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Measurement of tension release during laser induced axon lesion to evaluate axonal adhesion to the substrate at picoNewton and millisecond resolution

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Abstract:	The formation of functional connections in a developing neuronal network is influenced by extrinsic cues. The neurite growth of developing neurons is subject to chemical and mechanical signals, and the mechanisms by which it senses and responds to mechanical signals are poorly understood. Elucidating the role of forces in cell maturation will enable the design of scaffolds that can promote cell adhesion and cytoskeletal coupling to the substrate, and therefore improve the capacity of different neuronal types to regenerate after injury. Here, we describe a method to apply simultaneous force spectroscopy measurements during laser induced cell lesion. We measure tension release in the partially lesioned axon, by simultaneous interferometric tracking of an optically trapped probe adhered to the membrane of the axon. Our experimental protocol detects the tension release with pico-Newton sensitivity, and the dynamic of the tension release at millisecond time resolution. Therefore, it offers a high-resolution method to study how the mechanical coupling between cells and substrates can be modulated by pharmacological treatment and/or by distinct mechanical properties of the substrate.
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Author Comments:	Dear Editor Dr. Larissa Jarzylo, herewith enclosed we are sending the paper by Vassalli M. et al. "Measurement of the tension release during laser induced axon lesion to quantify axonal adhesion to the substrate with picoNewton and millisecond resolution" (Ms # 50477), which has been revised according to reviewer suggestions. You can find attached to this communication a detailed response to reviewer concerns. Thank you for the prompt and thorough processing of our paper. We hope that the paper, in this new form, will be evaluated for publication in JOVE. Yours sincerely, Francesco Difato Italian Institute of Technology Via Morego 30, 16163, Genoa, Italy

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Measurement of tension release during laser induced axon lesion to evaluate axonal adhesion to the substrate at picoNewton and millisecond resolution

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KEYWORDS: Axon, tension release, Laser dissector, optical tweezers, force spectroscopy.

SHORT ABSTRACT: We measured the tension release in an axon that was partially lesioned with a laser dissector by simultaneous force spectroscopy measurement performed on an optically-trapped probe adhered to the membrane of the axon. The developed experimental protocol evaluates the axon adhesion to the culture substrate.

LONG ABSTRACT: The formation of functional connections in a developing neuronal network is influenced by extrinsic cues. The neurite growth of developing neurons is subject to chemical and mechanical signals, and the mechanisms by which it senses and responds to mechanical signals are poorly understood. Elucidating the role of forces in cell maturation will enable the design of scaffolds that can promote cell adhesion and cytoskeletal coupling to the substrate, and therefore improve the capacity of different neuronal types to regenerate after injury.
Here, we describe a method to apply simultaneous force spectroscopy measurements during laser induced cell lesion. We measure tension release in the partially lesioned axon, by simultaneous interferometric tracking of an optically trapped probe adhered to the membrane of the axon. Our experimental protocol detects the tension release with pico-Newton sensitivity, and the dynamic of the tension release at millisecond time resolution. Therefore, it offers a high-resolution method to study how the mechanical coupling between cells and substrates can be modulated by pharmacological treatment and/or by distinct mechanical properties of the substrate.

INTRODUCTION: Optical microscopy represents the less invasive imaging system to observe living cells. With the exploitation of effects such as radiation pressure (as in optical tweezers[1]), or high-energy photon flux (as in laser dissector[2]), this technology was extended to nano-manipulation. The optical imaging system furnishes a precise control to visualize and manipulate sub cellular targets[3]. At the same time, thanks to the accurate calibration of the delivered laser power, optical tools accomplish either soft or invasive sample manipulation with unprecedented reproducibility.

Several laboratories integrated, in the same experimental setup, optical tweezers and laser dissector in order to ablate organelles[4], to fuse together different cells[5], or to stimulate cells by optically driven cargos[6,7]. While optical tweezers, after calibration of the optical stiffness, allow

for the control of applied force to the cell on a pico-Newton scale, laser dissection systems can modulate optical manipulation, which ranges from membrane photo-poration to ablation of single organelles or dissection of sub-cellular structures. However, laser dissection calibration relies on qualitative assessment of the entity of optical manipulation respect to the energy delivered to the sample, mainly based on image analysis illustrating morphological changes caused to the specimen[8]. In the presented method, we demonstrate how to perform force spectroscopy measurement during the laser axonal dissection of a developing neuron, to quantify, on pico-Newton scale, the force produced by an altered equilibrium in the cytoskeleton structure of a sub-cellular compartment[9]. Cultured neurons adhere to the substrate, and polarize during development. The polarization phase occurs during the first five days *in vitro*. At stage two of polarization, one of the extruding neurites becomes longer, and it will differentiate to become the axon[10]. Axonal elongation in response to towing force at the growth cone has been previously modeled by Dennerl model[11]. Recently, such model has been extended[12] to include the role of neurite adhesion to the extracellular matrix substrates. This biophysical model, proposed after experimental observations[13], showed that pulling forces on growth cone, propagating along the neurite, are modulated by focal adhesions to the substrate. Likewise, axonal lesion produces a local release of tension propagating toward the cell body. Thus, we proposed that measuring such released tension in a location along the axon between the lesion and the cell soma offers the possibility to assess the dampening outcome of unaffected focal adhesions.

We calibrate the necessary energy photon-flux of the laser dissector to control the extent of the inflicted axonal damage, from complete transaction to partial lesion. Following the calibration, we repeated partial lesion to the axons of several differentiating neurons and developed the protocol to quantify the tension release, and thus obtaining a quantitative parameter to estimate the adhesion of the axon to the substrate[14].
In the present work, we describe in detail the developed protocol, which represents a precise experimental procedure to evaluate and compare with picoNewton sensitivity the axonal adhesion to the substrate in different experimental conditions such as chemical treatment[14], or different types of cell culture support.

PROTOCOL:

1) Optical setup. The entire optical system was described earlier[15]. Briefly, the optical tweezers system is based on an ytterbium continuous wave (CW) fiber laser operating at 1064 nm (IPG Laser GmbH). A spatial light modulator (SLM) (LCOS-SLM, model X10468-07 – Hamamatsu) varies the phase of the incoming IR laser beam to control the position of the trapping focus spot on the culture dish by computer generated holograms. The freely available Blue-tweezers software (web link on equipment table) generated holograms projected on the spatial light modulator. The interferometer for force spectroscopy measurements was based on a four-quadrant photodiode (QPD, S5980 with C5460SPL 6041 board – Hamamatsu) and a photodiode (PD, PDA100A-EC - Thorlabs).

The laser dissection source was a pulsed sub-nanosecond UV Nd:YAG laser at 355 nm (PNV-001525-040, PowerChip nano-Pulse UV laser – Teem Photonics). An acousto-optic modulator

(MQ110-A3-UV, 355nm fused silica –AA-Opto-electronic) controlled the power of the UV laser delivered to the sample.

The holographic optical tweezers and laser micro-dissector were integrated on a modified upright microscope (BX51 – Olympus) equipped with a 60x, 0.9 NA water dipping objective.
The stage of the microscope is composed of a 3-axis linear DC motor micro-positioning system (M-126.CG1, Physics-Instruments) carrying a separate 3-axis piezoelectric nano-positioning stage (P-733.3DD, Physics-Instruments) to combine coarse movement of the sample with the sub-nanometer resolution of the piezo-stage. The microscope stage system was equipped with two control loops synergistically acting to maintain the trapping focus spot at the right position, depending on the selected working mode (position or force clamp, static or dynamic)[16]. In particular, an internal feedback loop acts on a piezoelectric stage, to keep the bead at a selected distance from the trap center. The other external loop controls the position of motorized stage to exploit the region spanned by the piezo-actuator on a larger area than its available stroke[17]. When the piezo-stage reaches the limit of the available stroke in one direction, the external loop moves the micro stage in the opposite direction, thus the piezo recovers toward its central position because it is tracking the trapped bead adhered to the sample. When the piezo-stage reaches the central position of its course range, the micro stage halted. Further details of the system are reporte in Guiggiani et al[16,17].

A Peltier device (QE1 resistive heating with TC-344B dual channel heater controller – Warner Instruments) controls the temperature of the cell culture under the microscope (37°C). In the culture h, pH and humidity were maintained at physiological conditions by aerating a custom-designed polydimethylsiloxane (PDMS) sleeve (integrating the microscope objective) with humidified carbogen (95% O2, 5% CO2).

2) Cell culture preparation.
All the experimental protocols were approved by the Italian Ministry of Health. Primary cultures were obtained from hippocampi of mice (C57BL6J, Charles River) at embryonic day 18 (E18).

2.1 Neurons were plated at a concentration of 25000 cells/mL on glass-bottom Petri dishes (P35G-0-14-C – MaTek Corporation). The low concentration of cultured cells is needed to avoid the formation of a dense network already in the first days in vitro. At the mentioned cell concentration, the likelihood of finding isolated cells with a longer neurite not connected to other cells is much higher.

3) Bead coating.

3.1 Polymer microspheres (Ø 4 µm, COOH-terminated– Bangs Laboratories, product code PC05N/6700) were coated with poly-D-lysine following the procedure described in the polyLink Protein Coupling Kit (Polysciences, product code 19539). The beads are coated with the same molecule used to cover the culture support and favor cells adhesion.

4) Choose isolated neuron. Detach a bead from the culture substrate, trap and move it next to the neuron.

4.1 Put the culture Petri-dish on the microscope stage. After setting the focus on the sample by

stage motion, correct the position of the illuminating objective condenser to set Kohler-illumination. Aligning the illumination optics to set the Kolher-illumination condition is necessary to improve the bright field imaging quality. Using such alignment conditions, it is also necessary to maximize the IR laser collection efficiency (through objective condenser), from the scattering trapped probe, to perform its interferometric tracking by the QPD and PD.

4.2 Pipette few µL of the coated microsphere stock solution in the culture dish. Microspheres will deposit and adhere on culture substrate. The amount of µL injected in the culture dish depends on the microsphere concentration of the stock solution. The ideal condition is to have one-two microspheres per imaging field of view. When too many microspheres are added to the culture dish, they can already attach randomly to the cultured neurons.

4.3 Move around in the culture dish for searching an isolated neuron with a longer neurite (the axon), and save the position of the microscope stage.

4.4 Move around and search for a bead adhered to the culture support.

4.5 Turn on the IR trapping laser. At this stage, no holograms are projected on SLM, and the position of IR laser spot coincides with the UV laser spot position. Set the axial position of the IR spot 2-3 µm above the culture support surface, by microscope stage motion.

4.5 Set the UV laser power delivered to the sample to less than 1µW. The UV laser dissector has been previously calibrated[14]:

5-6 µW the glass support is ablated
4 µW a neuronal connection is completely dissected
2.5 µW a neurite is partially lesioned
‹ 1 µW a shock wave is produced in culture dish. The optical shock wave is enough strong to detach the bead from the glass support.

4.6 Turn on the UV laser, while leaving the IR laser on, and move the UV spot above the adhered bead by microscope stage motion. The bead detaches from the surface, and it is trapped by the IR laser. Then, turn off the UV laser.

4.7 Move the trapped bead several micrometers above the glass support (20-30 µm). In such a way when moving the bead toward the previously chosen neuron, it will not contact the other cells. Bring the bead to the previously saved stage position. Set the stage speed to a low value (10 µm/sec) so the drag fluid force does not exceed the optical trapping force.

5) Move the trap position with respect to the laser dissector spot and axon position by computer generated hologram, and calibrate the optical tweezers stiffness.

5.1 Move the bead toward the glass support to visualize the axon. The bead is held above the cell to avoid contact with it (about 5 µm above the glass support).

5.2 Move the microscope stage to move the UV focus spot on the center of the width of the axon. Save the current stage position.

5.3 Move the IR focus spot position by computer-generated hologram. In this step, the stage is halted in the position chosen in the previous step. When computer generated hologram is projected on the SLM, the trapped bead is moved with respect to the axon. We move the IR laser spot to have the trapped bead aligned on the center of the neurite, with the UV laser spot centered on the same neurite too. The IR spot and thus the trapped bead are positioned along the neurite 5-10 µm away from the UV spot. We move the position of the IR respect to the UV spot depending on the neurite geometry. In case the optical tweezers are not equipped with a SLM system, the position can be varied by tilting mirrors, or acoustic optic deflectors, on the IR optical path. The IR laser spot may be positioned 5-10 µm away from the UV spot, to avoid optical interaction of the dissecting beam with the trapped probe, which could influence its Brownian motion and alter the force spectroscopy traces.

5.4 After defining the new IR spot position respect to UV spot and axon position, move the stage to position the trapped bead away from the axon and avoid collision with it. Move the axial position of the trapped bead at about 2 µm above the cover glass.

5.5 Align the QPD to center the interference fringes on it: x and y QPD differential signals are zeros when the QPD is centered. Acquire 5 seconds of the Brownian motion of the trapped bead by the interferometer, at 50 KHz sampling rate. Obtain the stiffness (pN/nm) and sensitivity (V/nm) of the optical trap by power spectrum method[18].

6) Attach the bead to the axon. Perform axotomy and simultaneous force measurement.

6.1 Raise the trapped bead position about 4 µm above the cover glass, and move it to the previously saved stage position (see step 3.2).

6.2 Move the trapped bead down toward the axon until it contacts the neurite. The collision between the trapped bead and the axon is monitored by the z QPD signal detecting displacement of the trapped bead in the axial direction.

6.3 Wait 10 seconds with the trapped bead pushed against the axon to favor its adhesion to the neurite membrane.

6.4 Move the micro stage to displace the trapped bead from the axon, and thus verify its adhesion to the cell membrane. If the bead adhered, it escapes from the optical trap.

6.5 Move back the laser trap on the adhered bead and switch on the force-clamp loop with force condition equal to zero. In such a way, the piezo-stage repositions the bead, attached to the cell, on the optical trap center. If the system has not a feedback loop control, the laser trap position can be moved by the microscope stage to center it on the adhered probe. The center of the trap is reached once on the QPD we obtain the same signals

appearing when the bead is trapped and does not adheres to the cell (x and y QPD signals equal to zero, and the z QPD signal gives the same voltage sum of the four quadrants when the bead is trapped far from any surface) .

6.6 Set a force-clamp condition on the z axis, positioning the trapping laser slightly over the center of the bead (about 100 nm), to generate a pretension on the adhered trapped probe. In case the system is not equipped with a force-clamp control, the optical trap position can be raised up at steps of 25 nm by the microscope stage. The z QPD signal allows monitoring and thus set the position of the laser trap respect to the adhered probe. Such pre-tension is needed to sense the strain on the membrane after the axonal lesion, and the consequent decrease of Brownian motion of the adhered probe. Similar approach has been proposed to measuring the release of tension in a membrane tether by video imaging[19].

6.7 Switch-off the force-clamp feedback to measure the force on the adhered probe in position-clamp condition (the piezo-stage is blocked).

6.8 Start simultaneous recording of the trapped probe positions trough the interferometer (20 KHz sampling frequency), and the cell during laser axotomy time-lapse bright-field imaging (20 Hz frame rate).

6.9 Turn on the UV laser to deliver laser pulses until a lesion becomes visible on the image of the axon (usually are needed 200-400 optical pulses. Energy per pulse 25 nJ), then turn off the UV laser.

6.10 Continue recording by the interferometer for about 3 minutes, until the x, y and z QPD traces reach a plateau.

7) Quantify the total tension release.

7.1 Convert the recorded QPD traces (in Volts) by the calibrated sensitivity of the optical trap, to obtain the respective traces in nanometers.

7.2 Filter the x, y, and z displacement traces by a high pass filter with cut off at 10Hz.

7.3 Calculate the total variance of the filtered traces representing the Brownian motion of the trapped bead. Calculate the variance at 25 ms steps for overlapping time windows of 500 ms (10000 data points)[20]. The high pass filtering of traces excludes the changes of Brownian motion due to the membrane fluctuation or cortical actin motion. The Brownian motion of the bead is reduced by the optical forces, and the adhesion forces on the cell membrane. When the membrane is strained because of the release of tension its viscosity is increased, and thus the Brownian motion of the bead, detected immediately after axotomy, starts to decrease.

7.4 Define t0: the beginning of decrease of the Brownian motion variance, after the delivery of UV laser energy. The time instant t0 indicates the beginning of membrane strain.

7.5 Define t1: the end of decrease of the Brownian motion variance (when it reaches a plateau). The time instant t1 indicates the end of membrane strain.

7.6 Perform video tracking of debris or scratch on the cover glass support, to measure any drift of the sample during the force measurement. To track the particle on the glass support, we first apply thresholds to the bright-field images, to obtain a stack of binary images with the particle in white color on black background. Then, we track the particle center of mass with sub-pixel accuracy (using the freeware ImageJ software).

7.7 Subtract the measured drift to the displacement QPD traces. Multiply the drift-correct QPD traces by the respective calibrated optical stiffness (k_x, k_y, k_z) to obtain the Fx, Fy, Fz traces in pico-Newton.

7.8 Calculate the total force trace as $F_{tot} = \sqrt{(Fx^2+Fy^2+Fz^2)}$.

7.9 Calculate the total release of force between t0 and t1 as $F_{released} = F_{tot}(t1) - F_{tot}(t0)$. The force measured at t0 has to be subtracted because it is due to the displacement of the probe from the trap center, when the bead adheres to the neurite.

7.10 Calculate the neurite contact area between the trapped bead and the UV laser spot position: on the bright field image measure the long (L) and short (D) axes of the neurite between the trapped bead and the UV laser spot position. The axon contact area is $A_{axon} = L \times D$.

7.11 Normalize the total released force $F_{released}$ by the axon contact area A_{axon}.

REPRESENTATIVE RESULTS: The cell generates traction forces on the substrate by its focal adhesions. Force generated by cytoskeletal elements are in equilibrium with the counteracting force of the culture substrate. After laser induced lesion of the neurite, some of the tensed cytoskeleton cable are disrupted and their equilibrated tension is released, because the opposing force of the substrate adhesion is eliminated. The released tension is partially distributed on the unaffected focal adhesions, and the bead attached to the cell membrane, held in an optical trap, measures the portion of such release not counteracted by cytoskeletal elements anchoring the cell to the substrate (see schematic figure 1).

We report, in figure 2, a representative result of the above-described experimental protocol. The neuron, in figure 2a left panel, presents a longer neurite, which identifies the axon of the differentiating neuron. In figure 2a right panel, it is shown the same neuron after the induced axonal lesion (indicated by the white arrow). Figure 2b shows the recorded bead displacement traces in x, y, and z directions. Figure 2c reports the video tracking of a scratch on the glass support, to take into account and eliminate the stage drift during the measurement.

In figure 3a, on the left panel, we show the lesioned neurite, and the white line upstream the lesion site, where we calculate the kymograph of the neurite diameter during the tension release measurement. On the right panel, it is illustrated the kymograph showing how the neurite diameter slightly increases immediately after the lesion, and then becomes thinner due to the release of tension toward the cell soma. On figure 3b, we quantify the kymograph result. The neurite appears brighter respect to the background, since we positioned the attached bead in the focus center of the objective, and therefore the axon is slightly out of focus. Indeed, by reporting the sum of pixels intensity along the kymograph line, at each frame of the time-lapse video recording, we obtain an estimate of the neurite diameter during the release of tension. On panel 3c, we report the total variance of the attached bead during the tension release, calculated on the traces recorded by the interferometer (in figure 2b), after high pass filtering at 10 Hz cut-off frequency. We can observe that the variance increases with the neurite diameter during accumulation of material upstream the lesion site. Then, the variance starts to decrease (at t0 in figure 3c) when the membrane is strained, and the neurite diameter decreases too. The variance decrease reaches a plateau (at t1 in figure 3c), when the tension release ceases. After t1, the Brownian motion starts to increase because of membrane relaxation possibly due to exocytosis[21] (see figure 3c).

In figure 4a, the white box indicates the estimation of the neurite contact area A_{axon} with the culture support. A_{axon} is calculated between the UV laser spot position and the center of the trapped probe. Figure 4b reports the amplitude trace of the total released force $F_{released}$ obtained after multiplying the drift-correct QPD displacement traces (in figure 2b) by the respective calibrated optical trap stiffness (k_x, k_y, k_z). We calculate the difference between force measured at time t1 and t0 (ΔpN in figure 4b), and then we divide by A_{axon}, to obtain the tension released in terms of $pN/\mu m^2$.

By repeating the same protocol on different neurons in the sample, we can start sessions of experiments on several cultures treated with chemical factors or plated on different supports, and finally compare the average value obtained in the distinct experimental conditions. In figure 5, we show the release of tension after axonal lesion is dampened by focal adhesions on the substrate, thus higher tension release value means less adhered axon to the substrate. Because we induce a partial, and not complete, lesion of the axon, we investigated the dependence of the measured tension release on the total energy delivered, and on the neurite contact area between the lesion site and the trapped bead. We supposed to destroy more cytoskeletal elements by delivering a higher number of light pulses, and consequently induce a higher release of tension. Otherwise, with an increased neurite contact area, we expected to measure a lower amount of tension release. In figure 5, we show not clear dependency on the two above-mentioned parameters. Therefore, we deduced that the induced partial lesion (with the previously calibrated low energy per pulse; see protocol step 4.5), is related to the dimension of the ablation spot[8], which is not varying when using the same microscope objective and the same energy per pulse at the sample. Moreover, it is not surprising the fact that the release of tension is not correlated to the contact area, because it is well known that such parameter is not representing a good estimate of the cell adhesion to the substrate as focal adhesions occupy only 1% of the basal surface[22].

Figure legends:

Figure 1. Graphical representation of the disrupted cytoskeleton equilibrium during laser axotomy. A cell adheres to a substrate by focal adhesions. Blue arrows specify traction forces generated by cytoskeletal elements (indicated by springs). Light-blue arrows indicate the counteracting forces generated by the rigid culture substrate. In a simplified scenario, before lesion, the cytoskeletal and substrate forces are pairwised and equilibrated. After laser-induced lesion of the neurite, the connections between some springs and the substrate are disrupted. Thus, the substrate is no longer counteracting the traction force of the cytoskletal element. The trapped bead, attached to membrane, tracks the direction of the released cytoskeleton forces.

Figure 2. Interferometric tracking of a trapped bead, attached to the membrane of the axon of a hippocampal neuron during laser induced lesion. (a) Bright field images acquired during ablation of an axon. Before the lesion on the left panel. After the lesion on the right panel. A poly-D-Lysine coated bead is attached to the membrane (polystirene bead,Ø4 µm) and held in an optical trap. Average power of the IR laser at the sample is 14 mW. White arrow indicates the lesion site. Bar is 5 µm. (b) Recorded traces by back focal plane interferometry of the bead position in the optical trap. Blue, green and red traces represent the bead position along x, y, and z axes, respectively. Sampling rate is 20 kHz. (c) Displacement of a scratch on the culture support measured by video tracking to monitor the stage drift. Blue and green traces are the x and y positions obtained by video tracking. Frame rate is 5.5 Hz.

Figure 3. Analysis of the axon diameter during the tension release, and the membrane strain of the lesioned axon. (a) Bright field image of the lesioned axon, on the left panel. The white line perpendicular to the axon indicate the position where a kymograph is computed. On the right panel, kymograph of the axon diameter, upstream the lesion site. Each row of the kymograph corresponds to 0.18 seconds. (b) Sum of pixels intensity of each row of the kymograph representing an estimate of the axon diameter during the tension release. (c) Total variance of the Brownian motion of the bead attached to the axon (t0 and t1 indicate respectively the beginning and the end of tension release in the axon after dissection).

Figure 4. Quantification of the tension released after axotomy. (a) Bright field image of the lesioned axon. The white box indicates the estimate of the neurite contact area between the lesion site and the center of the trapped bead. (b) Trace of the total amplitude of the force measured after axotomy ($Ftot= \sqrt{(Fx^2+Fy^2+Fz^2)}$). Fx, Fy, and Fz were calculated by the Hooke's law ($F=k \bullet x$), where the displacement traces are the ones shown in figure 2b. The stiffnesses, in the three orthogonal directions, of the optical trap were calculated by power spectrum method ($k_{x,y}$=7.9 pN/µm, k_z=2.3 pN/µm). ΔpN indicates the measured released force between the time instants t0 and t1.

Figure 5. Dependence of measured tension release on the amount of energy delivered to the sample, and on the neurite contact area. Data of 26 experiments performed on the axon of mouse hippocamapal neurons at 3 DIV. (a) Scatter plot of tension release versus neurite contact

area between lesion and trapped bead locations. (b) Scatter plot of tension release versus total amount energy delivered to the sample neurite contact area.

DISCUSSION: We report in this work a quantitative method to compare the neurite adhesion to the culture substrate, by performing simultaneous force spectroscopy measurement during laser induced cell lesion. The measured release of tension is related to the degree of adhesion of the cell to the substrate: cell with higher number of focal adhesion should release less tension. Measuring the release of tension in terms of picoNewtons allows to obtain a physical quantity to evaluate the axonal adhesion to the culture support in different experimental conditions[14].

Several laboratories integrated a laser dissector with an optical tweezers system, adopting distinct optical designs[23]. Our choice was a setup with fixed optical path and microscope stage motion. To accomplish it, we introduce a spatial light modulator in the IR laser beam path to move the trapping spot respect to the UV laser position. Although galvanometric mirrors allow stirring of the laser focus position without moving the sample, they can introduce mechanical noise in the system affecting the force spectroscopy measurements. Moreover, we decided to re-position the IR laser focus in the sample, since Brownian motion analysis permits a fast re-calibration of the optical stiffness. Moving the UV laser spot position on the sample can produce spherical aberrations affecting the quality of the UV focus spot itself, which are negligible only for small displacements of the beam from its central position. Therefore, modification of the optical properties of the laser dissector could require a *de novo* calibration of the delivered laser power versus damage entity.

By force spectroscopy measurement, we could observe that the tension release is composed of a fast phase of few seconds, and a slow phase that could last some tens of seconds. As reported in previous work[14], sometimes the slow phase could last few minutes. For that reason, we had to correct the force measurement by the video tracking of a scratch on the culture support. In future, a better approach would be to cancel stage drift with a feedback loop, which auto-corrects the microscope stage position by video or interferometric tracking of a bead attached to the cover glass. This type of configurations, already reported in literature, requires a second laser beam centered on the attached bead, and ensures stage stabilization with sub-nanometer precision[24,25].

In future, we want to apply the developed protocol to compare the axonal adhesion to the culture support at different stages of differentiation and in different pathological conditions to provide a quantitative assay for pharmacological treatment. Moreover, the same protocol could apply to design implantable scaffolds, to compare the neuronal adhesion to different types of materials, with distinct mechanical or chemical properties[26].

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DISCLOSURES

The authors declare that they have no competing financial interests.

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*Figure
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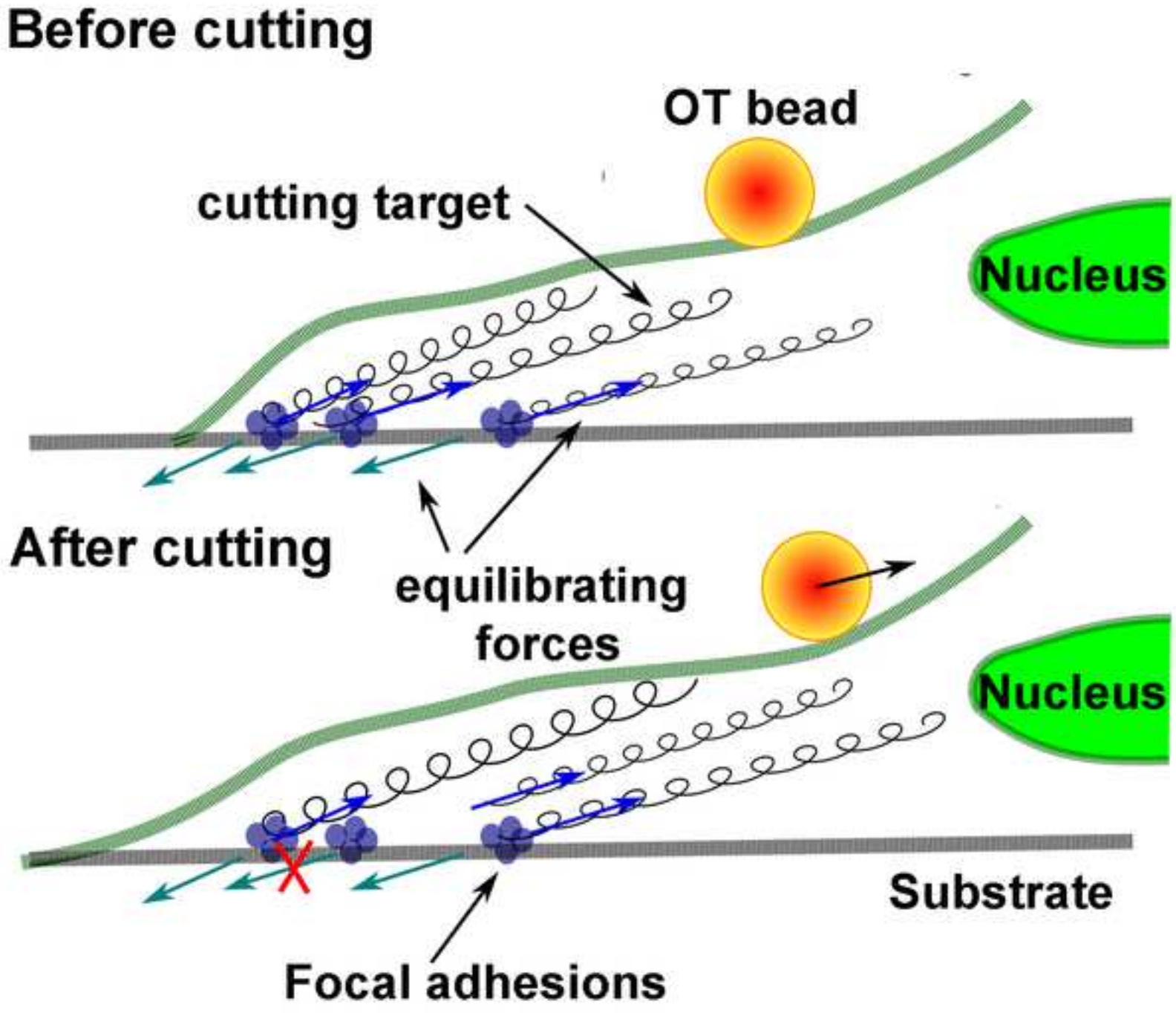

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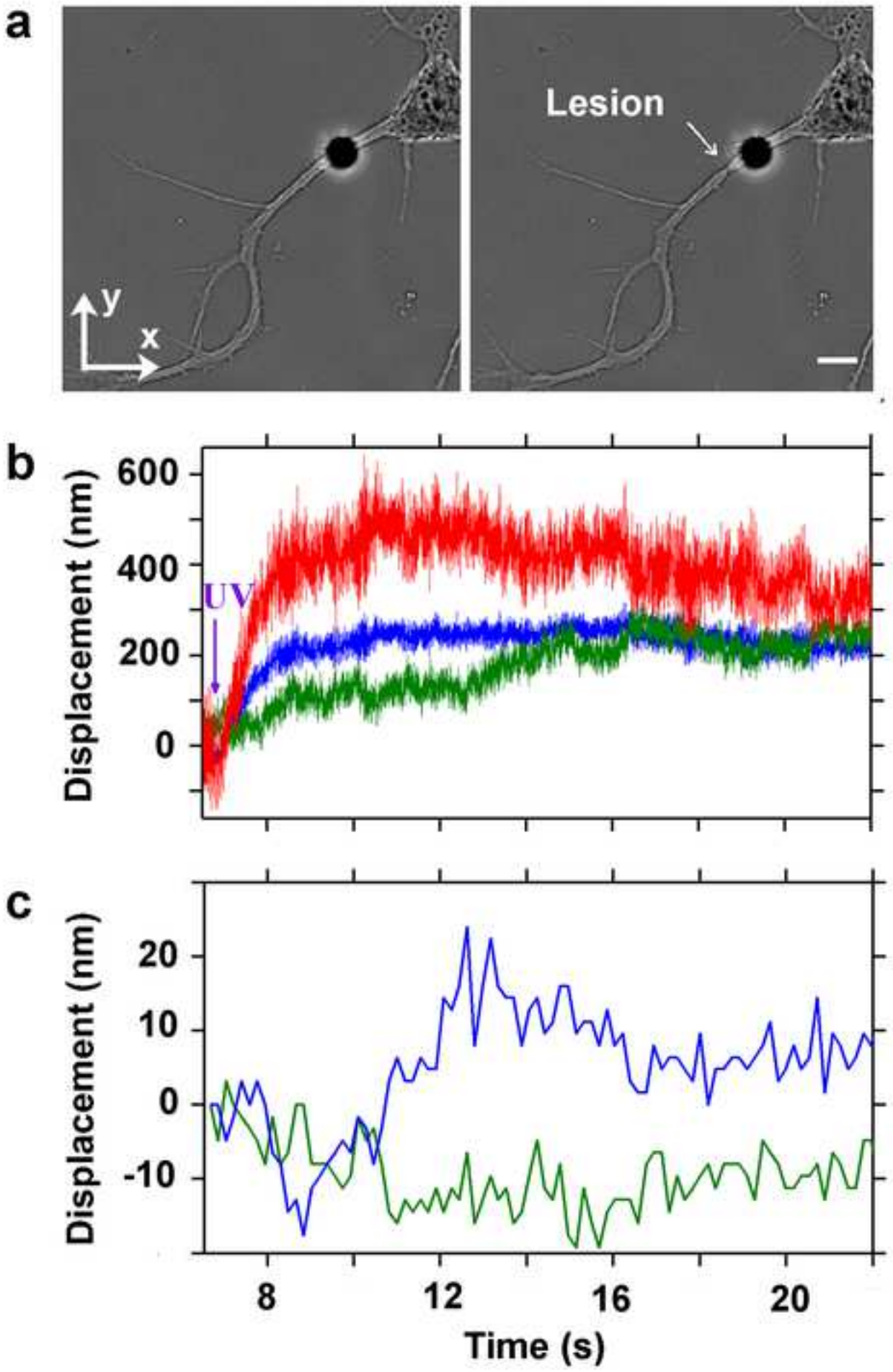

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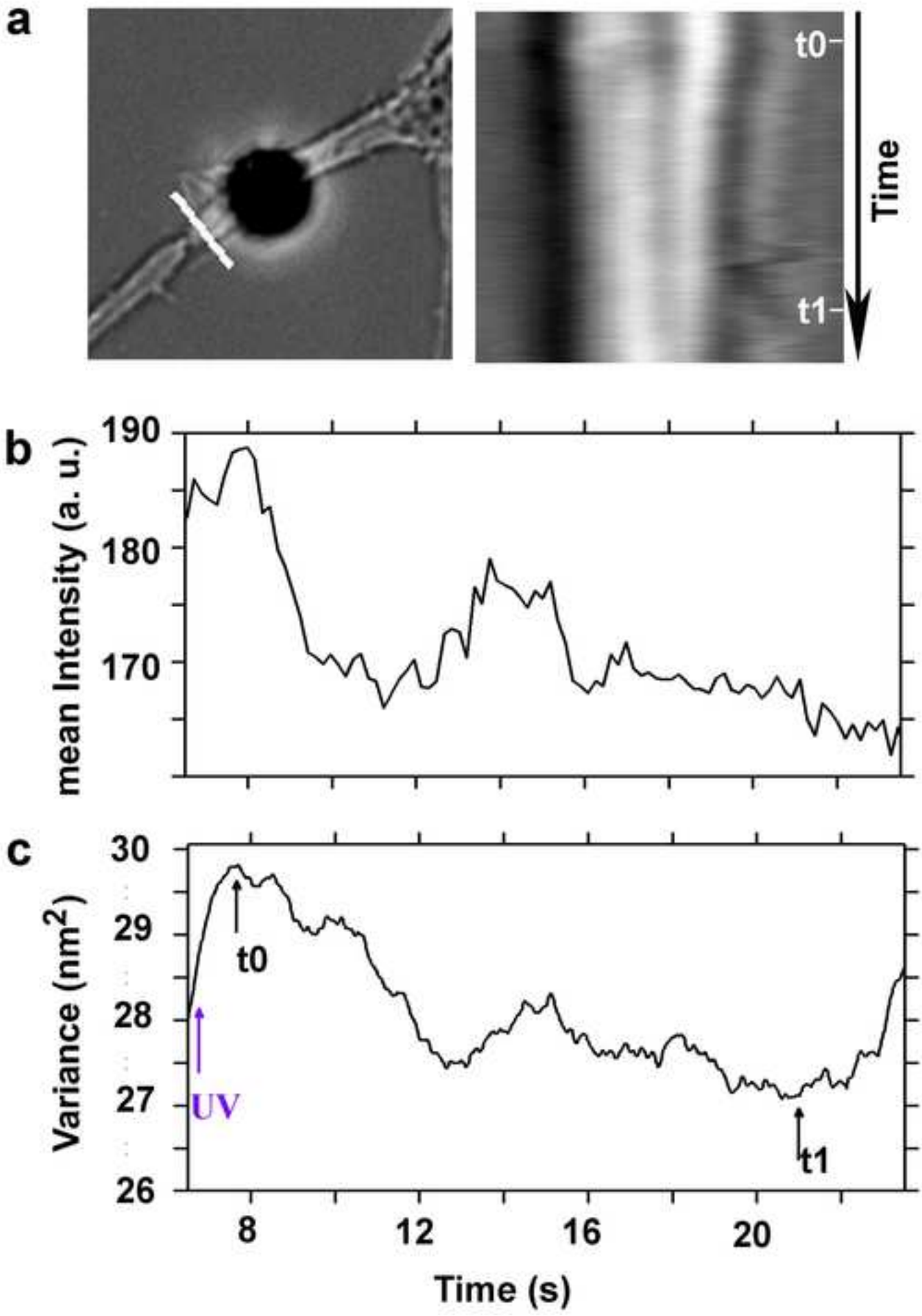

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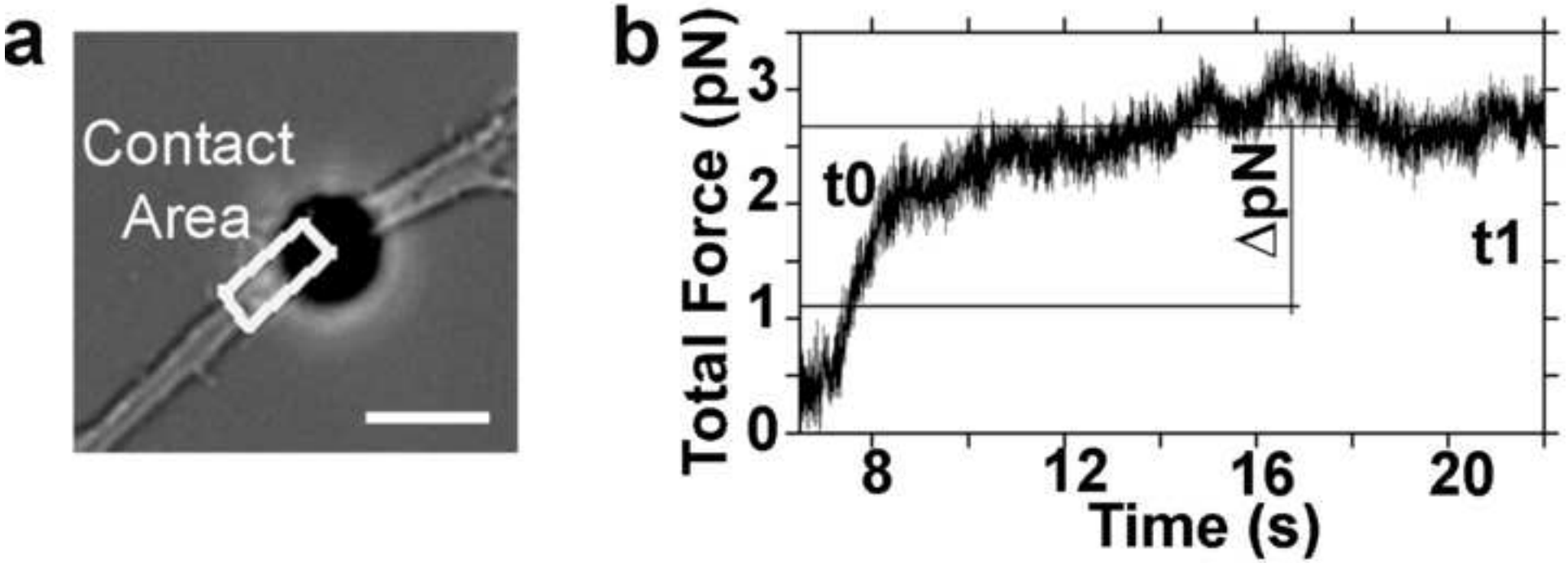

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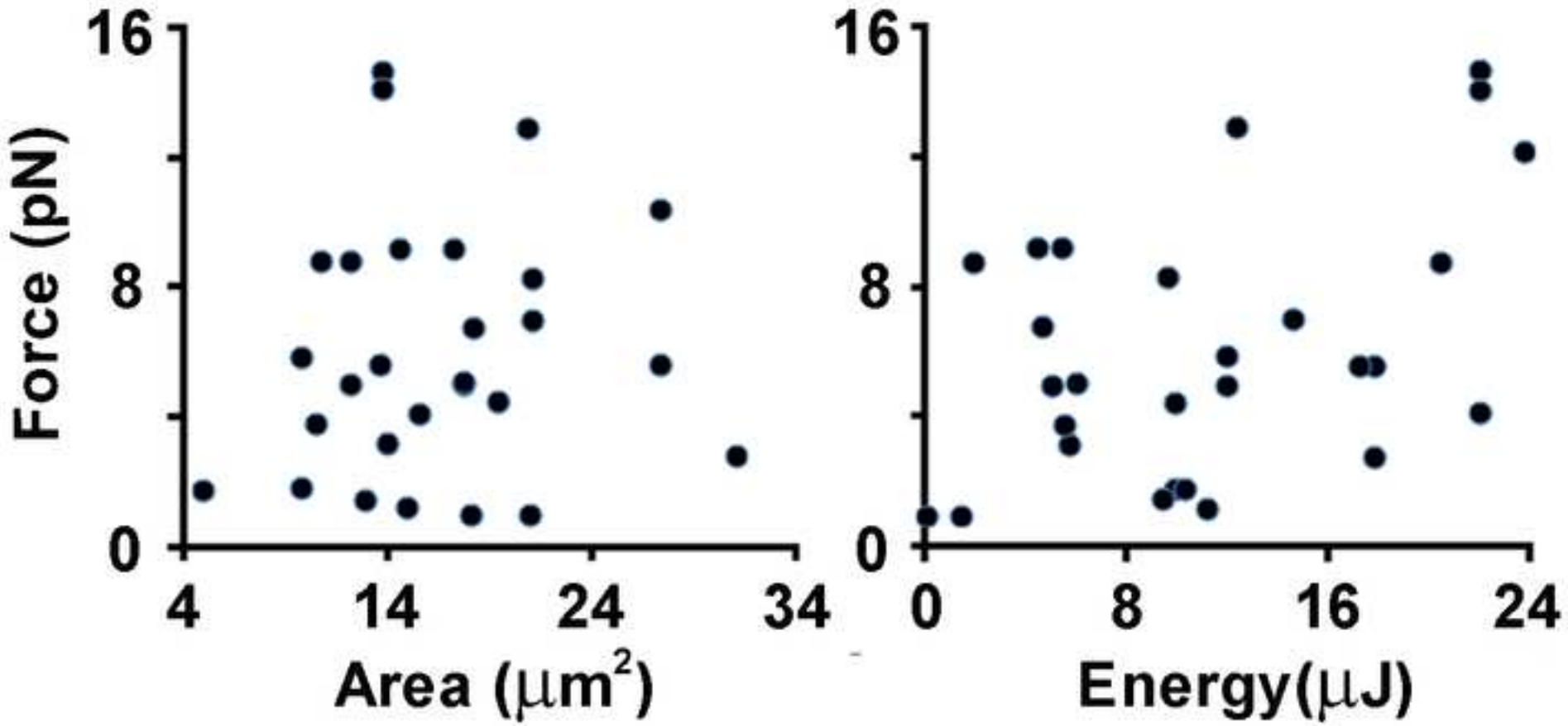

*Table of Reagents/ Materials Used
Click here to download Table of Reagents/ Materials Used: Materials.xls

REAGENTS:

Name of Reagent	Company	Catalog Number	Comments
Polymer microspheres, Ø 4 µm, COOH coated	Bangs laboratories	PC05N/6700	
PolyLink Protein Coupling Kit	Polyscience	19539	

EQUIPMENT:

Material Name	Company	Catalog Number	Comments
IR laser	IPG Laser GmbH	YLM-5-SC-LP	ytterbium continuous wave (CW) fiber laser operating at 1064 nm, with linear polarization
Spatial light modulator	Hamamatsu	LCOS-SLM 10468–07	
Blue-tweezers software	Optics group, University of Glasgow	Free downloadable software	http://www.physics.gla.ac.uk/Optics/projects/tweezers/slmcontrol/
ImageJ	Hamamatsu	Free downloadable software	http://rsbweb.nih.gov/ij/
QPD	Thorlabs	S5980 with C5460SPL 6041 board	Four quadrant photo-diode to measure x, y trapped probe displacement
PD	Teem Photonics	PDA100A-EC	Photodiode to measure z trapped probe displacement
nano-Pulse UV laser	AA-optoelctronics	PNV-001525-040	Pulsed UVA laser, pulse length 400 ps

Acoustic Optic Modulator	Olympus	MQ110-A3-UV, 355nm fused silica	
Upright microscope	Andor	BX51	Equipped with a 60, 0.9 NA, water dipping objective
CCD	Warner Instruments	V887ECSUVB EMCCD	
Peltier device	Physic Instruments	QE1 resistive heating with TC-344B dual channel heater controller	
Microscope stage: micro+piezo stage	National Instruments	Three linear stages M-126.CG1 carrying a separate 3-axis piezoelectric nano-positioning stage P-733.3DD	
Daq		NI PCI-6229	Acquiring the x, y, z position of the trapped probe, and sending feedback loop signals to microscope stage
Linux Real Time Application Interface (RTAI) machine			Real time feedback loop system, to control stage position, developed on a dedicated PC desktop

*Rebuttal Comments
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Dear Editor Dr. Larissa Jarzylo,

herewith enclosed we are sending the paper by Vassalli M. et al. "Measurement of the tension release during laser induced axon lesion to quantify axonal adhesion to the substrate with picoNewton and millisecond resolution" (Ms # 50477), which has been revised according to reviewer suggestions. You can find attached to this communication a detailed response to reviewer concerns.
Thank you for the prompt and thorough processing of our paper.
We hope that the paper, in this new form, will be evaluated for publication in JOVE.

Yours sincerely,

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E-mail: francesco.difato@iit.it

Reviewers' comments:

Reviewer #1:

Major Concerns:

- The authors claim in the abstract that the described method "offers a quantitative method to study how the mechanical coupling between cells and substrates can be modulated by pharmacological treatment and/or by distinct mechanical properties of the substrate"; in the short abstract: "The developed experimental protocol quantifies the axon adhesion to the culture substrate"; in the main text, pag. 2: "Since, the release of tension induced in the lesioned axon is dampened by the unaffected cellular focal adhesions, we can obtain a quantitative parameter to compare the adhesion of the axon to the substrate in different experimental conditions"; end of text before figure legends on pag. 7: "The release of tension after axonal lesion is dampened by focal adhesions on the substrate, thus higher tension release value means less adhered axon to the substrate"; beginning of discussion, pag. 8: "We report in this work a quantitative method to measure the neurite adhesion to the substrate culture". All such claims are not supported by experimental data in the present article. Are the authors referring to experimental results found in the literature? All such claims cannot be stated without experimental demonstration that there is a direct dependence between the strength of axon adhesion to the substrate and the measured tension release after laser axotomy. Also the title should be changed, for example by removing "to quantify axonal adhesion to the substrate".

We changed the title and we included explicit reference to a paper in which those aspects are theoretically described and experimentally confirmed, showing that the propagation of forces, applied on a neurite, is dampened by focal adhesion to the substrate. We exploited such model to interpret our data (description is provided in introduction section). In a previous published article (reference 14), we already showed that the change of neurite adhesion can be measured, when treating neurons with the neurotrophic factor BDNF.

- The authors should provide reference to previous works, where applicable, and clearly indicate which results are new (unpublished) and what refers to previous studies. For example, they do not cite their previous article by Difato et al., "Combined optical tweezers and laser dissector for controlled ablation of functional connections in neural networks", J Biomed Optics vol 16 pag 051306 (2011). This article should be cited, since some of the methodologies reported in the manuscript were already reported in that article. For this reason, the authors cannot state that "To the best of our knowledge, this is the first report of simultaneous force spectroscopy measurement during laser induced cell lesion" (first paragraph of the discussion, pag.8).

We included the suggested reference to our previous work, and we removed the sentence: "To the best of our knowledge, this is the first report of simultaneous force spectroscopy measurement during laser induced cell lesion" that was misleading. In the manuscript, we report a detailed description of the experimental procedure previously published, and we clarify this point in the last paragraph of the Introduction section.

The protocol lacks important details that should be specified:
1) end of pag. 2: the authors should clarify how the two feedback loops work. What does it mean internal and external loop? If the feedback on the piezo stage keeps the bead at constant distance from the trap center, the feedback works in force-clamp regime. What does it mean that the feedback on the motorized stage works synergistically with the feedback on the piezo stage?

We added an overview on how the two feedback loops work, and we provided the references to a paper in which a detailed description of the system is reported.

2) pag. 5, point 4.4 and 5.1: in point 4.4 the authors state that the measurement is performed under force-clamp (by moving the piezo actuator), unless the system is not equipped with force-clamp and a position measurement using z QPD signal is performed. However, in point 5.1 and below, they refer only to QDP measurements. Why? What to do in case of force-clamp measurement? How do data look like and how they should be interpreted?

We used the force-clamp system to reposition the laser-trapping center on the bead attached to the cell membrane. Then, we switched-off the force-clamp and performed force measurement in position clamp condition. A more detailed description of the procedure is provided in protocol steps 6.4 to 6.7

3) pag. 5, point 5.3: "Thus, the decrease of Brownian motion detected immediately after axotomy is an effect of the increased membrane viscosity due to its strain.". What does it mean? In case there is a decrease in the signal variance, there must be an increase in the stiffness of the system. Please clarify this point.

The Brownian motion of the bead is reduced by optical forces and adhesion forces to the cell membrane. When the membrane tension is released upon axotomy, the abrupt rearrangement induces an increase in the viscosity of the lipid layer, and thus the Brownian motion of the trapped bead start decreasing. We explain this point at protocol step 7.3, and we provide a reference to a similar literature approach.

4) pag. 5, point 5.4 and pag. 6, point 5.5: t0 and t1 are not well defined. t0 and t1 are qualitatively defined, not quantitatively. This means that the quantities whose definition is based on t0 and t1 (Freleased) are ambiguously defined. Can you provide a quantitative definition for t0 and t1? Moreover, such definition assumes that the signal variance after axotomy always follows the same behavior. Is such behavior always reproducible, or which frequency and types of behaviors are observed?

We defined t0 and t1 by visual inspection of the total variance trace. We defined t0 where we observe a clear decrease of the variance, and t1 where the decrease reaches a plateau. After the plateau, we observe a recovering of the Brownian motion due to membrane relaxation after tension release. Force spectroscopy measurement during axonal lesion allows to acquire Brownian motion of the bead with high temporal resolution, and thus calculating the variance of the bead motion (video imaging does not provide the same temporal resolution). We were able to observe that the release of tension does not start immediately after laser axotomy, and the membrane relaxation after the variance decrease reaches a plateau (see the new version of figure 3). The proposed protocol is a precise task of optical manipulation of a neuronal process requiring a high degree of control. However, we report in the new figure 5 a statistic of 26 experiments. The behavior of variance decrease is observed when a small pretension is applied on the attached bead as explained in protocol step 6.6.

5) pag. 6, point 5.6: How drift in the sample is measured from debris or scratches on the coverslide? Please provide details of experimental procedure, algorithm and accuracy of tracking.

We provide the description in protocol step 7.6

3) pag. 4, point 3.3: please specify in what direction the IR laser spot is moved with respect to the UV spot.

We described now the procedure in step protocol 5.3.

6) pag.6, end of first paragraph of "representative results": "the bead attached to the cell membrane, held in an optical trap, measures the undampened release of tension". What does it mean "undampened release of tension"?

The word “undampened” (misspelled for undamped) referred to the absence of cytoskeletal dampening effects. The sentence has been recast to clarify the concept.

11) Pag.8, 3rd paragraph: "a slow phase that could last few minutes". However, the authors only show a trace lasting for 20 s. Please provide experimental evidence for this sentence.

We corrected the sentence referring to the experiment shown in figure 3. We provide the reference of our previous work where we reported such experimental observation.

12) Pag.8, 3rd paragraph:"In future, a better approach would be to cancel stage drift with a feedback loop, which auto-corrects the microscope stage position by interferometric tracking of a bead attached to the cover glass. This type of configuration, already reported in literature, requires a second laser beam centered on the attached bead, and ensures stage stabilization with sub- nanometer precision17.". There are also simpler methods that do not require a second laser beam and ensures sub-nanometer stabilization. For example, video tracking of a bead attached to the coverslide (Capitanio et al., J. Eur. Phys. J. B vol. 46, pag. 1-8 (2005)).

We thank the reviewer for pointing out such simpler methods that were mentioned in the text along with the appropriate reference.

13) Fig. 3a, right panel: please indicate t0 and t1 on the time axis.

We indicated t0 and t1 on fig. 3a, right panel.

Minor Concerns:

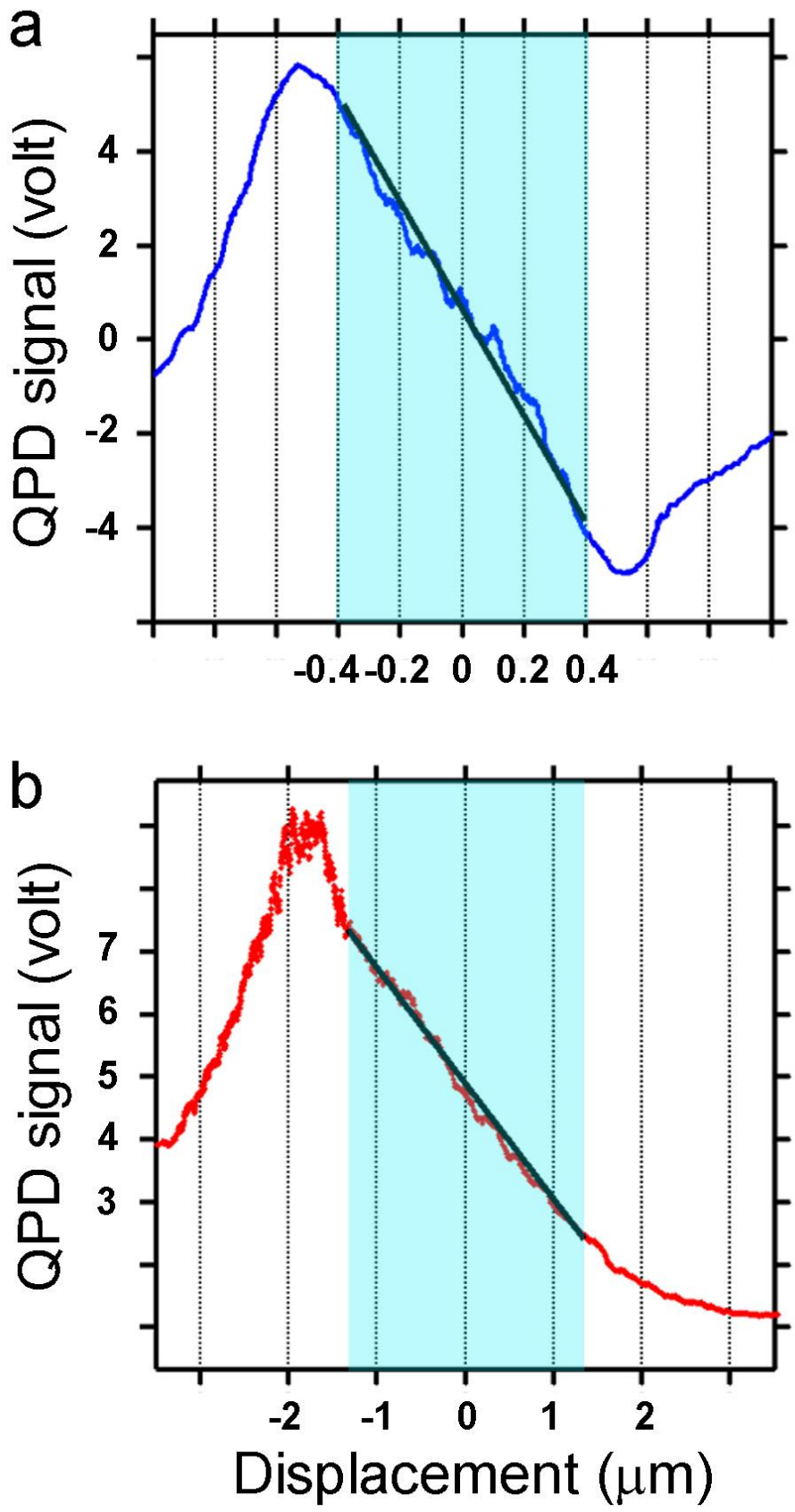
We thank the reviewer for careful reading of the paper. All minor concerns and suggestions were adopted in the new version of the text.

Reviewer #2:

Major Concerns:

1.) Figure 2b shows a displacement curve recorded using an interferometer. What is the displacement range of the interferometer? Is it possible to detect a displacement larger than a half-wavelength? The curve shows the measured displacement is around 500 nm.

We have a linear range (volt/nm) in the x and y axis of about 400 nm, and in z direction of about 1 µm. We provide here, the calibration curves obtained by scanning the laser-trapping beam on bead attached to the coverglass:


a
QPD signal (volt)
4
2
0
-2
-4
-0.4 -0.2 0 0.2 0.4
b
QPD signal (volt)
7
6
5
4
3
-2 -1 0 1 2
Displacement (μm)

Blue and red traces represent respectively the x and z directions. The bright blue shadow marks the linear range. Standard calibration curves are reported here for reference, but not included in the text.

2.) Item 7 of Step 5 of the protocol states that the drift is subtracted from the displacement data. Is it possible to subtract drift/disturbance directly? That should be valid only when the displacement and the noise is correlated. It will be better if this point is clarified.

As better clarified in protocol steps 6.4 to 6.7, we are performing position clamp experiment, thus the stage is held in a fixed position and any further low frequency displacement is only associated to thermal drift, also influencing the measurements, that can be directly subtracted.

1.) Protocol Step 1 states the lasers used for the setup. It will be better to state the requirement on power levels of the lasers employed for the setup.

We included the average power of the trapping laser in the legend of figure 2.

2.) What is the spot size of the trapping beam? How sensitively should the spot size be controlled?

The spot size of the trapping beam depends on the quality of the laser beam (i.e. M^2 factor, polarization, and laser wavelength), its collimation at the pupil entrance of the microscope objective, and on the NA of the microscope objective. In our system, we have all these parameters fixed and we measured about 900 nm spot diameter. When we project a hologram on the SLM, we introduce some spherical aberration, which could slightly enlarge the spot size. However, we calibrate the optical trapping stiffness before each experiment to take into account such aberrations as explained in discussion section.

Reviewer #3:

Major Concerns:

The quantification of cell adhesion is obtained by the measurement of the tension released after partial lesion, but this tension is proportional to the amount of lesion induced on the axon, the more cytoskeleton is disrupted, the more tension is released. Therefore, whenever it is going to be evaluated the adhesion force for different pharmacological treatments versus the controls, I see a potential problem comparing these populations. The authors should justify better this point.

We clarified the raised issue, at the end of representative results section, and we added a figure (figure 5), showing the tension release dependence on delivered energy and neurite contact area.

Minor Concerns:

We thank the reviewer for careful reading of the paper. All minor concerns and suggestions were adopted in the new version of the text.

Reviewer #4:

Minor Concerns:

1.) The long abstract included in the text ant the abstract in the cover page should be identical.

We followed the template article of JOVE journal.

2.) the numbering of the protocol section is confusing: after paragraphs 1,2,3,4 the text comes back to 3; the sub-numbering of any paragraph should be possibly clearly different from the main numbering (e.g. 4.1 4.2).

We apologize for the mistyping; we corrected the numbering of protocol steps.

3.) The authors claim that they are reporting the first experiment of force measurement during laser induced cell lesion; even if their set-up is innovative, similar approaches were tested in literature (see e.g. J.Scrimgeour, E.Eriksson, M.Goksör, Methods in Cell Biology 82:629-646, 2007) and some additional comment and comparison with literature should be included.

We added the reference to the suggested work, and we include a work of Martin Berns published on the same Journal book series (reference 8). We mentioned two times in the papers (in introduction and discussion sections) that several laboratories integrated, in the same experimental setup, optical tweezers and laser dissector. We proposed as an achievement the simultaneous force spectroscopy measurement during laser ablation procedures. However, we removed from the text our misleading claim as first experimental report of the protocol.