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# Signalling effect of NIR pulsed lasers on axonal growth

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ABSTRACT

In this work we show that a pulsed laser light placed at a distance is able to modulate the growth of axons of primary neuronal cell cultures. In our experiments continuous wave (CW), chopped CW and modelocked fs (FS) laser light was focused through a microscope objective to a point placed at a distance of about 15 μm from the growth cone. We found that CW light does not produce any significant influence on the axon growth. In contrast, when using pulsed light (chopped CW light or FS pulses), the beam was able to modify the trajectory of the axons, attracting approximately 45% of the observed cases to the beam spot. Such effect could possibly indicate the capacity of neurons to interpret the pulsating NIR light as the source of other nearby cells, resulting in extension of processes in the direction of the source.


## 1. Introduction

Axonal guidance and navigation is a complex processes which has been extensively studied. Controlling such process is one of the standing goals in neurosciences as it could be the key to solve many biomedical problems including neuronal damage. Growing axons have a highly motile structure, the growth cone (Ramón y Cajal, 1904; Tessier-Lavigne and Goodman, 1996; Kandel et al., 2000; Pantaloni et al., 2001; Suter and Forscher, 2000; Borisy and Svitkina, 2000), which follows a precise pathway towards its target by "scanning" the extracellular milieu using the filopodia. This pathway is influenced by both signals in the extracellular matrix and membrane-anchored proteins (Mitchison and Kirschner, 1988; Jay, 2000; Goldberg, 2003). The process of axonal guidance is controlled by a variety of axonal guidance molecules. These guidance cues fall into several classes of molecules (Dickson, 2002; Huber et al., 2003). The progressing knowledge of these molecules has resulted in some of these being used externally to achieve control over neurite growth (Gallo and Letourneau, 1998; Naka et al., 2004).

In the last twenty years there have been significant efforts in finding alternative guidance cues (other than biomolecules) that can manipulate and enhance the intrinsic natural growth process. Several methods have thus been suggested. These include the use of electric fields (Patel and Poo, 1982), use of micropatterned substrate structures (Fromherz et al., 1991; Lee et al., 2007; Kaehr et al., 2004) or mechanical forces (Fischer et al., 2005; Lamoureux et al., 1992). In addition to all the aforementioned a number of works have highlighted the use of light as a potential tool to achieve control on axon guidance. For example laser photolysis of caged $Ca^{2+}$ ions was used to induce a tropic turning of the axon (Zheng, 2000). Here, the effect was attributed to an increase in the $Ca^{2+}$ ion concentration in a localized region (laser focus) of the growth cone. Light's ability to impart mechanical forces has also been explored to achieve axon guidance in different cell lines (Ehrlicher et al., 2002; Mohanty et al., 2005; Carnegie et al., 2008; Stevenson et al., 2006). In these experiments a near infrared continuous wave (CW) laser beam was made to fall on the edge of the growth cone to optically drag the neuron towards a certain course. The main hypothesis attributed the guidance of the neuritis to light's ability to gather actin monomers to provide nucleation sites for the actin polymerisation which drives cellular growth. Neurite outgrowth of PC12 cell lines has also been observed under LED illumination (Higuchi et al., 2007). Notably, the underlying mechanisms responsible for the light–cell interactions described above, have not been fully understood or are not known.

Cell–light interaction have been explored in many applications. It has been reported that photo-irradiation can generate significant biological effects such as cell proliferation, collagen synthesis, the release of growth factors from cells (Sommer et al., 2001) and building up of plasma membrane (Henkel et al., 2006; Kumbalasiria and Provencio, 2005). In addition to this, photo-irradiation, using near infrared (NIR) wavelengths, has already been used for a variety of "photobiomodulation therapies" such as spinal cord nerve repair,

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wound healing (Desmet et al., 2006), promoting functionality of primary neurons (Wong-Riley et al., 2005) and stroke treatments (in both rabbits and humans) (Lapchak et al., 2004, 2007; Zivin et al., 2009).

A very interesting aspect of cell–light interaction is the ability of light to act as a signalling agent to remotely communicate with cells. For instance, 3T3 cells were shown to extend pseudopodia towards single microscopic infrared light sources at a distance (Albrecht-Buehler, 1991, 1995, 2005). It hence seemed very likely that light could be detected by neurons and this could induce similar signalling effects on them.

In fact, our preliminary studies showed that when femtosecond-pulses were used, filopodia show a clear tendency to orient towards the direction in which the NIR laser is focused (Mathew et al., 2008). The direction of the orientation of filopodia is the fundamental indicator of the most probable direction of axonal growth. In this work hence, we further explore if the observed light-based signalling mechanism could also be able to exert an influence on the whole axonal growth. This was investigated for different conditions of temporal regimes of the beam: continuous wave (CW), chopped CW and femtosecond (FS) pulses. In agreement with our previous results (Mathew et al., 2008), we observed significant axonal growth towards the laser spot when the laser was pulsed, either in FS or chopped CW regimes. This steering effect on the axon, however, was not evident when CW laser light (without pulsing) was utilised.

## 2. Materials and methods

### 2.1. Cell culture

OF1 embryos (Iffa Credo, France) were used. The mating day was considered embryonic day 0 (E0). After anaesthesia of the dams, embryos were dissected out and collected in 0.1 phosphate-buffered saline (PBS) and 0.6% D-glucose. Low-density dissociated cultures were established from the cerebral cortex of CD1 mouse embryos at 15 days gestation (E15). The dissected tissue was trypsinised (0.05% trypsin for 9 or 15 min respectively at 37 °C) and dissociated by trituration. All neurons were plated on a poly-ornithine/laminin substratum in 35 mm diameter tissue culture Petri dishes. The cerebral cortex neurons were grown on Neurobasal supplemented with 10% normal horse serum, L-glutamine, $NaHCO_3$, D-glucose and supplement B27 (all from Gibco Life Technologies). Purified recombinant NGF (4 ng/ml) (Sigma) was added to the cultures at the time of plating. All neurons were grown for 18 h in a 5% $CO_2$, 95% humidity incubator at 37 °C.

### 2.2. Experimental set-up

The experimental set-up was build around a commercial inverted microscope (Eclipse TE 2000-U, Nikon). The neurons were imaged using brightfield illumination provided by the microscope, and intermediate 1.5 magnification factor and with a 60×, 1.4 NA planapochromatic oil immersion objective. The behaviour of the neurons was time-lapse recorded (1 frame each 5 s) using a CCD camera (pixel size of 4.5 μm × 4.5 μm) attached to one of the output ports of the microscope. A chamber with an automatic heat control system was built around the microscope to keep the neurons at 37 °C. A minichamber was provided within the main chamber covering the culture dish that could be purged with the 5% $CO_2$. The neuron cultures were maintained in 2 ml of culture media in a closed glass bottom Petri dish and buffered with HEPES solution.

A Ti:sapphire laser oscillator (Mira 900f, Coherent), operating either in CW, chopped CW or modelocked femtosecond (fs) pulse regime was coupled into the microscope through the back port. This was achieved using a pair of galvanometric mirrors (used to steer beam during the conventional laser scanning modality for nonlinear microscopy), conventional relay optics and a 45° hot mirror to direct the beam through the microscope objective and towards the sample plane. The microscope objective focused the input laser beam (to a spot size radius of around 0.4 μm) on the sample plane. A planar two-photon fluorescent sample was used to draw up a calibration curve between pixel coordinates in the CCD camera image and the galvanometric voltage controlling the laser spot position. This allowed (using control software programmed in Labview) positioning the laser spot at any location on the image of the sample plane by a simple mouse click on the screen. A coloured dot in the image was automatically drawn, indicating the position where the beam lies (the colour of this dot was set to change, depending on whether the laser shutter was open or closed). The focal position of the laser beam spot was adjusted by varying the height of the objective lens. Once the beam spot position was set, this remained unchanged (axially and transversally) throughout the whole experiment.

The laser central wavelength was fixed to 800 nm ($\Delta\lambda$ = 10 nm in the fs pulsed regime). For the CW and chopped CW experiments the laser was operated using its auxiliary cavity (without the pair of prisms) to avoid spontaneous modelocking operation. For the chopped CW experiments an optical chopper (MC 100, Thor labs), operating at 20 Hz, was placed in the beam trajectory outside the laser. In the fs pulsed regime, the laser was operating at a repetition of 76 MHz and delivering pulses of 160 fs duration the measured pulse duration at the sample plane was 240 fs, which implies a net group delay dispersion introduced by the microscope of $D = 2100\ fs^2$ (Thayil et al., 2008). Neutral density filters were used to attenuate the beam average power (in pulsed, chopped CW and CW regimes) down to 3 mW at the sample plane of the microscope. This yields 40 pJ energy per pulse and an irradiation of 24 $mJ/cm^2$.

### 2.3. Axon observations

For consistency, in this work we reproduce the methodology followed for analysing filopodia behaviour under the influence of a focused beam of light (Mathew et al., 2008). However, axon dynamics are much slower than filopodia (Mitchison and Kirschner, 1988; Jay, 2000; Goldberg, 2003; Dickson, 2002). Cortical axons *in vitro* demonstrate great variability in growth rates but typically they are expected to grow at a rate of few tens of μm/h. In the present work, the observation time of axons is extended up to 3 h (time deemed sufficient enough for axons to grow). Otherwise, the methodology was the same (Mathew et al., 2008): Neurons were recorded under the influence of a focused laser spot. The spot was placed at a distance of up to 20 μm in front of the growth cone and at a certain angle (within ±90°) measured from the orientation axis defined by the axon (Fig. 1). The position of the beam was decided at the beginning of the experiment and was maintained fixed during the whole

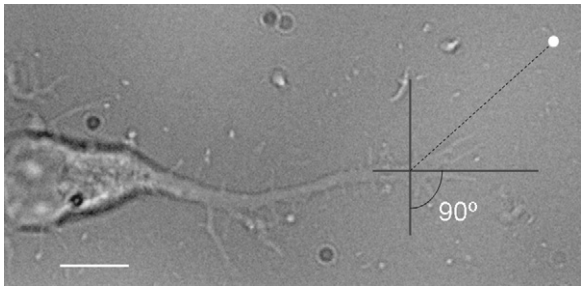


**Fig. 1.** Snapshot of an axonal growth cone in which the position of the beam spot, represented by the white dot, is indicate in relationship to the orientation axis of the growth cone. Scale bar is 10 μm.