Axonal regeneration of cultured mouse hippocampal neurons studied by an optical nano-surgery system.

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ABSTRACT

During development, the axons of neurons in the mammalian central nervous system lose their ability to regenerate after injury. In order to study the regeneration process, we developed a system integrating an optical tweezers and a laser dissector to manipulate the sample. A sub-nanosecond pulsed UVA laser was used to inflict a partial damage to the axon of mouse hippocampal neurons at early days in vitro. Partial axonal transections were performed in a highly controlled and reproducible way without affecting the regeneration process. Force spectroscopy measurements, during and after the ablation of the axon, were performed by optical tweezers with a bead attached to the neuronal membrane. Thus, the release of tension in the neurite could be analyzed in order to quantify the inflicted damage. After dissection, we monitored the viscoelastic properties of the axonal membrane, the cytoskeleton reorganization, and the dynamics of the newly formed growth cones during regeneration. In order to follow cytoskeleton dynamics in a long time window by tracking a bead attached to the neuron, we developed a real-time control of the microscope stage position with sub-millisecond and nanometer resolution. Axonal regeneration was documented by long-term (24-48 hours) bright-field live imaging using an optical microscope equipped with a custom-built cell culture incubator.

Keywords: Digital holography; optical tweezers; laser dissection; position-clamp force spectroscopy; force-clamp interferometric tracking; neuronal connection; axonal regeneration.

1. INTRODUCTION

Optical microscope became an indispensable tool in neuroscience to observe structures and function in living cells. Ongoing advances in optical systems enabled to observe the nervous system at spatial scale beyond the diffraction limit, to record electrophysiological activity of a cell with sub-millisecond and sub-micron precision, of many cells with hundreds Hz bandwidth. Recently optical imaging has been applied to perform fluorescence-guided surgery, for tumor removal with hundred micrometers accuracy. Moreover, the possibility to exploit the light not only as a sensing means, but also as an actuating tool, opens the way to high-resolution functional and structural imaging, and precise non-invasive sample manipulation in sterile conditions.

One possibility to achieve light actuation is represented by photo-activation of caged compounds or genetically engineered opto-genetics probes. However, most of the caged compounds produce undesired side effects and there is yet no possibility of caging most of the neuro-active molecules. Opto-genetics probes, usually employed in in vivo studies, require genetic modification of cells. Development of nano or micro carriers to encapsulate molecules in combination with their manipulation by optical tweezers, offer a valid alternative for time and spatial controlled protein delivery, and for studying cell response to physiological (instead of optical) stimuli in in vitro systems.

Recently, optical tweezers have been used to mechanically stimulate a single cell by manipulating a bead adhered to the cell membrane and measure or modulate the local membrane tension, or, alternatively, with the bead posed next to the cell, to optically actuate micro-fluidic shear stress. Combining chemical with mechanical stimulation allows studying the role of the extracellular matrix (ECM) in cellular processes as differentiation and migration. While the chemical stimuli elicit specific biochemical cellular pathway, the mechanical stimulus given by the trapped probe could be used to mimic ECM biophysical properties, or to measure with unprecedented sensitivity the cytoskeleton dynamic.
The combination of the optical tweezers with a laser dissector14 give the possibility to touch15, stretch, convey compounds16 and cut the cell17,18, opening the way for nano-surgery on a cellular experimental model with sub-cellular resolution.

Tunable ultra-short pulsed laser allows for fluorescence imaging, optical trapping and three-dimensionally confined ablation with the very same light source19. While fluorescence imaging and laser surgery have been already applied in tissues or living organism by exploiting the infrared (IR) light deep penetration in scattering sample20, optical manipulation in thick specimen require further technological development21. Moreover, although the use of a single laser source for the several tasks mentioned above simplify the optical layout, it does not allow to control the optical surgery tools independently and to use them simultaneously to obtain a more accurate manipulation of the sample, without an increase of the system costs22.

In the present work, we demonstrate the possibility to apply laser ablation on single neuronal processes and connections, assisted by force spectroscopy measurements in position-clamp condition, or interferometric tracking in force clamp condition9. In this way, it is possible to study the membrane and cytoskeleton dynamics during regeneration of a lesioned axon, or regeneration of a connection in a neural network, during the first days of neuronal development in vitro23.

2. MATERIAL AND METHODS

2.1 Nano-surgery optical setup.

The entire optical system was described earlier14,22. Briefly, the trapping source was an ytterbium continuous wave (CW) fiber laser operating at 1064 nm (IPG Laser GmbH). The phase of the IR laser beam was modulated through a spatial light modulator (SLM) (LCOS-SLM, model X10468-07 – Hamamatsu). The interferometer for force spectroscopy measurements was based on a four-quadrant photodiode (QPD, S5980 with C5460SPL 6041 board – Hamamatsu) and a photodiode PD2 (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).

The holographic tweezers and laser micro-dissector were integrated on a modified up right microscope (BX51 – Olympus) equipped with a 60x, 0.9 NA water dipping objective.

2.2 Microscope stage feedback control.

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 nanopositioning 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 control was built on a Linux Real Time Application Interface (RTAI) machine24. It is based on a modified Linux kernel that allows for the execution of tasks with strict temporal constraints, called real-time targets. A core functionality of an RTAI-based machine is given by RTAI-XML project, which adds the possibility for the real-time target to communicate with a remote client over TCP/IP. The real-time control target used in the experiments, running on a RTAI machine, was structured with nine analog channels, which were acquired and sampled at 2 kHz rate. Three of these channels were used for interferometric photodiodes measurements of bead displacement along the three axes. Another three channels were employed for capacitive sensors measurements of piezoelectric stage, and the last three were devoted to external, dynamic references coming from wave generators. The system was equipped with two control loops, synergically acting to maintain the system at the right position, depending on the selected working mode (position or force clamp, static or dynamic). In particular, an internal loop acts on a fast response – short dynamics piezoelectric stage, to keep the bead at a selected distance from the trap center. A second external loop controls the position of a slower motorized stage, aimed at maintaining the region spanned by the piezo-actuator in the central portion of the available stroke. Both these feedbacks were of proportional/integral (PI) type, with integral anti-windup compensation. Four signals were sent as output to the D/A interface: three as control signal, one for each piezoelectric stage axis; the fourth acted as a trigger for the camera to synchronize video acquisition with data logging. A second machine, through a custom user interface, allowed visualization and saving of the measurements, and provided a graphical front-end for editing of control parameters (e.g. proportional and integral gains). Alongside with data and parameter handling, the interface managed automatic micro-stage recoveries to extend the piezo-stage range25.

2.3 Long-term imaging optical setup.
Long-term bright field time-lapse experiments (2-3 days) were performed on a commercial inverted microscope (Eclipse Ti E; Nikon Instruments Inc.) with laser-based autofocus and motorized stage for multipoint sequential image acquisition. The microscope was equipped with a charge-coupled device (CCD) camera (Andor DU-897D-C00), Plan Fluor 40x, 0.75 NA DIC objective and imaging software (Nis elements AR; Nikon Instruments Inc.). For long-term time-lapse imaging, the neurons must be kept at stable temperature, pH and osmolarity. The temperature was kept at 35°C by a feedback system coupled to a thermocouple in contact with the sample holder (TC-324B, Warner Instruments Corporation). To maintain the physiological pH, 5% CO₂ balanced with air was obtained by a flow meter (CO₂BX, Okolab) and injected in the sample holder. To minimize the evaporation of the medium and stabilize its osmolarity, the gas was bubbled through a water reservoir warmed through a load resistance (10 W, 12 V) before being fluxed into the stage incubator. Moreover, a film of oil (polydimethylsiloxane 200 Fluid, 0.913 g/ml, Sigma Aldrich), permeable to CO₂ but not to water, was deposited on the culture media. This same system was adapted to an upright microscope, where pH and humidity were controlled by aerating a customdesigned polydimethylsiloxane sleeve, which enclosed the objective and the culture support.

2.4 Cell cultures.

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). Embryos were removed and dissected under sterile conditions. Hippocampi were dissociated by enzymatic digestion in trypsin (0.125% for 20 min. at 37°C). Trypsin activity was blocked by adding complete media (Neurobasal2 (Gibco) supplemented by B27 (2%, Gibco), alanyl-glutamine (2 mM, Gibco), penicillin/streptomycin (both 1 mM, Sigma) containing 10% fetal bovine serum (FBS, Gibco). After trypsinization, tissues were rinsed in complete media without FBS, and dissociated with a plastic pipette. Neurons were plated at a concentration of 0.25–1 x 10⁵ cells/ml on glass-bottom Petri dishes (P35G-0-14-C – MaTek Corporation). After 2 hrs in the incubator to allow cell attachment, the dishes were filled up to 2/3 of their volume with serum-free medium.

Silica beads (Ø 4 mm, COOH-terminated– Bangs Laboratories) were coated with poly-D-lysine or fibronectin following the procedure described in the PolyLink Protein Coupling Kit (Polysciences).

2.5 Measurements.

Previously fibronectin or poly-D-lysine coated beads were optically trapped and moved into close proximity to the neurite (IR power at the sample 35 mW). The position of the trapped probe with respect to the neurite geometry and position of the UV laser focal spot was controlled by displaying a dynamically-generated hologram on the SLM. The trapped probe was positioned 2 mm above the coverslip and about 10 m away from the UV laser spot. The interference fringes on the back focal plane of the condenser were imaged onto a QPD and PD, the light was converted to voltage outputs filtered and digitized at 5 and 10 kHz, respectively, to calibrate the trap stiffness kₓₙₙ, z (kₓ, ky, kz) and the detector sensitivity using the power spectrum method. The calibration of the trap stiffness was repeated for every new position of the trap determined by the SLM, before the attachment of the probe to the neurite. After calibration, the bead was brought into contact with the neurite to be damaged. After 15 seconds to ensure fibronectin-integrin attachment, the recording of QPD and PD signals (digitized at 4 kHz and low-pass filtered at 2 kHz) was started simultaneously with the image acquisition by a CCD. About 200–300 UVA laser pulses with energy of 25 nJ per pulse were delivered to inflict damage to the neurite, and force spectroscopy was performed in parallel. The release of tension was quantified as the difference in the total force measured at t1 and t0, by interferometric tracking, and corrected by the drift of the stage²³. For long-term time lapse imaging, axons of neurons cultured on a gridded Petri dish (MatTek) were laser-damaged, and the dish was then moved to the inverted microscope. The injured neurons were localized on the grid, and bright field time lapse imaging was continued for about 2 days (with one image every 2 minutes).

3. RESULTS AND DISCUSSIONS

In figure 1 is shown a schematic drawing of a holographic optical tweezers (OTs) (red box), and a laser microdissector (LMD) (violet box), integrated on a commercial upright microscope (green box). This system was used to monitor the mechanical perturbation produced by laser damage of a neurite and to study the regeneration of cellular processes in a developing neural network in vitro.
The OTs is based on a CW IR laser, and the SLM controls the position of the optical trap in respect to the position of the laser dissection focus spot by modulating the wave front of the beam. This is necessary to adapt the position of the trapped sensing probe to the geometry of the neuronal process.

Figure 1. Nano-surgery optical setup. A) Schematic optical layout of the instrument. Color box legend: Upright optical tweezers (red), a laser microdissector (violet), and a back-focal plane interferometer (brown) are operated on an Olympus BX51 microscope (green). AOM: acousto-optic modulator; BS50/50: beam splitter 50/50%; CCD: charged coupled device camera; DM: dichroic mirror; F: filter; \( \lambda/2 \): half-wave plate; L: lens; ND: neutral density filter; PBS: polarizing beam splitter; PD: photodiode; QPD: quadrant photodiode. B) Motorized Microscope stage control architecture. Overview of the control architecture, with real-time feedback PI control of the Piezo-stage, and automatic recoveries of the Micro-stage. Numbers in the black circles indicate the essential components of the systems: (1) Optical trapping laser, (2) QPD based detection system, (3) Micro-stage, (4) Piezo-stage.

The laser ablation system is based on a sub-nanosecond pulsed UVA laser source. The ablation efficiency depends on several parameters, such as the optical pulse duration, the laser wavelength, the pulse energy, and the wavelength of the light; the selected laser source in our experiments can create a high-energy fluency at the focus with an average power of only a few microwatts. As a result, the ablation process result confined on a three dimensional volume, and the release of energy, necessary to overcome the material threshold, occur in a short time interval confining the unwanted laser
induced thermal effects at the sample. The UV laser spot is shifted on the sample by the motion of the microscope stage. Although galvanometric mirrors allow stirring of the laser focus position without moving the sample, they can introduce spherical aberration in the focus spot, which result negligible only for small displacement of the beam from its central position. Such aberrations modify the optical properties of the LMD, and could require a de novo calibration of the LMD system. Taking into account that small changes in the power delivered at the sample (about 2 μW) may induce a shift from a partial to a complete dissection of the neurite, to scratching of the culture support and damage of the adhesion molecules, the power calibration is fundamental to perform well-defined damage on the sample

The same problem is present when the focus spot position of the OT is changed27. Nevertheless calibration of the optical stiffness should be repeated before any experimental session28 (changes in temperature, viscosity of the media, laser power fluctuation, and surrounding noise affect the optical stiffness and consequently sub-picoNewton force sensitivity), and it require few minutes when actuated by an interferometric tracking system (brown box, figure 1a).

Moreover, to perform long-term interferometric tracking of the trapped probe during regeneration of the partially dissected neurite, the OTs was provided with feedback loop to control the position of the probe, with nanometer and millisecond tracking capability on an extended spatial range. In such a way it is possible to follow the rearrangement of the membrane and cytoskeleton of a motile growth cone.

3.1 Position clamp optical tweezers: quantification of the release of tension in damaged neurites.

Fibronectin-coated beads were optically trapped and moved into close proximity of the neurite14. The position of the trapped probe with respect to the neurite and to the focal spot of the UV laser dissection was controlled by displaying a dynamically generated hologram on the SLM. Thus, after calibration of the optical trap by power spectrum method, the bead was brought in contact with the neurite allowing its adhesion on the membrane of the neuronal process (figure 2a).

Figure 2. Measurements of tension released during laser ablation of hippocampal axons. A) Bright field images acquired during axonal ablation of a mouse hippocampal neuron (2 DIV). A fibronectin-coated bead is attached to the membrane (silica bead, Ø4 mm) and held in an optical trap. Average power of the IR laser at the sample is 30 mW. Bar is 5 μm. Numbers indicate seconds. The violet arrow indicates the lesion site. White arrows indicate a structure retracting after the ablation of the axon. Green arrows indicate a structure moving toward the tip of the neurite. 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 axis, respectively. The UV depicted arrow indicate the time of UV laser energy delivery (300 UVA laser pulses with energy of 25 nJ per pulse). Sampling rate is 4 kHz. The trapping stiffness was calibrated by the power spectra method: \( k_x, k_y = 70 \ pN/\mu m, k_z = 14 \ pN/\mu m \). (C) Total variance of the Brownian motion of the bead attached to the axon (\( t_0 \) and \( t_1 \) indicate respectively the beginning and the end of tension release in the axon after dissection).
Back focal plane interferometry was performed simultaneously during delivery of UV laser energy to quantify the released tension in the damaged neurite with nanometer and sub millisecond resolution. The nanometer resolution provided sub-picoNewton sensitivity in the measure of the released tension, and the sub millisecond resolution allowed quantifying the changes in Brownian motion of the attached and trapped bead, by monitoring the variance of the bead motion.

Figure 2b shows the traces of the force measurements (blue, green, red traces are the x, y, z components, respectively), and figure 2c indicates the total variance of the Brownian motion of the trapped bead. The variance of the bead was calculated on the filtered component traces shown in figure 2b (high pass filter with cutoff at 10 Hz as previously described\(^{13}\)). The total variance of the bead position in the trap was determined in 50 ms steps for overlapping time windows of 500 ms (2000 data points)\(^{29}\).

The released forces after laser dissection cannot be due to actin assembly or actin retrograde flow because the measurements are performed at the dissection time, and therefore it is related to the disrupted balance in the cytoskeletal traction forces. Immediately after ablation, a small neurite structure (indicated by white arrows in figure 2a) was detected retracting toward the cell body, and was measured a corresponding decrease in variance of bead Brownian motion (figure 2c). Few minutes after the UV energy deliver, new material moving toward the tip of the neurite appeared (indicated by green arrows in figure 2a), corresponding to the plateau reached by the variance decrease (figure 2c).

The changes in Brownian motion could be due to the detachment of the bead from the membrane (increased motion) or to a tighter coupling between the surface and the bead (decreased motion). The decrease of Brownian motion observed immediately after ablation suggests a higher viscosity in the membrane induced by the strain, as it is highly improbable that there is a tighter coupling of the bead to the surface during laser ablation. Therefore, the variance of the Brownian motion of the bead indicated the beginning (decrease of the variance, t0 in figure 2c) and the end (start of the plateau, t1 in figure 2c) of the membrane strain. Considering that the cell exerts traction forces on the substrate, and that focal adhesions occupy only 1% of the basal surface area, discontinuity in the variance drop could correlate with discrete detachment and disruption of focal adhesion units.

The force measured at t0 is related to the displacement of the probe from the trap centre, due to the bead contact with the neurite, and it is subtracted from the measured force at t1. The total amplitude of the released force was quantified as 

\[ F_{\text{tot}} = \sqrt{F_x^2 + F_y^2 + F_z^2}. \]

3.2 Force clamp optical tweezers: quantification of cytoskeleton dynamics during regeneration.

Figure 3a shows a connection between two neurons where is attached a poly-D-lysine coated bead held in an optical trap. As previously described, the optical trap position was varied by a diffractive optical element displayed on the SLM.

In figure 3b are the frames of a video registered during UV laser induced lesion (violet arrows) of the neuronal connection shown in figure 3a at lower magnification. By force spectroscopy measurement in position clamp conditions (as described in section 3.1), was quantified a release of tension of about 2.4 picoNewton due to a displacement of the bead toward cell soma (about 150 nm displacement on the x direction) on the right side of the field outside the frame. To perform interferometric tracking of the bead attached to the neurite in force clamp condition (force clamp equal to zero) was activated the feedback loop on the microscope stage. The attached bead represents an ECM site with known stiffness (equal to the calibrated stiffness of the optical trap), which supply a mechanical constrain to the cells during regeneration of the lesioned connection.

Time-lapse bright field imaging was performed during interferometric tracking; in figure 3c are shown frames from a video registered during regeneration of the neuronal connection. The formation of lamellipodia-like structures moving toward the lesion site is evident. In the first 400 seconds, such structures originate on the left side of the lesion, (black arrows numbered 1, 2, 3) and are completely reabsorbed at 450 seconds. After 600 seconds two lamellipodia-like structures originate from the right side of the lesion site (black arrows numbered 4, 5).

Figure 3d shows the interferometric tracking of the bead (blue and green traces are the x, y components, respectively) indicating that the trajectory of the bead is composed of alternating advancing and retracting phases. During the first 400 seconds, the overall motion of the bead was directed toward the upper right corner and, from 600 seconds on, the bead moved toward the lower left corner (figure 3c).

Therefore, by interferometric tracking of the bead, and simultaneous bright field time-lapse imaging, we could monitor the cytoskeleton and membrane dynamics of the connected neuronal processes during the regeneration.
Figure 3. Force-clamp interferometric tracking of a bead attached to a neuronal connection, during regeneration after partial lesion by UV laser ablation.  A) A poly-D-lysine coated bead attached to a connection of a developing neural network of mouse hippocampal neurons (4 DIV). The white box marks the field of view shown in B) and C). Bar is 10 μm. B) Frames of the neuronal connection shown in A) during partial lesion induced by UV laser. Violet arrows indicate the site of lesion. Numbers indicate seconds. Bar is 5 μm. Simultaneous force spectroscopy on the attached bead, held in the optical trap, was performed in position-clamp condition. Average power of the IR laser at the sample is 10 mW. Sampling rate is 10 kHz. C) Tiles of the neuronal connection shown in A) during regeneration after the partial lesion induced by UV laser. White numbers indicate seconds. The black arrows and numbers indicate the growth cone like structures moving along the axon. Bright field images were acquired at 1 Hz. Simultaneous interferometric tracking of the bead attached to the neuronal connection was performed in force-clamp condition. Average power of the IR laser at the sample is 7 mW. Sampling rate is 2 kHz. D) Traces of the x, y coordinates of the tracked bead attached to the lesioned neuronal connection during regeneration (in blue and green, respectively). Axes origin is at the left lower corner of the field of view in A). Traces are sampled at 2 KHz.

3.3 Long term imaging of a lesioned axon during regeneration

The axons of mouse hippocampal neurons at 2 and 3 DIV were partially damaged by the LMD system, and then moved to a microscope equipped with a custom-built cell incubator. The cell incubator system allowed the continuous long-term monitoring (24-48 hrs) of axonal regeneration. The lamellipodia-like structures similar to a growth cone, also called actin waves, formed at the cell soma and travelled along the lesioned axon to the tip (figure 4, black arrows). Actin waves have been described as part of a mechanism needed to transport material from the soma toward the tip of the neurite during its elongation\(^{30}\). The observation that actin waves form after axonal lesion indicate their role also in the process of regeneration and re-growth\(^{23}\).
Figure 4. Growth cone like structures formation during axonal regeneration. The axon of mouse hippocampal neuron at 2 DIV was damaged by UVA laser with an average power of 2.5 mWatt at a pulse rate of 100 Hz. The white arrows in the frames at 10 seconds and 14 minutes indicate the ablation site. The black arrows indicate the formation of a growth cone like structure travelling along the axon, passing the lesion site, and reaching the tip of the axon. Frame rate 1 Hz. Bars are 5 μm.

4. CONCLUSION

We present a nano-surgery system based on light tools for the manipulation and dissection of axons and neuronal connections, in sterile conditions. Mouse neurons at early days of development in vitro, were employed to perform axonal damage in the proximity of the cell body. The back focal plane interferometry system was applied to monitor the disruption and the re-establishment in the cytoskeleton equilibrium during wound healing. In the future, this system could be applied on knockout and transgenic mice models, to investigate the role of single molecules involved in neuronal regeneration. The development of chemical carriers manipulated by the optical tweezers will allow targeted pharmacological treatment of neural injury.

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5. REFERENCES


