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Deliverable Nature	
R = Report, P = Prototype, D = Demonstrator, O = Other	R
Dissemination Level	
PU = Public PP = Restricted to other programme participants (incl. the Commission Services) RE = Restricted to a group specified by the consortium (incl. the Commission Services) CO = Confidential, only for members of the consortium (incl. the Commission Services)	PU

A. Abstract / Executive Summary

We have constructed a multimodal imaging methodology for performing femtosecond laser ablation and at the same time, be able to assess the collateral damage induced by the laser. This is demonstrated by dissecting single axons within a living *Caenorhabditis elegans* (*C. elegans*). This relies on the observation of the tissues surrounding the targeted region using a combination of different high resolution microscopy modalities. All the above is done using a single instrument: multimodal microscopy setup that allows simultaneous imaging in the linear and non-linear regimes and femtosecond-laser ablation.

B. Deliverable Report

1 Introduction

Ultrashort pulsed lasers employing near infrared wavelengths have been shown to be the ideal tool when a very controlled and precise modification is required. These lasers have the ability to induce nonlinear photoionization and thereby to confine interaction to the focal volume (<1 femtoliter) of a tightly focused beam. This enables controlled incisions with submicrometer precision. In addition to this, the use of near infra-red (NIR) light provides much higher tissue penetration due to a reduced (linear) absorption and scattering of the sample. This is opening up a whole new window for laser nanosurgery as a non-invasive powerful surgery technique that can be applied in a vast range of biomedical areas.

Although the precision of the femtosecond laser scalpel in biological samples is widely accepted to be superior to other surgical techniques, the complex nature of the interaction light-sample calls for stringent tests on how precise this tool is. A logical way to perform these tests is to “observe” how much damage is sustained in the structures surrounding the target of surgery and, specially, in a specific biological sample. Assessment of this collateral damage is not just important to validate the precision of the surgical tool but it would also help to understand how a specific biosample responds to the surgery (chemically, structurally and behaviorally). Such determination of collateral damage is not trivial when the surgery is performed at the subcellular level.

A very interesting case of the use of femtosecond lasers for subcellular surgery is the dissection of axons, or axotomy, of the nematode *Caenorhabditis elegans* (*C. elegans*).

C. elegans D-type motoneurons are particularly attractive for the study of collateral damage induced during a femtosecond laser axotomy due to several reasons. First, these neurons extend axons that are extremely thin (couple of hundred nanometers) and reside in a complex environment surrounded by cuticle (above) and muscle (below). This means that even a very tiny mis-targeting or inaccuracy in the use of the laser surgery tool would show up as a collateral damage in the surrounding structures. Secondly, they provide an effective method towards the understanding of the triggered responses after axotomy that lead to axon regeneration [5]–[9]. Therefore, having a system that produces very precise cuts and has the capability to readily detect any unwanted/collateral damage is very important in the understanding of the mechanisms of neuronal regeneration.

2 Objectives

To demonstrate the ability of our multimodal imaging and nanosurgery instrument by cutting a set of axons of *C. elegans* D-Type motoneurons using femtosecond unamplified MHz pulses.

To provide a new approach for a rigorous, detailed and more precise assessment of the collateral damage induced to the surrounding tissues.

To present an integrated multimodal optical workstation to monitor collateral changes induced around the operated axon.

3 Work performed / results / description

A multimodal microscope was built around a commercial confocal (Nikon C1–Si) inverted microscope (Nikon Eclipse Ti–E) using a Kerr-lens mode-locked Ti:Sapphire oscillator producing 150 fs pulses at a repetition rate of 76 MHz. This microscope incorporate two independent pairs of galvanometric mirrors, one for continuous-wave visible laser (within scan head of Nikon C1–Si) and the other (placed externally) for the ultrashort pulsed laser. Both laser sources were coupled through 2 different input ports in such a way that both the confocal and multiphoton sections could work independently and simultaneously. This multimodal unit could, therefore, be used in the linear and nonlinear regime with a variety of different techniques working in a simultaneous way. In particular, the nonlinear input could be used for performing the nanosurgery in the axons of the worm while the confocal unit could be used to visualize axotomy and the collateral damage at the same time.

Nanosurgeries I the axons of the worm (juIs76 [unc-25::GFP]) were performed on the commissures of the D-type motoneurons on the most ventral/dorsal part of the axon just after its association with the cords (~1–5 µm away). The position of the precise focal plane for cutting was found based on simultaneous TPEF and SHG imaging of the axon and the body wall muscles, respectively.

A different number of axotomies per worm (in different axons) were performed resulting in a total of sixty-one surgeries. Axotomies were performed by parking the tightly focused laser beam on the target point while controlling the irradiation time with an electronic shutter. We employed an oil immersion 60×1.4 NA microscope objective that focuses the beam to 0.45 µm in the transversal direction and 1.2 µm in the axial one.

To find the appropriate laser power for cutting, the exposure time was set to 200 ms and the laser power was increased up to the value that led to an effective disruption of axons. This methodology yielded an optimal average power of 90 mW (energy per pulse of 1.2 nJ) which corresponds to a peak intensity of approximately 3×10^{12} W/cm^2 at the sample plane.

The methodology used for the damage assessment is the following. First, the area surrounding the point of surgery was imaged pre-surgically using TPEF and SHG microscopy. The axotomy was then performed and recorded. Immediately after the surgical procedure, a second set of nonlinear (TPEF and SHG) images were taken, at several focal planes around the point of surgery, to correct for any changes in the focal plane over the tissue relaxation time.

Sixty-one axons were laser cut and simultaneously imaged using confocal microscopy. Fifty six of the 61 operated commissures were successfully severed. The inflicted collateral damage was determined using different imaging techniques using our multimodal optical workstation.

Figure 1 shows a complete multimodal imaging performed at the region closer to the axotomy. Figure 1 (a–c) and (g–i) show the multimodal images taken before axotomy, whereas Figure 1 (d–f) and (j–l) are the resulting images after the procedure. In this case, no collateral damage is observed either in the confocal image (Figure 1 (d)) or in the TPEF image (Figure 1 (j)). Minimum collateral cuticle damage is observed in Figure 1(e). The surgical tool has made an efficient cut in the axon with minimum damage to the cuticle, however, changes have been induced to the muscle as can be observed in the Figure 1(k): reduction of the SHG signal in a small region around the dissection point and transition from Single-Band to Double-Band (SB to DB) signal structure of the muscle sarcomeres. This clearly illustrates the power of multimodal imaging (Confocal+LT+TPEF+SHG) in making a thorough assessment of collateral damage.

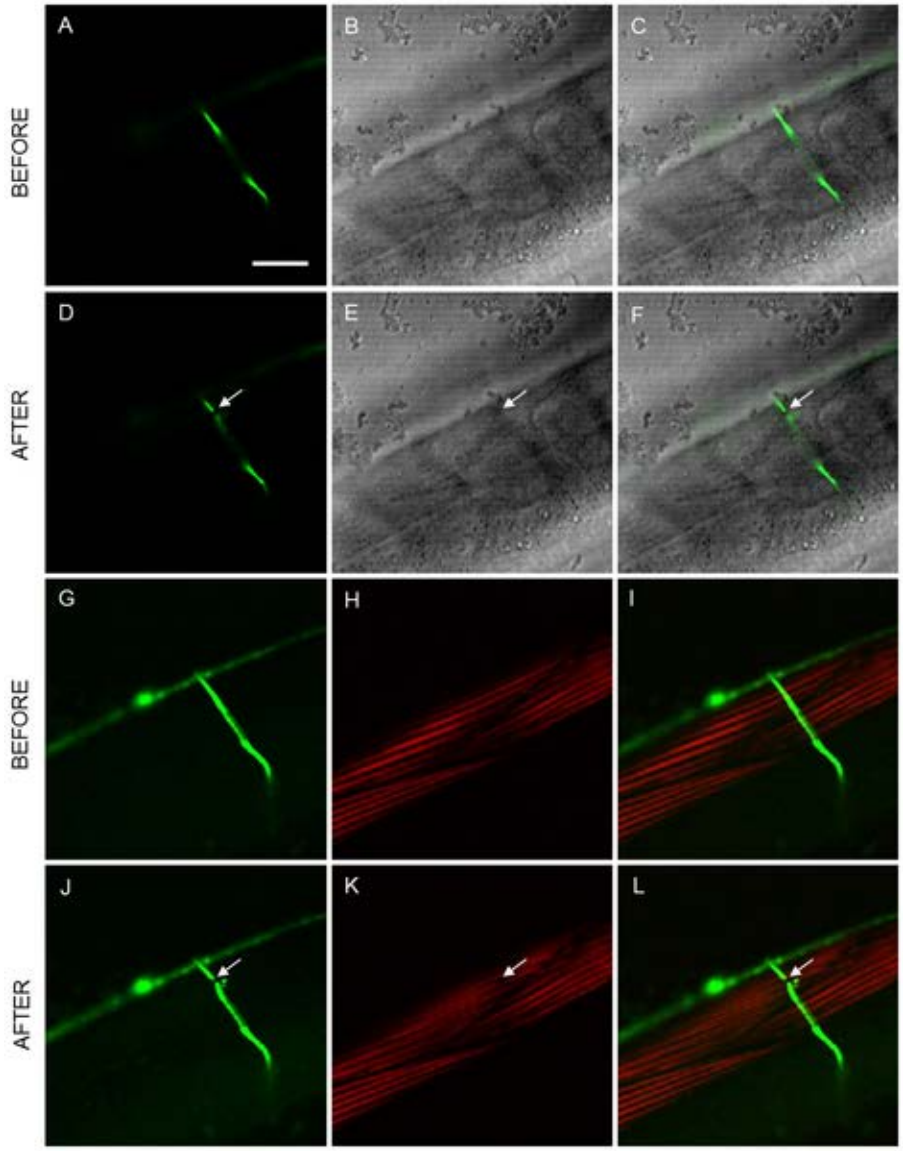

Figure 3. Collateral damage assessment using linear and nonlinear imaging techniques. Linear: a) confocal, b) transmitted light and c) combined images of the region surrounding the axon before the laser dissection; d–f) show the same region after the surgery (Media S2). Nonlinear: g) TPEF, h) SHG and i) combined images before the laser dissection; j–l) show the same region after the surgery. No collateral damage was observed in CFM while damage in muscle is evidenced with SHG microscopy. Arrows point to the place of the laser axotomy. Scale bar 20 µm.

When the femtosecond laser tool is properly tuned and perfectly focused at the center of the axon, incisions can be induced without any collateral damage. Such a case was analyzed using TPEF from the axons (GFP labeled) revealing a successfully cut axon (shown as a gap that interrupts the continuity of the axon) with no other apparent damage. Here a highly confined (with a few pixels) decrease of SHG signal near the axotomized neuron was observed (see Figure 2). In this case, our PSHG analysis (unpaired two tailed t- test (n = 200 pixels for both sets)) did not find any significant difference ($p>0.05$) with intact tissue. This suggests that no damage has been produced or that this has been propagated in a negligible way after the axotomy procedure.

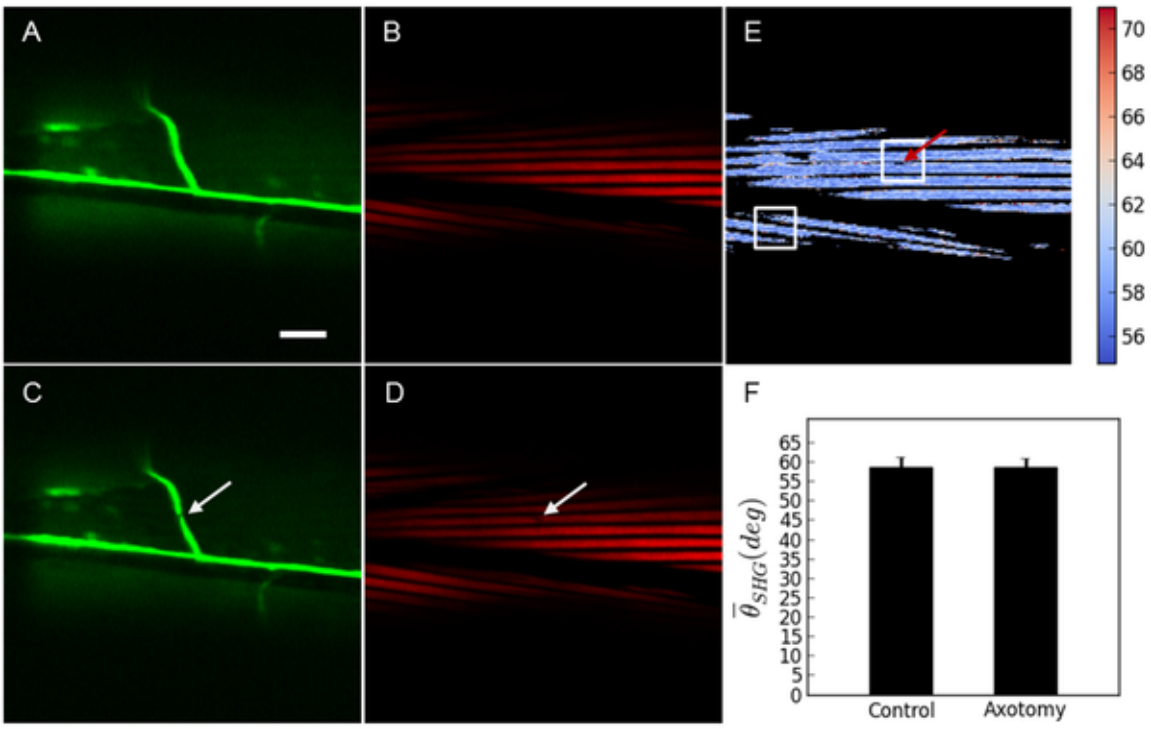

Figure 2. Comparison between PSHG and fluorescence for the muscle damage assessment after the axotomy. Minimum collateral damage. Before the axotomy: a) TPEF image of muscles and axons; b) SHG image of the muscles in the same region. After the axotomy: c) TPEF reveals a successfully cut axon, in the shape of a gap that interrupts the continuity of the axon, but no other damage is apparent; d) SHG image of the body wall muscle shows a small signal decrease at the targeted point on the axon. No other change or structural transformation is evident. PSHG analysis: c) Post-surgical pixel-resolution mapping of myosin θ_{SHG} at the muscle (color bar in degrees); d) θ_{SHG} (mean±1 standard deviation) for the muscles in the region surrounding the cut and in the control region (white squares). The control region was selected in the adjacent muscular cell far away from the axotomy. Unpaired two tailed t- test (n = 200 pixels for both sets) yields $p>0.05$ meaning that θ_{SHG} mean is not significantly different between the two regions. Arrows point to the place of the laser axotomy. Scale bar 10 µm.

4 Conclusions

This study shows a multimodal imaging platform that can be effectively used to perform high precision nano-surgery and provides an alternative approach for a practical and comprehensive damage assessment. The application of the above mentioned techniques can be extended to other biosamples. The use of combined imaging techniques in one imaging set-up is an unconventional approach to *in vivo* studies of different structures and tissues that can prove to be an extremely valuable tool to the examination of induced damage by laser surgery tools.

5 References/Publications

Santos SICO, Mathew M, Olarte OE, Psilodimitrakopoulos S, Loza-Alvarez P (2013) Femtosecond Laser Axotomy in Caenorhabditis elegans and Collateral Damage Assessment Using a Combination of Linear and Nonlinear Imaging Techniques. PLoS ONE 8(3): e58600. doi:10.1371/journal.pone.0058600

http://127.0.0.1:8081/plosone/article?id=info:doi/10.1371/journal.pone.0058600

Also highlighted in the magazine: BioPhotonics: June 2015, “Femtosecond Pulses: Control Is Key to New Discoveries” by Marie Freebody, Contributing Editor.