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TITLE:

Ultrafast Force-Clamp Spectroscopy as a Guide to Dissect Mechanoenzymatic Properties of Processive Myosins under Load

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SUMMARY:

Presented here is a comprehensive protocol to perform ultrafast force-clamp experiments on processive myosin-5 motors, which could be easily extended to the study of other classes of processive motors. The protocol details all the necessary steps, from the setup of the experimental apparatus to sample preparation, data acquisition and analysis.

ABSTRACT:

Ultrafast force-clamp spectroscopy (UFFCS) is a single molecule technique based on laser tweezers that allows the investigation of the chemomechanics of both conventional and unconventional myosins under load with unprecedented time resolution. In particular, the possibility to probe myosin motors under constant force right after the actin-myosin bond formation, together with the high rate of the force feedback (200 kHz), has shown UFFCS to be a valuable tool to study the load dependence of fast dynamics such as the myosin working stroke. Moreover, UFFCS enables the study of how processive and non-processive myosin-actin interactions are influenced by the intensity and direction of the applied force.

By following this protocol, it will be possible to perform ultrafast force-clamp experiments on processive myosin-5 motors and on a variety of unconventional myosins. By some adjustments, the protocol could also be easily extended to the study of other classes of processive motors such as kinesins and dyneins. The protocol includes all the necessary steps, from the setup of the experimental apparatus to sample preparation, calibration procedures, data acquisition and analysis.

INTRODUCTION

In the last decades optical tweezers have been a valuable tool to elucidate the mechanochemistry of protein interactions at the single molecule level, due to the striking possibility of concurrent manipulation and measurement of conformational changes and enzymatic kinetics [1,2]. In particular, the capability to apply and measure forces in the range of those exerted by molecular motors in the cell, together with the capacity to measure sub-nanometer conformational changes, made optical tweezers a unique single-molecule tool for unraveling the chemomechanical properties of motor proteins and their mechanical regulation.

Ultrafast force-clamp spectroscopy (UFFCS) is a single-molecule force-spectroscopy technique based on optical tweezers, developed to study the fast kinetics of molecular motors under load in a three-bead geometry (**Figure 1a**)[3,4]. UFFCS reduces the time lag for force application to the motor protein to the physical limit of optical tweezers, i.e., the mechanical relaxation time of the system, thus allowing the application of the force rapidly after the beginning of a myosin run (few tens of microseconds)[3]. This capability has been exploited to investigate the early mechanical events in fast skeletal [3] and cardiac[5] muscle myosin to reveal the load dependence of the powerstroke, the weak- and strong-binding states, as well as the order of biochemical (Pi) and mechanical (powerstroke) events.

Although a three bead geometry is usually employed to study non-processive motors, a single bead geometry with a force-clamp has been commonly used to investigate processive non-conventional myosins such as myosin Va[6]. However, there are several reasons to prefer a three-bead UFFCS assay also for processive myosins. First, the rapid application of load right after actin-myosin binding allows the measurement of the early events in force development as in non-processive motors. In addition, in the case of processive motors it also allows an accurate measurement of the motor's run lengths and run durations under constant force all through their progression (**Figure 1b**). Moreover, because of the high rate of the force feedback, the system can maintain the force constant during fast changes in position, such as the myosin working stroke, thereby guaranteeing a constant load during motor stepping. The high-temporal resolution of the system allows the detection of sub-ms interactions, opening the possibility of investigating weak binding of myosin to actin. Finally, the assay geometry guarantees that the force is applied along the actin filament, with negligible transverse and vertical components of the force. This point is of particular relevance since the vertical force component has been shown to influence significantly the load-dependence of motor's kinetics[7,8]. By using this technique, we could apply a range of assistive and resistive loads to processive myosin-5B and directly measure the load dependence of its processivity for a wide range of forces[4].

As shown in **Figure 1a**, in this system a single actin filament is suspended between two polystyrene beads trapped in the focus of double optical tweezers (the "dumbbell"). An imbalanced net force F= F1-F2 is imposed on the filament, through a fast feedback system, which makes the filament move at constant velocity in one direction until it reaches a user-defined inversion point where the net force is reversed in the opposite direction. When the motor protein is not interacting with the filament, the dumbbell is free to move back and forth in a triangular wave shape (**Figure 1b**, bottom panel) spanning the pedestal bead on which a single motor protein is attached. Once the interaction is established the force carried by the dumbbell is very rapidly transferred to the motor protein and the motor starts displacing the filament by stepping under the force intensity and direction that was applied by the feedback system at the time of the interaction, until myosin detaches from actin. Being the displacement produced by the stepping of the motor dependent on the polarity of the

trapped actin filament, according to the direction of the applied force the load can be either assistive, i.e., pushing in the same direction of the motor displacement (push in **Figure 1b** upper panel), or resistive, i.e., pulling in the opposite direction with respect to the motor displacement (pull in **Figure 1b** upper panel) making it possible to study the chemomechanical regulation of the motor processivity by both the intensity and the directionality of the applied load.

In the next sections all the steps to measure actin-myosin-5B interactions under different loads with an ultrafast force-clamp spectroscopy setup are fully described, including 1) the setting up of the optical setup, optical traps alignment and calibration procedures, 2) the preparations of all the components and their assembly in the sample chamber, 3) the measurement procedure, 4) representative data and data analysis to extract important physical parameters, such as the run length, the step size and the velocity of the motor protein.

PROTOCOL:

1. Optical setup

NOTE: The experimental setup is composed of double optical tweezers with nanometer pointing stability and < 1% laser intensity fluctuations. Under these conditions, stability of the dumbbell at the nanometer level is guaranteed under typical trap stiffness (0.1 pN/nm) and tension (1 pN - few tens of pN). **Figure 2** shows a detailed scheme of the optical setup.

1.1. Optical tweezers design and construction [9,10,11].

1.1.1. Place all the components of the setup on an optical table according to the scheme in **Figure 2**. Note that the optical table includes active isolators to minimize mechanical vibrations. Additionally, the microscope structure is mounted on elastomeric isolators to absorb acoustic noise and mechanical resonances.

1.1.2. Insert an optical isolator close to the laser source ("OI" in **Figure 2**), to avoid random amplitude fluctuations due to optical feedback.

1.1.3. Seal the whole path in a closed box to reduce air current and turbulences that could impact the laser pointing stability.

1.1.4. Create the double optical tweezers by dividing the main laser source (Nd:YAG laser, 1,064 nm wavelength in **Figure 2**) into two branches with orthogonal polarizations by the use of polarizing beam splitters (PBS) . Time-shared traps should be avoided because they induce oscillation of the dumbbell under tension[12].

1.1.5. Use two acousto-optic deflectors (AODs in **Figure 2**) driven by Direct Digital Synthesizers (DDSs) to allow fine and rapid movements of the two traps and precise regulation of the actin tension by directly driving the DDSs through the digital outputs from the field-programmable gate array FPGA board (see **Figure 2**).

NOTE: The overall feedback response time must be <10 μs to rapidly correct and maintain constant force on both traps during measurements, including the unbound state, myosin interaction and movement. To this end, position detectors must have a >=100kHz bandwidth and data must be

acquired at >=200-kHz sample rate. For each data point acquired (5 µs acquisition time), proportional corrections for the two traps are calculated by the FPGA and sent to the two DDS driving the AODs. The AOD response time must be below 5 µs to satisfy the required feedback response time.

1.1.6. For nm detection of the trapped beads position, put two Quadrant Photodiode Detectors (QPDs in **Figure 2**) in the back focal plane of the condenser. Accurate alignment of the QPDs in a plane conjugated to the back focal plane of the condenser, as well as the AODs in a plane conjugated to the back focal plane of the objective, will assure that QPDs signals will be independent of the AODs frequency.

1.1.7. Mount the AOD on a linear translator with micrometer drive and displace it until the crystal edge gets close to the laser beam. Then, replace the objective with an iris, centered on the threaded objective housing, and regulate its aperture to fit the objective back aperture size.

1.1.8. Move the translator towards the laser beam until the portion of the beam blocked by the piezo crystal is visible after the iris, turn the translator slightly backwards to get the beam to completely fill the iris aperture again.

1.1.9. Repeat steps 1.1.7-1.1.8 for the second AOD. Check the response time of the feedback loop by measuring the time lag from the QPDs while rapidly moving a beam on a bead stuck on the surface of the coverslip[13].

NOTE: The above three steps (1.1.7-1-1-9) lead to the careful alignment of AOD crystals. These steps are important to optimize the time response of both the beam deflection and the feedback[13].

1.2. By means of a photodiode measure the intensity fluctuations of the trapping laser at the microscope entrance which must be below 1%. Note that the photodiode bandwidth must be larger than the imaging rate.

1.3. Check the pointing stability of both traps.

1.3.1. Prepare silica beads in phosphate buffer (PB) by diluting 20 µL of silica beads (1.2 µm, 10% solids) in 1 mL of acetone, sonicate for 30 s, vortex briefly, and centrifuge for 2 min at 19,000 x *g*.

1.3.2. Remove the supernatant, resuspend in 1 mL of acetone, and repeat the wash. Resuspend in 1 mL of 50 mM PB, wash 2 times. Finally, resuspend in 100 µL of 50 mM PB.

1.3.3. Perform optical tweezers calibration with silica beads by sticking a coverslip onto a microscope slide with a double-sided tape (about 60 µm thick) to build a flow chamber. Fill the chamber with a 1 mg/mL BSA (Bovine Serum Albumin protein) and wait for 3 min.

1.3.4. Flow a 1:1000 dilution of silica beads in PB into the chamber. Use a syringe filled with silicon grease to carefully seal the chamber. Trap a single bead in each trap and apply the power spectrum method[14] to calibrate them.

1.3.5. Prepare silica beads in pentyl acetate (at room temperature) by dissolving 20 µL silica beads (1.2 µm diameter, 10% solid) in 1–1.5 mL of acetone, vortex and sonicate for 30 s.

1.3.6. Centrifuge at 18,500 x *g* for 2 min. Discard the supernatant and resuspend in 1 mL of acetone, then repeat the wash. Resuspend in 1 mL of pentyl acetate and repeat wash (centrifuge and resuspension) in pentyl acetate 2 times. Resuspend the pellet in 100 µL of nitrocellulose 1% and 900 µL pentyl acetate. Store at 4 °C for 2 months.

1.3.7. Take a 24 x 24 mm glass coverslip and clean it carefully with paper soaked with pure ethanol. Then, while holding it with clean tweezers, rinse it a second time by washing directly with pure ethanol. Let it dry under a gentle flow of nitrogen. If needed, repeat this operation to remove all visible residues on the glass surface.

1.3.8. Take the silica beads stock, vortex it and sonicate it briefly for ~30 s.

1.3.9. After cleaning a second coverslip (24 x 60 mm), use it to smear 2 µL of silica beads solution on one surface of the coverslip, and wait for it to dry.

1.3.10. Clean carefully a microscope slide (26 x 76 mm) that will be used to create the flow chamber.

1.3.11. Cut two lines of double sticky tape (~3 mm large, 60-100 µm thick) and attach them on one side of the microscope slide, as shown in **Figure 3**.

1.3.12. By using clean tweezers, close the chamber (about 20 µL final volume) by putting the coated coverslip (1.3.9) in contact with the sticky tape lines, with the nitrocellulose + beads layer facing the inside of the chamber, as shown in **Figure 3a**. Fill the flow chamber with 50 mM phosphate buffer and seal it with silicon grease.

1.3.13. Image a single silica bead in brightfield microscopy at >200x magnification using a charge-coupled device (CCD) or complementary metal–oxide–semiconductor (CMOS) camera with >1.4 megapixels. Use a feedback software to move the piezo stage (with nanometer accuracy or better) to compensate for thermal drifts[10].

1.3.14. Overlap the center of the left trap with the center of the bead (x-y signal levels from the QPD should match those from the calibration). Then measure the position noise and standard deviation of the position signals for this trap.

1.3.15. Repeat the previous step for the right trap[15].

1.4. Trap position calibration: MHz to nm

1.4.1. Prepare a flow chamber with silica beads attached on the coverslip surface (1.3.5-1.3.12) and floating polystyrene beads (use α-actinin conjugated beads prepared as in the following section 2.1).

1.4.2. Focus a silica bead on the coverslip surface slightly decentered (~5 µm) from the center of the field of view (FOV) and acquire an image of the FOV. Move the bead by 10 µm towards the FOV center using the piezo stage and acquire a second image. Calculate the center of the bead in the two images using a centroid algorithm or similar and calculate the distance in pixel between the two beads to obtain the nm/pixel calibration of the brightfield camera.

1.4.3. Trap a single floating particle in one trap. Then, move the trap using AOD in small steps (0.2 MHz) and acquire an image of the particle and the corresponding frequency of the AOD for each step. Calculate the position of the particle in the FOV by using the centroid algorithm as before and convert it in nm by using the nm/pixel calibration obtained in the previous step.

1.4.4 Perform a linear fit to the frequency-position data and calculate the calibration constant in nm/MHz.

1.4.5 Repeat calibration for the second trap

1.5. Trap power and stiffness calibration (MHz vs W), QPD (MHz vs pN/nm)

1.5.1 Prepare a flow chamber with silica beads attached on the coverslip surface (1.3.5-1.3.12) and floating polystyrene beads (use α-actinin conjugated beads prepared as in the following section 2.1) and trap a single particle in one trap. Then displace both traps through AODs in small steps (0.2 MHz) and record a Brownian motion of the particle in both traps with QPD and the corresponding frequency of the AODs.

1.5.2. Calculate an average power on the detectors at each position and obtain trap stiffness and QPD calibration constant beta by fitting a Lorentzian function to a power spectrum of the recorded Brownian motion[13].

2. Sample preparation

2.1. Prepare α-actinin conjugated fluorescent beads**:**

2.1.1. Perform conjugation[16]: Take amino-functionalized polystyrene beads (1 µm diameter, 2.5% solids), wash them twice in 500 µL distilled water and resuspended in 500 µL of PBS (pH 7.0). Add 1 mM HaloTag succinimidyl ester O_2 ligand and incubate at room temperature for 1 h. Wash three times with 500 µL of PBS and resuspended with 100–200 µM HaloTag α-actinin. Incubate for 1 h at 37 °C and wash three times in 500 µL of PBS (use beads within 1.5 weeks or flash-frozen in liquid nitrogen and store at −80 °C).

2.1.2. Labeling: Incubate 200 µL of beads solution with Rhodamine-BSA at 5 µg/mL final concentration for 10 min. Wash with 50 mM PB three times and resuspend in 500 µL of PB 50 mM. This can be stored in aliquots at – 80 °C for months).

2.2. Express and purify biotinylated Myosin-5B as described previously[4,17].

2.3. Polymerize and label F-actin[13]:

2.3.1. F-actin polymerization: Mix 69 µL of ultrapure water, 10 µL of actin polymerization buffer 10x (100 mM Tris HCl, 20 mM $MgCl_2$, 500 mM KCl, 10 mM ATP, 50 mM guanidine carbonate pH 7.5), 20 µL of G-actin 10 mg/mL, and 1 µL of DL -Dithiothreitol (DTT) 1 M. Leave it on ice for more than 1 h.

2.3.2 F-actin labeling with rhodamine (Ex/Em: 546/575 nm): Take 25 µL of polymerized F-actin and add 19.5 µL of ultrapure water, 2.5 µL of actin polymerization buffer 10x (100 mM Tris HCl, 20 mM

$MgCl_2$, 500 mM KCl, 10 mM ATP, 50 mM guanidine carbonate pH 7.5), 1 µL of 1 M DTT, and 2 µL of 250 µM rhodamine phalloidin. Leave it on ice overnight. For trapping experiments rhodamine F-actin can be stored on ice and used within a week.

2.4. Sample assembly

2.4.1. Take the flow chamber (1.3.5-1.3.12) and incubate 1 mg/mL biotinylated BSA for 5 min. After washing with AB buffer (25 mM MOPS ,25 mM KCl, 4mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, pH 7.2) incubate 1 mg/mL streptavidin for 5 min and wash again with AB buffer. Incubate biotinylated myosin-5B heavy meromyosin at 3 nM concentration in M5B buffer (10 mM MOPS pH 7.3, 0.5 M NaCl, 0.1 mM EGTA, 3 mM NaN_3) with 2 µM CaM for 5 min. Wash with three volumes of 1 mg/mL biotinylated BSA supplemented with 2 µM CaM in AB and incubate for 3 min.

2.4.2. While incubating prepare the Reaction Mix (RM): 0.005% α-actinin functionalized beads (section 2.1), 1 nM rhodamine F-Actin (section 2.3) in Imaging Buffer (IB: AB buffer with 1.2 µM Glucose-Oxidase, 0.2 µM catalase, 17 mM glucose, 20 mM DTT, 2 µM CaM and ATP at the concentration needed for the experiment).

2.4.3. Wash with RM and seal the chamber with silicone grease. The sample is now ready to be watched under the microscope.

3 Measurement

3.1. Assemble the dumbbell.

3.1.1. Look for the floating α-actinin beads by moving the sample using long-range translators, switch on one trap and trap one bead.

3.1.2. Once the first trap is occupied, move the translator to position the trapped bead close to the coverslip surface to avoid trapping of multiple beads, and trap another bead in the second trap.

3.1.3. Adjust the two traps to equal stiffnesses by adjusting the power of the AODs acoustic waves. Stiffnesses are usually set between 0.03 and 0.14 pN/nm. The smaller the stiffness the better the sensitivity, in particular at low forces.

3.1.4. Then flip the motorized mirror M (**Figure 2**) to switch to fluorescence microscopy, and look for an actin filament floating in solution by displacing the sample through long-range translators[13]. Prefer long filaments (>5 µm) since the processive myosin will displace it and move it for a few microns before detaching.

3.1.5. Move the sample to let one of the trapped beads approaching one end of the filament until they attach to each other. Then, adjust the beads distance to the approximate filament length and create a flow in the direction of the unbound second bead by moving the stage in its direction. The filament will be stretched by the flow and it will eventually bind to it[13]. The bead-actin-bead complex is called “dumbbell”.

3.2. Establish actin-myosin contact.

3.2.1. Gently separate the two traps far apart to pre-tension the filament up to about 3 pN and probe the rigidity of the dumbbell by making one trap oscillate in a triangular wave by changing the frequency of one of the two AODs and verifying the consequent transmission of the motion to the trailing bead through its position signal.

3.2.2. Move the stage to put the dumbbell in proximity of a pedestal silica bead and allow the contact between the filament and the protein attached onto bead surface by adjusting the height of the trapped bead centers slightly below the silica bead diameter. Then position the center of the silica bead in between the trapped beads.

3.3. Force clamp and nm stabilization feedback:

3.3.1. Switch on the ultrafast force-clamp with 2–3 pN force and 200 nm oscillation and scan the pedestal bead in discrete steps of about 20–30 nm in the direction perpendicular to the actin filament. Wait for interactions to occur at each position (few seconds), then step ahead if no interaction is observed. As the protein-filament interaction is established, look for the position where interactions are more frequent.

3.3.2. Ensure that when the processive myosin moves towards one end of the actin filament (usually the + end), the trapped bead attached to the + end moves towards the silica bead. Move the stage towards the -end of the filament so that the silica bead lies as close as possible to the trapped bead attached to the -end of the filament when myosin is not bound and start the nanometer-stabilization feedback. In doing so, the probability that the trapped bead attached to the +end of the filament crashes into the silica bead is minimized.

3.4. Record data.

4. Data analysis[4]

NOTE: The analysis method that is described allows for the detection and measurement of processive runs and fast stepping events based on changes in the dumbbell velocity, as caused by myosin stepping. Analysis of processive runs is performed based on a data analysis method for non-processive motors described in references[3,4,13].

4.1. Set a threshold for velocity changes to allow for stepping event detection. Since in this case both forward and backward steps are expected, the crossing of the threshold is accepted in both directions.

4.2. Assign each step to the corresponding run: if the time interval between two consequent steps is shorter than 3 ms and the amplitude of the steps is < 90 nm, steps are assigned to the same run, otherwise steps are assigned to different runs[4].

4.3. Correct run length for assistive forces[4].

4.3.1. Correct run lengths under assisting forces by calculating the real run length value RL from the measured average run length value $\langle RL_m \rangle$ from the following equation, where D is the oscillation range:

$$< RL_m > = \int_{-\infty}^{+\infty} x \cdot p(x)\, dx = \frac{1}{D}\left[RL^2\left(e^{-\frac{D}{RL}} - 1\right) + RL \cdot D\right]$$

NOTE: Details on the derivation of this equation can be found in reference[4].

REPRESENTATIVE RESULTS:

Representative data consist in position records over time as shown in **Figure 4**. In the position record two kinds of displacement are visible. Firstly, when the myosin motor is not interacting with the actin filament the trapped beads are moving at constant velocity against the viscous drag force of the solution showing a linear displacement oscillating within the oscillation range set by the operator in a triangular wave[3] (not visible in **Figure 4** due to the long temporal scale). Second, once the myosin motor interacts with the filament the force carried by the moving filament is very rapidly transferred to the protein, the system velocity drops to zero (red lines in **Figure 4**) and stepping events occur under constant force till the end of its run. As shown in **Figure 5** the force is switched from the positive to the negative direction (and vice versa) by the feedback system, which switches the force direction when the bead reaches the edge of the oscillation range set by the user. In some cases, it can happen that, when the myosin binds and displaces the filament towards the positive direction, it pushes the bead towards the (upper) edge of the oscillation range. If this happens under assistive force (i.e., directed towards positive displacement, push, in **Figure 5**), the run of the myosin will be interrupted by the force direction inversion at the oscillation edge (arrows in **Figure 5**), thus limiting the length of the run to the amplitude of the dumbbell oscillation *D.* This requires a correction the run length in case of assistive force.

FIGURE LEGENDS:

Figure 1: Schematic of UFFCS applied to a processive myosin-5B motor. (**a**) A single myosin-5B molecule is attached to a glass bead pedestal through a streptavidin-biotin link. A single actin filament is trapped by suspending it between α-actinin coated beads (the so called "three-bead" geometry). Black arrows represent the force clamped on the right (F1) and left bead (F2), red arrow represents the net force (F) on the dumbbell. F is alternated back and forth to maintain the dumbbell within a limited oscillation range when myosin is not bound to actin. (**b**) Example trace showing displacement and force during the corresponding phases of dumbbell oscillation, myosin-5B attachment, and processive runs under assistive (push) and resistive(pull) loads. This figure has been modified from[4]. Raw data acquired at 200 kHz sample rate are plotted. Std. Dev. of force is about 0.27 pN.

Figure 2: Optical scheme of the experimental setup. The experimental setup consists of: halogen lamp (H), condenser (C), sample (S), piezo translators (x-y and z), objective (O), a low-magnification camera (CCD 200X) and a high-magnification camera (CCD 2,000X) used for the nm-stabilization feedback (BS is a 50:50 beam splitter cube). Double optical tweezers are inserted and extracted from the optical axis of the microscope through dichroic mirrors (D2 and D3) and comprise: Nd:YAG laser (1,064 nm), optical isolator (OI), λ/2 waveplates, polarizing beam splitter cubes (PBS), acousto-optic deflectors (AOD), 1,064 nm interferential filters (F1 and F2), quadrant detector photodiodes (QPD). Signals from QPDs were acquired through an analog-to-digital converter (ADC) and elaborated with a FPGA board. Two custom-built direct digital synthesizers (DDS) drove the AODs to control traps' position. Fluorescence excitation was provided by a duplicated Nd:YAG laser (532 nm) and the image projected on an electron multiplied camera (EMCCD). M is a movable mirror, F3 an emission filter. This figure has been modified from [3].

Figure 3: Flow chamber assembly. (**a**) Chamber preparation. A glass coverslip, smeared with silica beads, is attached onto a microscope slide through double sticky tape stripes to form a flow-cell about 20 µL volume. **b)** Top view of the flow-cell. Solutions are flown from one side of the chamber with a pipette and sucked from the other side through a filter paper to create a flow along the arrow direction.

Figure 4: Representative position recording. Position recording showing myosin-5B processive runs and the step and run detection algorithm. Detected beginning and end of each run are indicated by green and cyan vertical lines, respectively. Red horizontal lines indicate the detected steps. This figure has been modified from[4].

Figure 5: Force inversion during myosin runs. When myosin binds and moves the filament in the positive direction under assistive force (push), it can happen that it reaches the edge of the oscillation range where the force is reversed (indicated by the arrows), so that the myosin run under assistive force is interrupted. Contrary, under resistive force (pull), myosin processive stepping prevents the dumbbell from reaching the force inversion point. Therefore, in the latter case, run lengths are not limited by the oscillation range for resistive forces. This figure has been modified from[4]. Raw data acquired at 200 kHz sample rate are plotted. Std. Dev. of force is about 0.27 pN.

DISCUSSION

Although single molecule techniques, such as the three-bead assay, are technically challenging and low throughput, UFFCS improves the detection of molecular interactions thanks to the high signal-to-noise ratio of the data. UFFCS allows the study of the load-dependence of motor proteins, with the main advantages of applying the force very rapidly upon binding of the motor to the filament to probe early and very rapid events in force production and weak binding states under controlled force; maintaining the force constant all through the run and probing the motor dependence with full control on force directionality. Regarding the last point, the three-bead geometry as we use here is very efficient in applying and measuring forces along the filament direction, minimizing contributions from transverse or vertical components. However, when the motor protein is expected to actively produce transverse or vertical forces, or even torques, other configurations such as the single bead geometry are more appropriate[2,7,18]. Moreover, thanks to its spatial and time temporal resolution, UFFCS represents a unique tool for the understanding of basics of molecular interactions that would otherwise be hindered with conventional single-molecule techniques. In fact, UFFCS made it possible to investigate how assistive and resistive forces regulates the mechanical response of myosin-5B, thus giving new insight into its collective behavior within the actin mesh in the cell[4].

However, the success of these experiments relies on the fulfillment of some important requirements that must be addressed very carefully by following all the instructions found in this protocol: the precise alignment and isolation of the optical setup is fundamental to reach an optimal spatial resolution; careful calibration of the optical system is necessary to determine the values of the applied forces with high precision; the setting of a fast feedback system is necessary to reach the high temporal resolution; finally all the components that are assembled in the sample chamber must be prepared in a controlled environment, keeping them as sterile as possible, since any impurity in the sample chamber could compromise the experiment, and all indications about their optimal storage and handling should be strictly respected for the success of the experimental protocol. Importantly, data analysis should be carefully adapted to the different kinds of motor-filament interactions to properly interpret results and avoid artifacts.

In this protocol are included all the steps to perform ultrafast force-clamp experiments on processive myosin-5 motors, from the setup of experimental apparatus to sample preparation, measurement and data analysis, that could be conveniently adapted to study a variety of unconventional myosins and other classes of processive motors such as kinesins and dyneins.

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DISCLOSURES:

The authors declare no competing interests.

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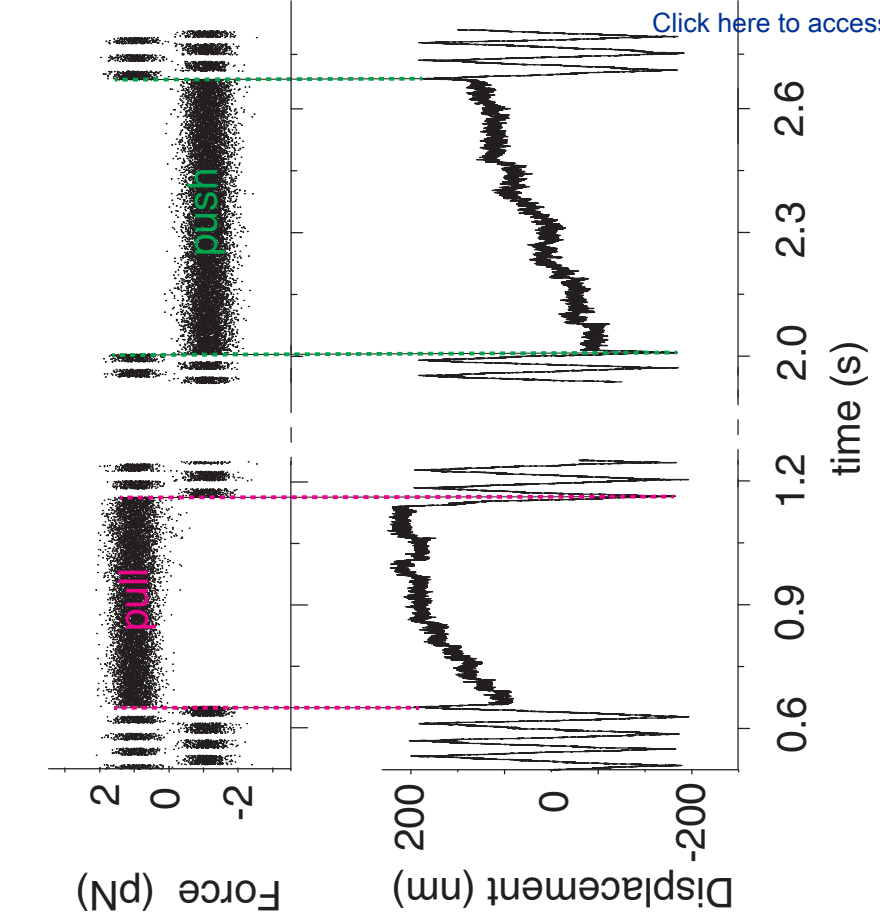
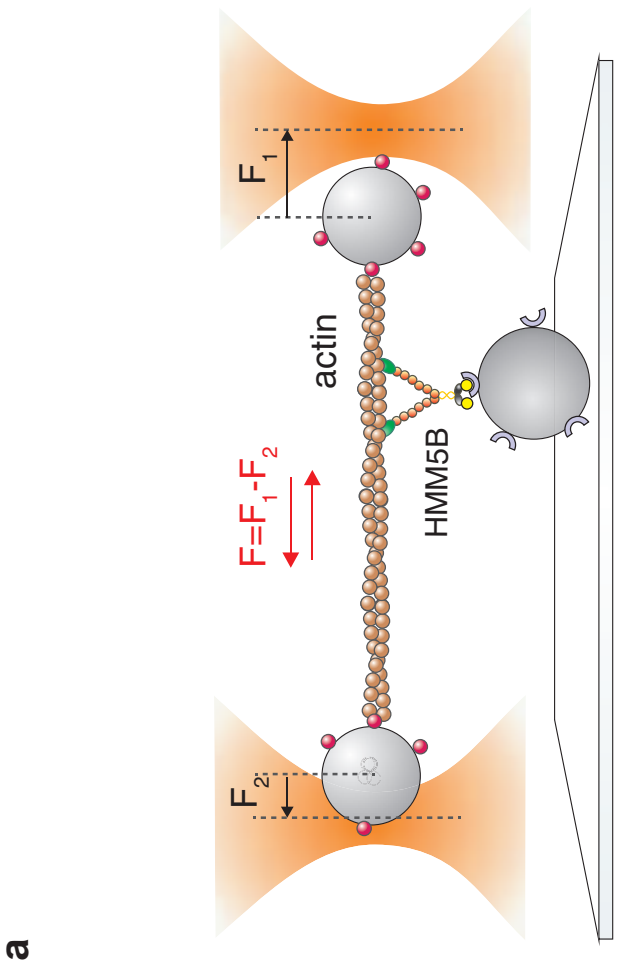
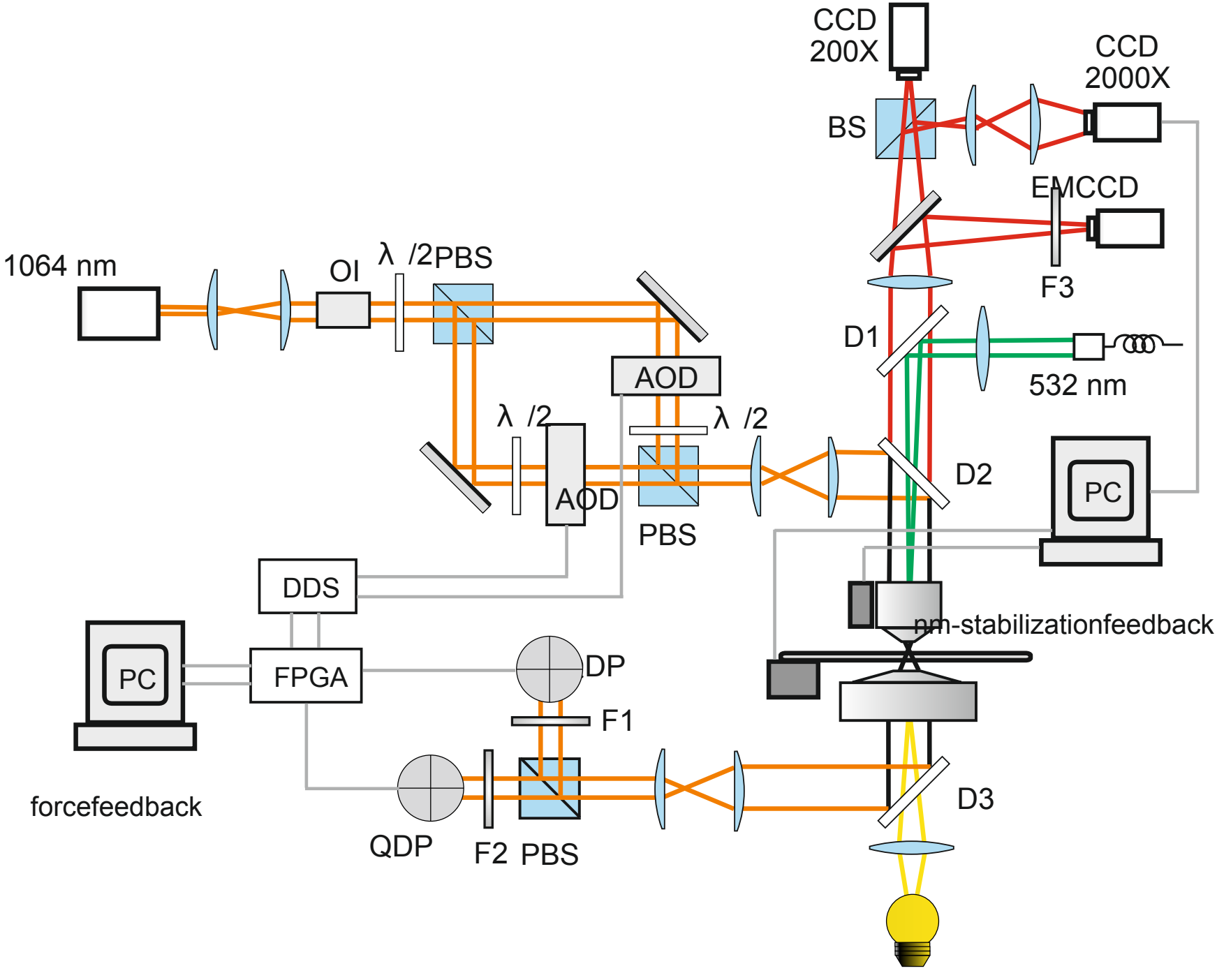
Figure 1



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AOD
PBS
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DDS
nm-stabilizationfeedback
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FPGA
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forcefeedback
QDP
F2
PBS
D3

Figure 3

Revised Figure 4

Click here to access/download;Figure;Fig.4_rev.eps

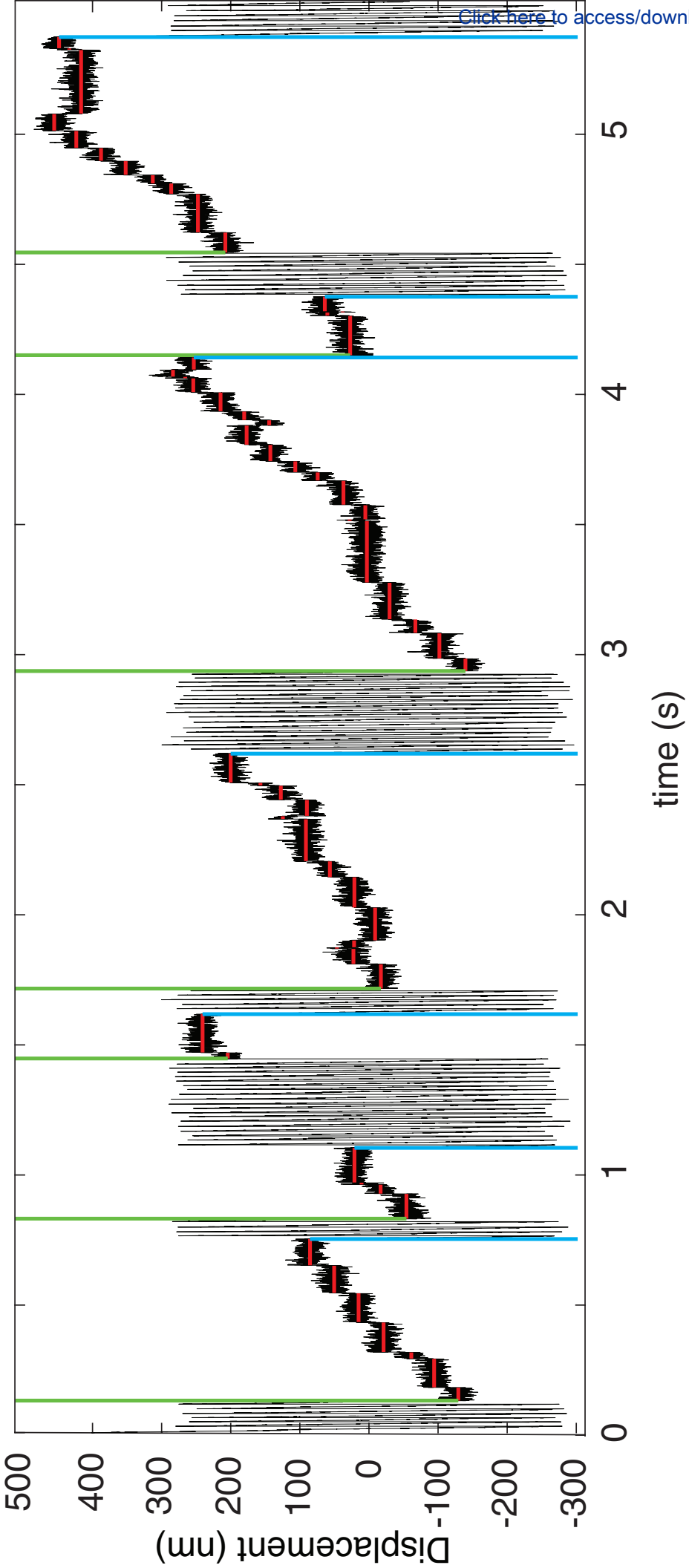

Figure 5

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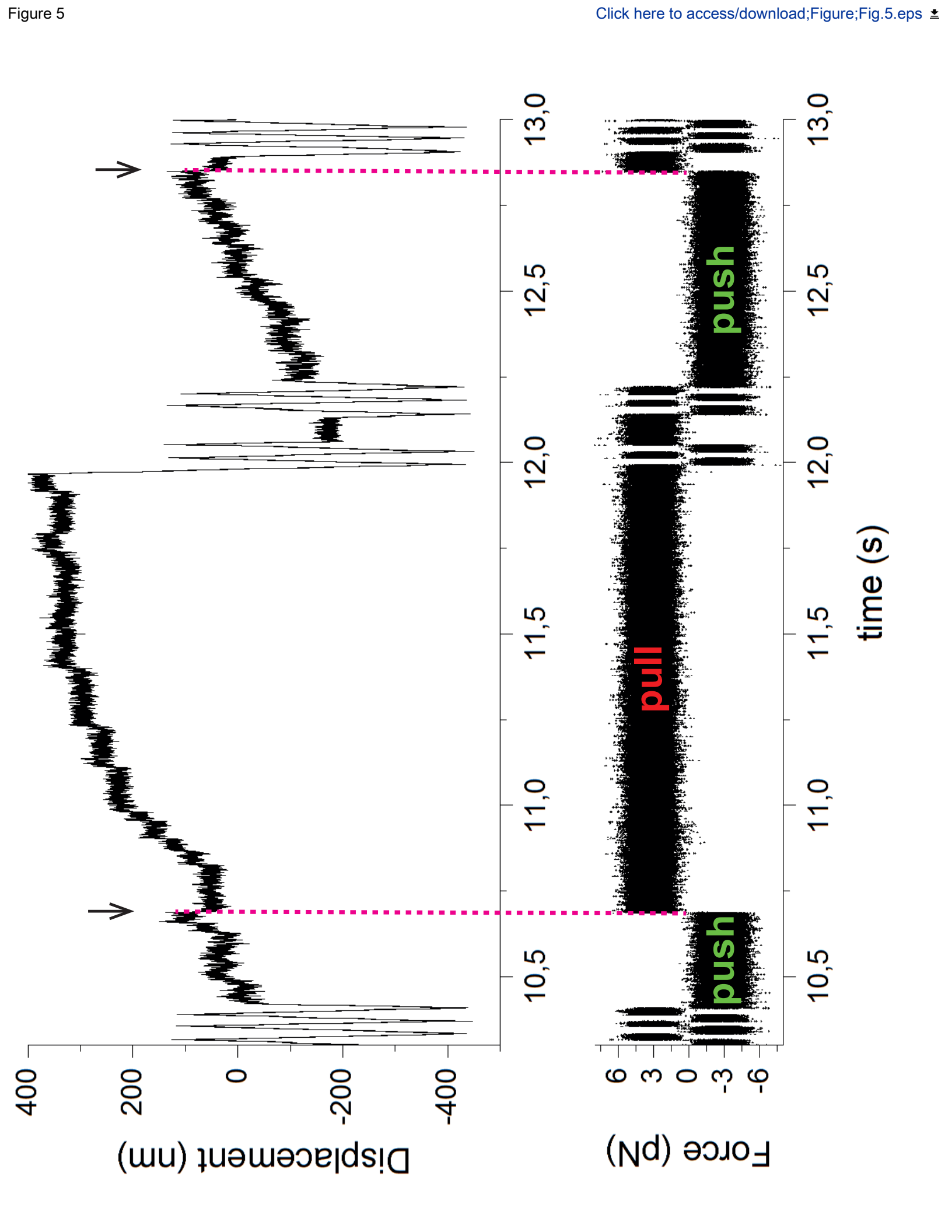

Name of Material/ Equipment	Company	Catalog Number
Aliphatic Amine Latex Beads	ThermoFisher	A37362
Acetone	Sigma	32201
Actin polymerization buffer	Cytoskeleton	BSA02
AODs (acousto-optic deflectors)	AA Opto Electronic	DTS-XY 250
ATP	Sigma	A7699
Biotinylated-BSA	ThermoFisher	29130
BSA	Sigma	B4287
Calmodulin from porcine brain	Merck Millipore	208783
Catalase from bovine liver	Sigma	C40
Condenser	Olympus	OlympusU-AAC, Aplanat, Achromat
Creatine phosphate disodium salt tetrahydrate	Sigma	27920
Creatine Phosphokinase from rabbit muscle	Sigma	C3755
DDs	AA Opto Electronic	AA.DDS.XX
DL-Dithiothreitol (DTT)	Sigma	43819
EGTA	Sigma	E4378
G-actin protein	Cytoskeleton	AKL99
Glucose	Sigma	G7528
Glucose Oxidase from Aspergillus niger	Sigma	G7141
HaloTag succinimidyl ester O2 ligand	Promega	P1691
High vacuum silicone grease heavy	Merck Millipore	107921
KCl	Sigma	P9541
KH2PO4/K2HPO4	Sigma	P5379/ P8281
Labview	National Instruments	version 8.1
Labview FPGA module	National Instruments	version 8.1
Matlab	MathWorks	2016
MgCl2	Fluka	63020
Microscope Objective	Nikon	Plan-Apo 60X
MOPS	Sigma	M1254
Nitrocellulose	Sigma	N8267
Pentyl acetate solution	Sigma	46022

Pure Ethanol	Sigma	2860
QPDs	UDT	DLS-20
Rhodamine BSA	Molecular Probes	A23016
Rhodamine Phalloidin	Sigma	P1951
Silica beads	Bangslabs	SS04N
Sodium azide	Sigma	S2002
Streptavidin protein	Sigma	189730

Comments/Description
1.0-µm diameter, 2% (w/v)

10X
Laser beam deflectors

NA 1.4, oil immersion

Two-channel digital synthesizer

Data acquisition
Fast Force-Clamp
Data analysis

NA 1.2, WD 0.2 mm, water imm.

0.45 pore size

D Position Detecto

1.21 μm, 10% solids

Editorial comments:

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

We did it.

2. Please revise the following lines to avoid previously published work: 43-45, 83-85, 96-97, 111-121, 145-147, 199-200, 210-211, 214-216, 220-221, 244-245, 251-254.

We did it.

3. Please define all abbreviations before use (CCD, CMOS, RT, AB, M5B, PB, etc.)

We did it.

4. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.)

We have removed all the pronouns and rephrased accordingly.

5. JoVE cannot publish manuscripts containing commercial language. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials: e.g., HaloTag, etc. We must maintain our scientific integrity and prevent the subsequent video from becoming a commercial advertisement.

We checked for commercial language throughout the manuscript and we didn't find any specific reference to commercial products. HaloTag is the name of a published (2008) worldwide used protein labeling technology for Cell Imaging and Protein Analysis (https://pubs.acs.org/doi/10.1021/cb800025k), thus it is not commercial language.

We checked the Table of Materials and we updated it with a couple of materials that were missing in the previous version and new material specifications on the optical setup hardware, we uploaded the new table as Table of Materials_Gardini_rev file.

6. Please use standard abbreviations when the unit is preceded by a numeral throughout the protocol. Abbreviate liters to L to avoid confusion. Examples: 10 mL, 8 µL, 7 cm2

We changed them.

7. Line 132: For time units, please use abbreviated forms for durations of less than one day when the unit is preceded by a numeral. Do not abbreviate day, week, month, and year. Examples: 5 h, 10 min, 100 s, 8 days, 10 weeks

We changed them.

8. Line 173- 177: Please include the details of the volume of PBS and distilled water used for each washing step.

We did it.

9. Please revise the Representative Results to include at least one paragraph of text to explain the Representative Results in the context of the technique you have described, e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc.

We added a sentence at the beginning of the Representative Results section describing more in detail the data obtained by the technique.

The paragraph text should refer to all of the figures which show representative results. Data from both successful and sub-optimal experiments can be included. Please discuss all figures in the Representative Results.

We moved a paragraph from section 4.2 to the first paragraph of Representative Results where we refer to figure 5 and described the showed results.

However, for figures showing the experimental set-up, please reference them in the Protocol.

Figure 2, describing the experimental set-up, was already referenced in the Protocol section dedicated to the optical setup (section 1 of the Protocol), but we added other references as you requested

10. Please move the Figure Legends to the end of the Representative Results section.

Done

11. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

We further revised the discussion according to the editor requests.

12. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al. Ensure that the Journal titles and book titles are not abbreviated. Please do not use &- sign or the word “and” when listing the authors.

We modified the references accordingly.

13. Figure 4: Please relabel “displacement” in the Y-axis with a capital “D” (Displacement) to make it consistent with the other figures.

We uploaded the updated version of Figure 4 as Figure 4-rev.

14. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in Figure Legend, i.e. “This figure has been modified from [citation].”

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Reviewers' comments:

Reviewer #1:

Manuscript Summary:

Authors generously provide an effective protocol to perform ultrafast force-clamp experiments on processive myosin-5 motors, which includes necessary steps, from the setup of the experimental apparatus to sample preparation, calibration procedures, data acquisition and analysis.

Several concerns are listed below.

Major Concerns:
1. The interpretation of the experimental data acquired in Ultrafast force-clamp spectroscopy seems to require a decent theory for the kinetic model of motors under investigation. As clearly shown in the paper by Dong and Chen JAM (2016), detachment rates would couple with transition rates in determining the breaking rates acquired in Ultrafast force-clamp spectroscopy. How would authors address such a challenge in their data analysis?

The goal of the present protocol is to describe in depth the experimental procedure, from the setup of the experimental apparatus to data analysis to allow other researcher setup the technique, acquire data, and extract kinetic and mechanical data from processive myosin motors. Regarding data analysis, we describe the procedure to identify myosin interactions and steps and precisely determine the duration of interactions, velocity and run length. This analysis has been described and validated through a number of peer-reviewed papers (Capitanio et al. Nature Methods 2012; Gardini et al. Nature Comm 2018; Tempestini et al. NAR 2018; Woody et al. eLife 2019). We don't go into the details of interpreting the kinetics through a motor model, which is a very interesting subject but beyond the scope of the present paper.

Minor Concerns:
1. In general, both the direction and the intensity of the applied load strongly affect the kinetics of motors. The authors stated in the paper that they were able to control the direction of the applied load, which, however, is unclear to us.

We added a more detailed description of the system in the Introduction, where we explained more in detail how the force intensity and directionality are controlled and can influence the motor protein processivity.

"As shown in Fig. 1a, in this system a single actin filament is suspended between two polystyrene beads trapped in the focus of double optical tweezers (dumbbell). An imbalanced net force F= F1-F2 is imposed on the filament, through a fast feedback system, which makes the filament move at constant velocity in one direction until it reaches a user-defined inversion point where the net force is reversed in the opposite direction. When the motor protein is not interacting with the filament the dumbbell is free to move back and forth in a triangular wave shape (Fig. 1b, bottom panel) spanning the pedestal bead on which a single motor protein is attached. Once the interaction is established the force carried by the dumbbell is very rapidly transferred to the motor protein and the motor starts displacing the filament by stepping under the force intensity and direction that was applied by the feedback system at the time of the interaction until myosin detaches from actin. Being the displacement produced by the stepping of the motor dependent on the polarity of the trapped actin filament, according to the direction of the applied force the load can be whether assistive, i.e. pushing in the same direction of the motor displacement (push in Fig. 1b upper panel), or resistive, i.e. pulling in the opposite direction with respect to the motor displacement (pull in Fig. 1b upper panel) making it possible to study the chemomechanical regulation of the motor processivity by both the intensity and the directionality of the applied load."

2. In the described assay, the load would be guaranteed to be applied along the length of the filament, with negligible transverse and vertical components, though transverse and vertical components of the load could strongly affect the kinetics of motors. But what if the active load generated by motors in vivo has such transverse, vertical components, or even is a torque?

This is an interesting subject of research that has been pursued by other groups. The three-bead geometry as we use here is very efficient in measuring forces along the filament direction, but it dissipates to a great extent mechanical work performed in different directions because of the

compliance of the filament along the other directions. Single bead assays are more effective for measuring transverse and vertical force components as well as torque. We have added a sentence in the discussion on this point.

Reviewer #2:

Manuscript Summary:

Gardini et al. present a detailed protocol for conducting ultra-fast force-clamp optical tweezers experiments and investigate processive myosin-5 motors. The authors present details on the assembly of their setup and a thorough protocol for sample preparation and data acquisition. I believe that the protocol includes sufficient information for reproducing the proposed experiments; therefore, I recommend it for publication on JoVE. However, I have some remarks that could improve the level of detail of the publication:

Major Concerns:

None

Minor Concerns:

* The authors highlight the importance of having force-clamp conditions, which are not common in most optical tweezers setups. Therefore, they could include further information on their force-feedback system. From 1.1.5, it could be understood that the force signal is processed at high-speed with the FPGA, but not much detail is given. The authors could include some technical details on their force-clamp strategy.

We have included details of the feedback system in section 1.1.5

* Why does the force fluctuate so much (Fig. 1B, Fig. 5)? It is surprising that, if the system corrects the force at 200 kHz, there are such large fluctuations in force (from the figure it seems 3+-2 pN!). The authors could include information on the force signal to demonstrate their force-clamp (e.g., distribution of the force signal, power spectrum). Does this affect the measurement, or is it too be sensed by the motor (much faster than the timescale set by the system's diffusion coefficient)?

Data reported in Fig. 1B and 5 are raw, non-filtered data. Thermal noise explores a large range of forces much faster than the about 100kHz response time of our feedback. Data in fig.5 are plotted on a large time scale (2.75 seconds) and each point in the graph is a position acquired with 5us sampling time. Therefore, there are 550000 points plotted in Fig.5, and each point has a finite size. Therefore, also the tails of the Gaussian at several sigma from the center of the distribution appears as a full black line. This gives the impression that the standard deviation of the force is large. Actually, direct calculation of the force standard deviation gives about 0.27 pN under typical experimental conditions (trap stiffness about 0.047 pN/nm and force in the range 0.5 - 2 pN). In the plot in Fig. 2B, which is on a shorter time scale, the force noise looks smaller than in Fig. 5, but again it's just because of the lower number of data points. We have added a sentence in the figure caption to clarify that raw data at 200kHz is plotted and we report a value of the force standard deviation to clarify this point.

* Including some technical information about the recordings on Fig. 1, Fig. 4, and Fig. 5 would be illustrative. Is the raw extension signal shown, or there is some filtering? At what frequency is the data acquired?

We have now included details on the acquisition rate in 1.1.5 (200kHz). We have added in the figure caption that raw data are plotted.

* From the instrumental perspective, it could be useful to know about the software/s employed for data acquisition, force feedback, data analysis, etc.

We have included details on the software used for data acquisition and feedback and data analysis in the Table of Materials (as JOVE politics we cannot mention commercial products in the article text)

Reviewer #3:
Manuscript Summary:
The authors Gardini, Kashchuk, Pavone and Capitanio present a comprehensive protocol for assembling an optimized dumbbell setup for optical traps. Furthermore they describe their recipes to prepare and assemble fluid cells and to setup the trap for measurements on myosin V. Data obtained from such measurements are evaluated exemplarily. Extensions to other molecules and advantages of their setup are discussed.

Major Concerns:
none

Minor Concerns:
none

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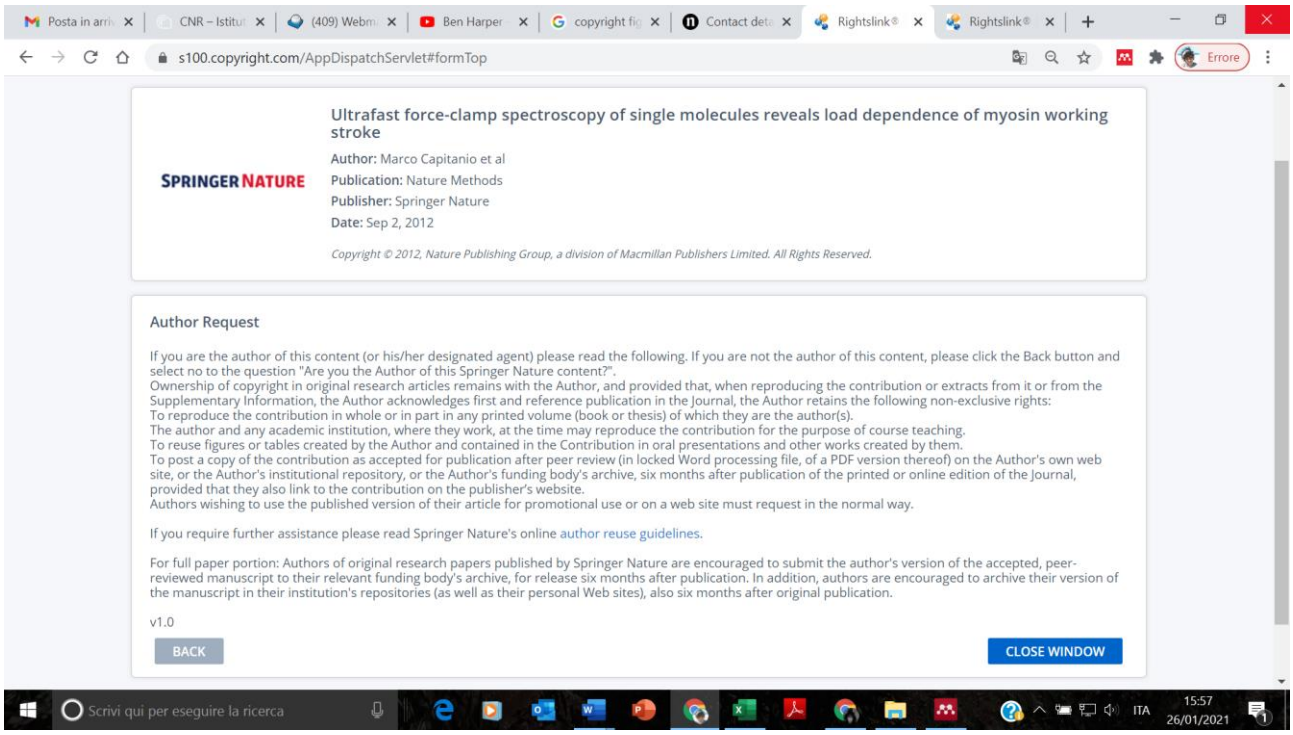

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