temperature sensor mounted on the flow chamber. Temperature stability is important due to the nature of the force transducer. The cantilever consists of a gold-coated glass strip, effectively making it a thermometer. Thus, the cantilever will bend with temperature changes.

We use a fast-step perfusion system (see **Table of Materials**) to control the movement of the theta-glass. This system allows for perfusion switches within 10 ms. Combining the method of temperature control and solution switching, makes this system particularly suitable to measure the kinetics of sarcomere contractility (rates of force development, tension redevelopment and relaxation) in myofibrils.

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Initially, the downside of using interferometry was the small usable range due to the necessity to use the linear part of the interference curve (λ/8, with the wavelength of the laser λ). However, recent innovation eliminated this need by combining wavelength modulation with a lock-in amplifier. Hereby, the system is not limited to a single linear part of the interference curve. This enables the measurement of infinite deflection of the cantilever .[14] Thus, the range of cantilever deflection readout of this system is greatly enlarged compared to traditional interferometry. Additionally, the force probes described are easy to replace and there are many cantilevers available, with stiffnesses ranging from 0.5 N/m to >20 N/m. Therefore, it is possible to quickly change between cantilevers of various stiffness and select the stiffness best suitable for the experiment to be conducted.

Challenges

The current system is a prototype based on a cardiomyocyte measuring system (see **Table of Materials**). Several components can be improved to provide a better user experience and data of higher quality. First, due to add-ons to the system, vibration and resonance can be an issue that will add noise to the signal. Also, the theta-glass holder and fast-step motor attachment method could be improved to make it less prone to vibration.

Second, it is desirable to replace the fast-step motor with a piezo length actuator to increase the speed of solution switching and to obtain a more consistent motion.

Third, the calcium solutions we previously used to activate single striated muscle fibers included propionic acid, but this solution absorbs near-infrared light, interfering with the force measurements. Calcium chloride was used to eliminate the need for propionic acid, which greatly reduced this effect. This issue is inherent to a system based on interferometry and not present when utilizing optical beam deflection.

Fourth, a custom flow bath was engineered to create a laminar flow, which matched the flow of the theta glass. This prevented backflow, due to turbulence, of the calcium rich solution. Therefore, the solution between the tip of the optical fiber and the cantilever remained constant. The coverslip with the myofibrils can move freely under the flow chamber and therefore, selection of suitable myofibrils is not confined to the small area of the flow chamber.

Reproducibility and variability

There are several elements of the system and protocol, which are important for the degree of reproducibility and variability of the data obtained.

First, the quality of the measurements strongly depends on the quality of the myofibril isolation. Identical protocols yield different quality and quantity of myofibrils in different biopsies. In some cases, biopsies barely yield usable myofibrils or none at all. Common consensus is the assumption that damaged myofibrils will break during contraction and thus do not enter the results.

Second, there is a uncertainty in the determination of the cross-sectional area of the myofibril. Due to technical constraints, it is possible to measure the width of the myofibril in only one plane. Therefore, to calculate the cross-sectional area we make the assumption that the width and depth are equal. When force is normalized to cross-sectional area to calculate maximal active tension, one should be aware of this assumption. Mounting of myofibrils due to myofibril mounting angle, position and the integrity of the glue. Although mounting angle and position can largely be controlled visually, small variations between myofibrils might be present. Glue integrity has not been investigated extensively. However, glue integrity can be verified by monitoring the sarcomere length in the myofibril before and after activation. When more sarcomeres are between the glue after a protocol, this suggests that slippage of the myofibril in the glue has occurred. Consequently, this myofibril should be excluded from the dataset.

Other applications of the setup: Calcium pre-activation in cardiomyocytes isolated from rat left ventricle

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In addition to assessing the contractile function of myofibrils, the system can also be used to measure cardiomyocyte mechanics. For example, **Figure 11** illustrates the use of membrane-permeabilized single cardiomyocytes isolated from rat left ventricle.[21] Contrary to the experiments described above, relaxing solution was changed and activating solution was kept constant. Each cardiomyocyte underwent five sets of activations, exposing it to a 2 μM free calcium solution for 1 s. The 1 s time constraint is chosen to mimic the time-limited nature of cardiac contractions, where the exposure to low [Ca^{2+}] solutions mimics the diastolic phase and the exposure to high [Ca^{2+}] solutions mimics the systolic phase of cardiac muscle contraction (**Figure 11A**). For each of the five sets diastolic calcium was varied (1, 80, 160, 250 and 400 nMCa^{2+}), while systolic calcium remained constant (**Figure 11A**). A set consisted of two sets of three activation-relaxation cycles at 1.8 μm versus 2.0 μm and 2.0 μm versus 2.2 μm for different experimental groups. Peak force was measured at 1 second from the switch of the pipet and averaged for the set of three activation-relaxation cycles. The high signal-to-noise ratio and the high dynamic range of this force transducer allowed us to measure both the small changes in diastolic force and the much larger systolic forces (**Figure 11B**). Increasing diastolic calcium resulted in a higher force at 2 μM Ca^{2+} relative to the first activation (**Figure 11B**). Wild-type (WT) rat cardiomyocytes were compared with heterozygous (HET) RMB20 rat cardiomyocytes. HET rats have a more compliant titin protein as compared to the WT rats, due to alternative splicing. The effect was exaggerated in HET cardiomyocytes at 80 and 160 μM Ca^{2+} (**Figure 11C**).

FIGURE LEGENDS:

Figure 1 – Schematic depiction and (electron microscopy) images of a skeletal muscle and its morphology. A) shows the structure of skeletal muscle and B) shows the structure of the sarcomere, the smallest contractile unit. These schematic images are adapted from Servier Medical Art. C) shows an image of a single muscle fiber, and D) shows an electron microscopy image of a muscle fiber revealing myofibrillar damage as well as preserved myofibrillar ultrastructure.

Figure 2 – Images showing a mounted myofibril, Θ-glass alignment and piezo mounting needle. A) A myofibril mounted at slack length between the glass fiber needles coated with shellac as seen through a 40X objective. B) Images of the position of the Θ-glass relative to the myofibril (highlighted with the white ovals) as seen through a 10X objective. (TOP) Aligned to the top channel (relaxing solution, pCa 9.0); (BOTTOM) Aligned to the bottom channel (activating solution, pCa 4.5) to perfuse the myofibril with calcium and induce contraction. C) Schematic depictions of the position of the Θ-glass relative to the myofibril. (TOP) Aligned with the top channel (relaxing solution, pCa 9.0); (BOTTOM) Aligned with the bottom channel (activating solution, pCa 4.5) to perfuse the myofibril with calcium and induce contraction. D) Mounting needle attached to the carbon rod of the piezo holder.

Figure 3 – Schematic representation of the setup. (bottom left) End part of the tissue flow chamber. In dark blue the tissue flow chamber made out of aluminum and in white the cavity in which the force probe and theta glass are shown in position; (center) myofibril attached between two glass fiber mounting needles attached to the force probe and piezo length motor. The theta glass is aligned with the myofibril. The theta glass can move up and down to expose the myofibril

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to the calcium solution. (right) The cantilever force probe in close-up. Indicated are the cavity size (or Fabry-Pérot cavity) d, reflection interfaces A, B and C and an example of a light wave emitted by the laser (red). The cantilever is mounted on the shoulder of the ferrule. The fiber that carries the laser from the interferometer exits the ferrule at the tip of the cantilever. A glass mounting fiber is fixed on the cantilever using wax. (top left) The interferometer (see **Table of Materials**) analyses the interferometer signal which is transmitted to the system controller software (see **Table of Materials**).

Figure 4 – Experimental setup and data from active tension experiments. A) Schematic representation of the perfusion setup and solutions used. Note that the first and last tubes (light blue) contain calcium free solution (relaxing solution). B) Example force traces of an active tension experiment with a myofibril isolated from human skeletal muscle tissue showing five activations from relaxing solution (pCa 9.0) to multiple activation solutions (pCa 6.2 – 4.5). C) A force calcium curve; force levels at the plateaus in panel B) are normalized and plotted against their respective calcium levels. D) Example force trace of a type II (fast twitch) myofibril isolated from human skeletal muscle activated with pCa 5.6 solution (blue) and subsequently with pCa 5.6 + BTS (a type II specific cross-bridge inhibitor, red). E) Example data trace of an active tension experiment with myofibrils isolated from mouse soleus skeletal muscle tissue with a rapid shortening-restretch protocol during activation to determine the rate of tension redevelopment (k_{TR}, yellow dashed line). Also, the activation and relaxation curve are fitted to determine the rate of activation (k_{ACT}, red dashed line) and relaxation (k_{REL}, green dashed line), respectively. F) shows a zoom of the relaxation phase (top left) highlighted in E. The fast step motor signal (bottom left) indicates the time point at which the solution is changed from activation solution (pCa 4.5) to relaxing solution (pCa 9.0). The relaxation phase consists of a linear, slow phase (top right) and a exponential, fast phase (bottom right).

Figure 5 – Example setting for the signal generator in the system control software (see **Table of Materials**). 1) Indicates the button to execute commands entered in the signal generator. A) Initialization of the piezo length motor. B) Initialization of the fast-step motor. C) Performing a fast-step to activate a myofibril for a duration of 5 s. D) Performing a rapid shortening-restretch of a myofibril to determine the k_{TR}. E) Performing a stepwise stretch of a myofibril to determine the viscoelastic properties.

Figure 6 – Valve controller software as used on the PC (see **Table of Materials**). A) Indicates the button to turn on to open valves 1 (Rx) and 6 (Act). B) Indicates the state of the buttons when all valves are closed.

Figure 7 – Measuring sarcomere length, myofibril length and myofibril width with the system controller software (see **Table of Materials**). A ruler is used as an example. A) Measuring the sarcomere length: the purple box is placed around the myofibril and the sarcomere length is shown in (1). B) Measuring the length: The cyan box is placed from beginning to end of the

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myofibril. C) Measuring with: After rotating the camera 90 C°, the cyan box is placed from side to side of the myofibril.

Figure 8 – Thermoelectric temperature controller software. A) Establish connection with the thermoelectric temperature controller. B) Expand temperature settings. C) Set desired temperature, in this case: 15 C°. D) Turn on thermoelectric temperature controller and send voltage to Peltier thermoelectric cooler module.

Figure 9 – Settings for the syringe outflow pump (see **Table of Materials**). A) Open connection to the pump by pressing (1). B) Initialize pump with pre-defined settings by pressing (2). C) Start outflow pumping by setting the 'Valve Commands' to 'Bath Valve (2)' and entering the 'Command Set Parameters' as shown. Execute the command by pressing (3). Commands can be terminated by pressing (4).

Figure 10 – Example data trace of a passive tension experiment with myofibrils isolated from human skeletal muscle tissue. A) Recording of the force (upper) and sarcomere length (lower) during a stretch and release protocol. B) Zoom of (A) showing the force (upper) and sarcomere length during the stretch phase of the myofibril.

Figure 11 – Experimental setup and data from cardiomyocyte calcium pre-activation experiments. A) Schematic representation of the perfusion setup. Note that the last tube (light blue) contains calcium free solution (relaxing solution). B) Superimposed curves of activation of a cardiomyocyte without (light blue) and with (dark blue) calcium pre-activation, with $[Ca^{2+}]$'s of 1 nM and 80 nM respectively. C) Comparison of calcium pre-activation in wild-type (WT) and heterozygous RBM20 (HET) cardiomyocytes isolated from rat left ventricle. This figure has been modified from Najafi et al. (2019).[21]

TABLE LEGENDS:

Table 1 – Table describing the various signal generator settings used in the system controller software to operate the piezo length motor and fast-step motor.

ACKNOWLEDGEMENTS:
This project was funded by AFM-Telethon and A Foundation Building Strength for Nemaline Myopathies. The authors wish to acknowledge the creator of the products mentioned in this article, IONOptix Inc.

DISCLOSURES:
Michiel Helmes is shareholder and co-owner of IONOptix Inc.

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Author: Aref Najafi, **Martijn de Locht**, Maike Schuldt, et al
Publication: Journal of Physiology
Publisher: John Wiley and Sons
Date: Jul 30, 2019
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