
CHAPTER 21

Optical Neuronal Guidance

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Abstract

We present a novel technique to noninvasively control the growth and turning behavior of an extending neurite. A highly focused infrared laser, positioned at the leading edge of a neurite, has been found to induce extension/turning toward the beam's center. This technique has been used successfully to guide NG108–15 and PC12 cell lines [Ehrlicher, A., Betz, T., Stuhmann, B., Koch, D. Milner, V. Raizen, M. G., and Kas, J. (2002). Guiding neuronal growth with light. *Proc. Natl. Acad. Sci. USA* 99, 16024–16028], as well as primary rat and mouse cortical neurons [Stuhmann, B., Goegler, M., Betz, T., Ehrlicher, A., Koch, D., and Kas, J. (2005). Automated tracking and laser micromanipulation of cells. *Rev. Sci. Instr.* 76, 035105]. Optical guidance may eventually be used alone or with other methods for controlling neurite extension in both research and clinical applications.

I. Introduction

A. Neuron Structure

In the central nervous system, there are two principal kinds of neuronal cells: glial cells and neurons. Glial cells vastly outnumber neurons in vertebrates, and they are much softer than neurons, suggesting that they may function as padding to protect neurons from mechanical trauma (Lu *et al.*, 2006); however, glial cells perform other unique functions. For example, Müller cells, a special kind of glial cells in vertebrate retinas, have been found to act as living optical fibers, guiding light from the vitreous body to the photoreceptor cells (Franze *et al.*, 2007).

Unlike glial cells, neurons are the information processing/transmitting neuronal cells, and are generally composed of two main regions: neurites and the soma. Neurites are further subdivided into multiple dendrites and a single axon. The dendrites (Greek: dendron = tree) are highly branched neurites, or extensions, which receive input stimuli and direct the excitation to the soma. The soma contains the cell nucleus and other organelles, and is the most rigid part of the cell (Lu *et al.*, 2006). Typically, the axon is the longest neurite, ranging from tens of micrometers to meters in length, depending on the type of neuron and the species of animals. Axons create the “forward-active” efferent structure of the network, meaning that electrochemical activities integrated from other neurons and sources are transmitted from dendrites, through the soma, and outward along the axon to the next cell in the path through a synapse.

B. Neuronal Cells in Development

During the development of the nervous system, neurons are rapidly interconnected via migrating neurites, which must be guided through a chemically noisy and physically crowded microenvironment. The highly dynamic structure at the tip of neurites that interprets guidance cues into a connection path is known as the growth cone (Fig. 1). As the growth cone is the focus of our study, its

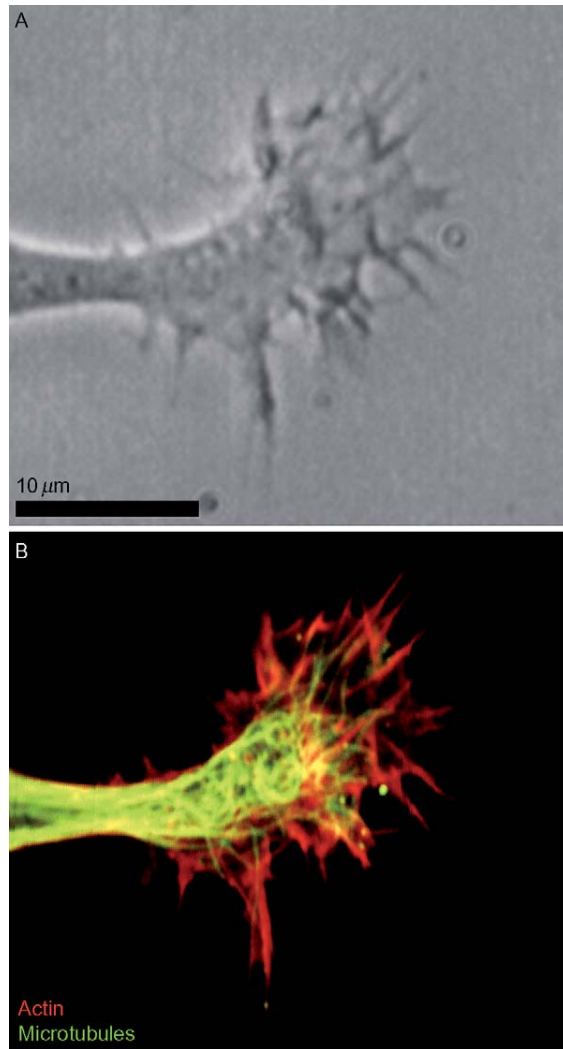


Fig. 1 Phase-contrast and fluorescence image of growth cone in a PC12 cell. The growth cone is imaged in phase-contrast (top panel) and fluorescence microscopy (bottom panel). Actin filaments are stained with fluorescent rhodamine-phalloidin and shown in red, microtubules are stained with indirect immunofluorescence and shown in green. One can see that while most microtubules are concentrated near the central stump, a few individuals are able to penetrate far out into the lamellipodium. The actin-rich projections emanating from the outer lamellipodial edge are filopodia. Additionally, one can see a strong correlation between the darker areas of the phase-contrast image and actin distribution in the fluorescence image. Scale bar is 10 μm .

properties are described in some detail. Actin filaments (red in Fig. 1) fill the peripheral region of the growth cone, known as the lamellipodium. The spike-like protrusions of actin bundles that protrude outward beyond the lamellipodium edge are known as filopodia (Fig. 1). The central region of the growth cone and the axon are filled with microtubules (green in Fig. 1), which extend into the growth cone and are largely restricted by the actin-rich lamellipodium (Dent and Kalil, 2001; Forscher and Smith, 1988; Kabir *et al.*, 2001; Schaefer *et al.*, 2002). This combination of actin filaments and microtubules, as well as other biopolymers known as intermediate filaments, molecular motors such as myosins and kinesins, and a host of accessory proteins, builds a dynamic cellular cytoskeleton. These cytoskeletal structures give neurons their structural integrity and their ability to generate forces for movement and morphological changes.

Growth cones respond to guidance cues in two- and three-dimensional environments. *In vitro*, it has been shown that nerve growth factor (NGF) is a potent neuronal chemoattractant, even at very low concentrations in three-dimensional gels (Rosoff *et al.*, 2004). Considering a 10- μm -wide growth cone exposed to a 0.1% gradient of 1 nM leads to about a thousand molecules of NGF in the vicinity of the growth cone. The growth cone shows an amazing ability to detect such small number of molecules and to differentiate directions based on even one single molecule (Rosoff *et al.*, 2004), making it a truly impressive natural detector. A statistical analysis of the movement of the growth cone's leading edge has recently revealed that stochastic fluctuations between extension and retraction may explain its ability to follow minute chemical gradients in an extremely chemically noisy environment (Betz *et al.*, 2006).

C. Growth Cone Movement and Guidance

The growth cone is characterized by highly dynamic movements of actin filaments, which continuously incorporate new actin monomer subunits at the leading edge of the lamellipodium. In one scenario, it has been proposed that as the membrane and filaments elastically fluctuate due to thermal energy, actin monomers can slip in between the tips of filaments and the membrane when the fluctuation creates a sufficient space for a 7-nm subunit. The polymerization of monomer subunits in turn exerts a force on the membrane, resulting in forward membrane protrusion in a process generally known as a “thermal ratchet” (Mogilner and Oster, 2003; Theriot, 2000).

Polymerizing actin filaments in the lamellipodium flow away from the leading edge at a fairly conserved rate in an actin and myosin motor-dependent process, which is known as centripetal actin flux or simply actin flow (Jay, 2000; Medeiros *et al.*, 2006). The term “retrograde flow” describes the variable movement of actin filaments rearward with respect to the substrate, as its movement is in opposition to the forward movement of the cell (Jurado *et al.*, 2005; Lin *et al.*, 1995). The migration speed of the growth cone, or a nonneuronal-migrating cell that shows similar processes, is approximately equal to the retrograde flow minus the centripetal actin flux, such that

when the retrograde flow is near zero, the cell approaches its maximum speed (Jurado *et al.*, 2005). Similar movements of actin have been found in many types of migrating cells such as fibroblasts, keratocytes, and single-cell organisms such as amoebas, although the molecular details differ between systems (Ponti *et al.*, 2004). In the growth cone, actin retrograde flow also fulfills the additional task of limiting microtubule extension into the peripheral region (Schaefer *et al.*, 2002). Microtubules emanate from the central region around the neurite, and individual filaments extend dynamically throughout the growth cone. It has been shown that microtubule extension is inversely proportional to actin flow since microtubules must extend radially outward against the inward flow of actin (Schaefer *et al.*, 2002; Williamson *et al.*, 1996).

A key difference between the growth cone and other motile systems is the stiff, long axon, or more generally neurite carried in tow behind the growth cone. The neurite is filled with bundles of microtubules, which derive their extreme rigidity from a tubular pipe-like assembled structure of tubulin. Intermediate filaments also contribute to the width of the neurite. Since the growth cone is not free to simply crawl, but must be followed by an neurite, the rate of growth cone extension is limited by the rate of neurite assembly and the rate of material transport to support neurite assembly (Martenson *et al.*, 1993). Manually pulling on growth cones, however, has been shown to increase the rate of neurite extension (Zheng *et al.*, 1991). Eventually, the transport efficiency can drop to a point where growth cone movement becomes significantly diminished.

Although not unique to neurons, filopodia are more prominent in growth cones than in many other cell types in two-dimensional culture. They are believed to serve as extracellular chemical and mechanical sensors, relaying information about the external environment to the cell (Challacombe *et al.*, 1996; Chien *et al.*, 1993). Filopodia are not simply passive sensors. They can generate active pulling forces with a magnitude of ~ 1 pN per filopodium (Heidemann *et al.*, 1990). Furthermore, microtubules have been observed to target the “focal complexes” (sites of cell adhesion) at filopodia ends precisely (Bershadsky *et al.*, 2006; Gordon-Weeks, 1991; Kaverina *et al.*, 2002), and filopodia movement appears to guide microtubule extension (Schaefer *et al.*, 2002). Since local pharmacological stabilization or depletion of microtubules in the growth cone leads, respectively, to neurite turning toward or away from the drug (Buck and Zheng, 2002), microtubules and filopodia are likely to form a key partnership in axonal turning. However, growth cone guidance may involve additional mechanisms. For example, reducing centripetal actin flux (via myosin Ic inactivation) caused a more significant turn response in growth cones than disabling filopodia (via myosin V inactivation), suggesting that traction forces exerted by filopodia alone are probably not the dominant element in growth cone turning (Diefenbach *et al.*, 2002); however, it is noteworthy that in this study the growth cone somata were detached from the substrate (Wang *et al.*, 2003). Additionally, myosin II inactivation has been shown to decrease retrograde flow rates (Medeiros *et al.*, 2006), but conflictingly also to increase retrograde flow rates in other studies (Brown and Bridgman, 2003). From these results, it is

obvious that the critical mechanisms for propelling and turning the growth cone have not yet been clearly identified, which poses a challenge in developing strategies to guide growth cones.

D. Existing Guidance Methods

There exist numerous techniques, natural and synthetic, to guide a neuron to a particular target. In general, complex conditions must be fulfilled in order to obtain the right neuronal response to guidance cues. Researchers have explored various approaches to control neuronal growth, primarily *in vitro* and additionally *in vivo* during nerve regeneration (Chierzi *et al.*, 2005). Even in the 1920s, experiments showed that extracellular electric DC fields could direct nerve outgrowth (Ingvar, 1920). Electric fields reversibly increase neurite growth toward the cathode and reduce extensions in the direction of the anode (Patel and Poo, 1982; Schmidt *et al.*, 1997). Furthermore, they also increase and direct neurite branching (McCaig and Rajnicek, 1991), and the electric fields induced by conducting polymer layers can enhance growth speed (Schmidt *et al.*, 1997).

The search for neuronal guidance techniques *in vitro* has resulted in a variety of methods, many of which use the general phenomenon of contact guidance and surface micropatterning strategies to regulate neuronal growth. For example, surface topography with pits and connecting grooves has been used to control the outgrowth and synapse formation of snail neurons (Merz and Fromherz, 2002). Other micropatterning strategies utilize differential adhesion, whereby a growth cone leads a neurite by sticking preferentially to substrates of stronger adhesiveness (Rudolph *et al.*, 2006; Vogt *et al.*, 2003). Adhesive properties of surfaces have been controlled by polymer patterning (Bohanon *et al.*, 1996; Takezawa *et al.*, 1990; Yamada *et al.*, 1990) or by patterning chemical properties such as hydrophobicity (Dewez *et al.*, 1998) or surface charge (Branch *et al.*, 2000). A more physiological methodology relies on the patterning of cell adhesion proteins (Blawas and Reichert, 1998), peptide sequences derived from these proteins (Herbert *et al.*, 1997; Patel *et al.*, 1998), or extracellular matrix proteins such as laminin and fibronectin. In addition, assays based on patterned surfaces have been used to identify new adhesion factors (Walter *et al.*, 1987a,b). The surface patterns in these studies are typically generated by photolithography or microcontact printing (Clark *et al.*, 1993; Fromherz *et al.*, 1991; Prinz and Fromherz, 2000; Chapter 5 by Spatz and Geiger, and Chapter 19 by Lele *et al.*, this volume).

In vivo, axons find a trail to their targets by exploiting many different guidance cues. A family of conserved chemical cues known as netrins can cause attractive or repulsive responses of growth cones (Livesey, 1999). Once the initial group of axons is in place, other axons can follow the pathfinders' trail by using several cell-cell adhesion molecules (CAMs) to which they selectively adhere (Walsh *et al.*, 1997). Guidance channels have also been used in a clinical context for the regeneration of peripheral nerves (Rivers *et al.*, 2002; Valentini, 1995).

All of the techniques above either directly or indirectly modulate the activity of the cytoskeleton, which as described earlier is the structure responsible for moving the growth cone and changing its shape. In optical guidance, we propose to bypass signaling processes and directly, physically, modify the activity of cytoskeleton to steer the growth cone's movement. The physical principles are similar to those in optical tweezers or the optical stretcher (Chapter 17 by Lincoln *et al.*, this volume), except that in optical guidance the leading edge of the growth cone is the target for the optical forces.

II. Apparatus

A laboratory equipped with optical tweezers will require only a minimal amount of construction to perform optical neuronal guidance. In essence, all that is needed for optical cell guidance is an infrared (IR) laser, a microscope with a high numerical aperture objective [as would be found in a typical optical tweezers setup (Svoboda and Block, 1994)], and a system for ensuring the viability of cells on the microscope. Of course, these three principal components can be incorporated in a myriad of different ways, depending on the researcher's focus, skills, time, and budget. Here, we will present a detailed description of our chosen setup, with some thoughts about other accessible options.

A. Laser Light Sources

An essential part of any experiment with optical forces lies with the laser and optics. In general, a near-IR wavelength of 800 nm is preferable when working with cells due to the low absorption of water at this wavelength (Peterman *et al.*, 2003; Svoboda and Block, 1994). Currently, this wavelength limits the use of high-power, cost-effective diode/fiber options; however, new products are emerging and other near-IR wavelengths may be equally effective (Mohanty *et al.*, 2005). For example, with collaborators we have successfully guided primary embryonic chicken neurons with a ytterbium fiber laser at 1064 nm (Stuhrmann *et al.*, 2006), although heating due to increased water absorption at this wavelength deserves attention, as it is approximately five times higher than using 800-nm laser of comparable power (Svoboda and Block, 1994). Other collaborators have also demonstrated that wavelengths of 780 and 1064 nm optically guide neurons equally well (Stevenson *et al.*, 2006). In addition to potential cell damage, the applied power is also limited by the objective's damage threshold, which is usually between 500 and 1000 mW, with phase-contrast objectives typically occupying the lower part of this range. We have chosen a Coherent 890 Ti:Sapphire (1.6 W at 800 nm), which is pumped by a Coherent Verdi V-10 diode-pumped ring laser (10 W at 532 nm). This Ti:Sapphire has the added convenience that its wavelength can be tuned between 690 and 1100 nm. To maintain a clean Gaussian intensity profile, we use the TEM₀₀ mode of the laser. Additionally, we use the laser only in

continuous wave (CW) mode, as opposed to mode-locked (pulsed) operation, which would cause significant morphological damage by essentially cooking the cell with high-intensity pulses.

B. Laser Light Control Elements

The laser must then be directed to and optically coupled into the microscope. In Fig. 2, one can see that we first expand the laser to roughly the size of the objective's back aperture (6 mm) using a collimating two-lens system (telescope). We then split the beam (not depicted in figure), to allow the use of a single-laser source in two setups, at a ratio of 60/40 to compensate for the differences in the efficiency between the two paths. In the first setup, the laser enters the acousto-optical deflectors (AODs; AA Opto-Electronic, St. Remy les Chevreuse, France; 6.7-mm aperture, 31-mrad maximum scan angle at 800 nm, 10- μ sec random access time, 80% maximum transmission efficiency). An AOD is a solid-state device that is able to change the transmitted laser's deflection and intensity in response to an input radio frequency (RF) signal (AA Opto-Electronic: 91–118 MHz, 2-W max output power, 1- μ sec sweep time). The device is aligned such that the Bragg configuration of its internal crystal transmits a first-order diffracted beam whose angle is related to the RF signal's frequency and whose intensity at that angle is controlled by the amplitude of the RF.

The second setup is largely identical to the first, apart from the use of rapidly moving galvo mirrors (Magnet Optical Scanner, VM-1000C, accessible angle of $\pm 45^\circ$, 0.8-msec full step access time; GSI Lumonics, Billerica, Massachusetts), instead of AODs as the laser-steering component. Additionally, since the galvo mirrors only control the laser angle, an acoustooptical modulator (AOM; AA Opto-Electronic: AA.MT.110/A1-ir) is included to control the incident power. While the AODs offer a much faster scan rate than with galvos, they are optimized to operate at a specific wavelength ± 50 nm, and are less efficient, transmitting only $\sim 80\%$ of the incident light. Both systems allow the implementation of multiple optical traps and arbitrary radiation patterns by quickly rastering between different points on the sample plane, achieving similar results as those done with holographic optical tweezers (HOTs) (Grier and Roichman, 2006), but due to their much faster scan rate, AODs typically produce more time-continuous radiation patterns. Galvos, however, reflect a broad spectrum of light with highly reflective mirrors that are very efficient, and have a much larger angular range. We find that the AODs suit our purposes better since we have ample incident power and have only used a single wavelength of 800 nm to date, but the preference between these steering devices will vary with researchers and applications.

After passing through the steering device, the laser beam is focused through another telescope to ensure that the beam hits the center of the objective back aperture for any angle of deflection. The laser is coupled into the objective via reflection off a narrow bandwidth dichroic mirror, with $>90\%$ transmission from 410 to 660 nm and high reflectivity at other wavelengths (675DCSX, Chroma

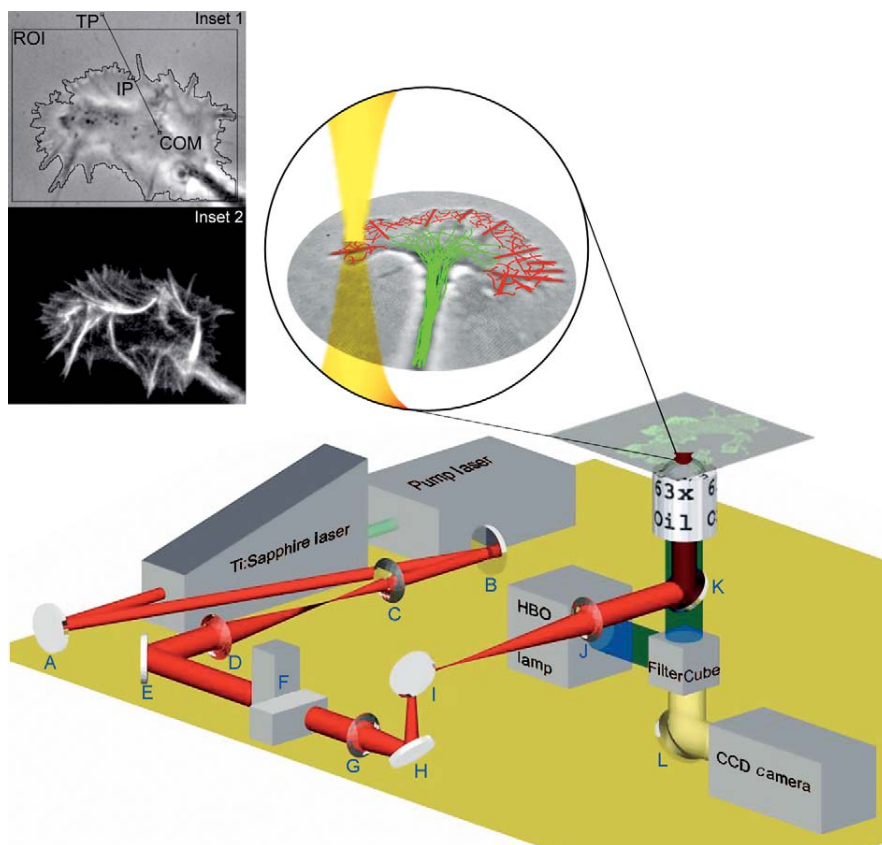


Fig. 2 Illustration of experimental setup. The laser system is based on a Ti:Sapphire laser (Coherent, 890) pumped by a 10-W solid-state laser. The laser beam is repositioned by mirrors A and B and then collimated and expanded to 6-mm diameter in a telescope (C, D). It is deflected by a mirror (E) into a pair of acoustooptical deflectors (F), which control x - and y -deflection as well as the intensity of the beam. The beam is then coupled into the microscope beam path via additional mirrors (H, I), lenses (G, J), and a dichroic mirror (K). The dichroic mirror only reflects infrared light of the laser and thus allows the combination of laser irradiation, fluorescence microscopy, and phase-contrast imaging (recorded with a CCD camera as shown). Circular insert shows a diagram of the focused laser beam positioned with respect to growth cone. Rectangular insert shows automated tracking by the LabVIEW program (top) and the corresponding fluorescence image of GFP actin (bottom). Tracking is computationally done by an edge-detection algorithm within the user-defined region of interest (ROI). The laser beam is automatically targeted just outside the leading edge (target point; TP), and the guidance action presumably takes place near the intersection point (IP), between TP and the center of mass (COM) of the growth cone.

Technology Corp., Rockingham, Vermont). This mirror is inserted in the nose-piece (Fig. 2, element K) and is aligned to reflect the laser straight through the objective via a homebuilt arrangement with micrometer screws, as shown in

Fig. 2. Since this mirror reflects 800-nm laser light but transmits the rest of the visible spectrum, phase-contrast, fluorescence, or DIC imaging can be conducted simultaneously with optical guidance.

C. Microscope Irradiation and Imaging

Our system is built on a Leica DM IRB inverted microscope; however, most modern microscopes should be adequate, provided there is a video port and some way to couple in the external laser. Due to safety concerns, particularly due to the laser's near-IR emission not being readily visible, we use a CCD camera (Cohu 4910 monochrome CCD camera, $6.4 \times 4.8 \text{ mm}^2$ chip with 768×576 pixels resolution; San Diego, California) as opposed to microscope eyepieces to observe the sample, and record the images to a computer. A modest computer by modern standards (Pentium 4, 1.8 GHz, 1-GB RAM), with an analog-to-digital converter card for image capture (National Instruments IMAQ PCI/PCX-1407 Framegrabber; Austin, Texas), and an analog voltage output card for controlling the AODs (National Instruments PCI-6711 analog voltage output device), is adequate for our applications. Image recording, recognition, and laser control are all performed with LabVIEW programs written by our group (available on request). One of these programs was developed specifically for optical guidance of cells and is detailed by [Stuhrmann *et al.* \(2005\)](#). Our system allows the recording of images with or without showing the laser beam, by using a wide band-pass filter which blocks the laser's IR light to the camera while allowing other light to pass (FM01 Wide Band hot mirror: 90% reflectance 750–1200 nm, Thorlabs GmbH Dachau/Munich Germany). This filter is moved in or out of the imaging light path with an actuator, which is also computer-controlled through LabVIEW. Even for images including the laser, we use a filter that blocks $\sim 99\%$ of the light to prevent the laser's intensity from dramatically overexposing the image.

D. Cell Culture System

We principally use two cell lines, PC12 and NG108-15, which are both immortalized cells. PC12 cells were originally derived from a transplantable rat pheochromocytoma (tumor of the adrenal gland) ([Greene and Tischler, 1976](#)), and behave as not fully differentiated precursors of nerve cells, but reversibly become more “neuron-like” when treated with nerve growth factor (NGF). In the undifferentiated proliferative state, they adhere weakly to plastic dishes and tend to grow in small clusters with a doubling time of ~ 92 h. They are cultured in a specially formulated medium:

- 85% RPMI-1640 (ATCC 30-2001, containing 2-mM L-glutamine, 10-mM HEPES, 1-mM sodium pyruvate, 4500-mg/liter glucose, 1500-mg/liter sodium bicarbonate)
- 10% horse serum (ATCC 30-2040)

- 5% fetal bovine serum (ATCC 30-2020)
- 100-U/ml penicillin and 100- μ g/ml streptomycin (Sigma P0781).

This medium is refreshed every 2 days, and cells are resuspended and subcultured prior to confluency, or about every 5–7 days. To induce the neuronal phenotype, NGF (Sigma N6009) is added at 25–50 ng/ml for at least 24 h.

NG108-15 is an immortalized mouse neuroblastoma cell line, which was developed in 1971 by [Hamprecht *et al.* \(1985\)](#) by fusing mouse neuroblastoma cells with rat glioma cells in the presence of inactivated *Sendai virus*. NG108-15 cells grow neurites spontaneously in culture and are also known to form synapses that are at least functional on the presynaptic side. They require a different medium from PC12 cells:

- 90% DMEM (ATCC 30-2002, containing 4-mM L-glutamine, 110-mg/liter sodium pyruvate, 4500-mg/liter glucose, 1500-mg/liter sodium bicarbonate)
- 10% fetal bovine serum (ATCC 30-2020)
- 100-U/ml penicillin and 100- μ g/ml streptomycin (Sigma P0781)
- 10-mM HEPES, diluted from 1-M stock solution.

Unlike PC12 cells, no further chemicals are necessary for neurites to extend. However, 1-mM cyclic AMP (Sigma D0627) is required for presynaptic activities ([Chen *et al.*, 2001](#)). In addition, both cell lines seem to show decreased proliferation and increased neurite extension with reduced serum in the medium (total serum $\sim$ 2.5%).

We have also worked with primary rat and mouse neonatal cortical cells, which are taken directly from freshly euthanized animals. Primary cells are of course the most physiologically relevant cells, especially for neuronal networks. Nevertheless, since neurons with long, well-established neurites are preferred, cell lines are much easier to use on a regular basis than primary cells, which are only recommended for the most advanced stage of study. Primary mouse and rat neonatal cortical cells require a special culture medium:

- Neurobasal NB (Gibco 21103-049)
- 2% B27-supplement (Gibco 17504-044, serum-free supplement)
- 2-mM L-glutamine (Sigma G7513)
- 1% penicillin–streptomycin–neomycin (PSN) antibiotics (Gibco 15640-055).

Cells are usually maintained in an incubator at 37 °C with 5% CO₂ to balance the pH and a humidity close to 100% to prevent evaporation. Without stable cell viability, the described optical guidance experiments are simply impossible. In addition, challenges of cell culture are compounded when cells are transferred to a stage-based culture system.

There are many commercial and homebuilt systems for maintaining mammalian cells on the microscope stage. However, since an oil objective is typically used to create a tightly focused laser trap, the system must heat not only the stage but also

the objective as it is in direct thermal contact (through the oil) with the cell substrate. We initially used a rather expensive, commercially available system (Bioptechs FCS2 Focht Live-Cell Chamber and objective heater; Butler, Pennsylvania), which has excellent temperature control and offers an open or closed design. However, pH control and pressure fluctuations from the peristaltic pump were problematic. We have since developed our own stage culture system, which is in our opinion generally easier and more flexible to use.

We modify standard 2-in. tissue culture dishes (Nunc Brand, Denmark), by cutting a 26-mm-diameter hole from the bottom-center, and then gluing a 40-mm-diameter, 170- μm -thick glass coverslip (VWR) from the outside of the dish with silicone adhesive (General Electric, RTV 108) to cover the hole. The substitution of the coverslip for the existing plastic bottom is critical, since the latter is thicker than the oil objective's working distance. Other consumer silicone sealants may also be used for attaching coverslips, although it is preferable to use adhesives with nontoxic solvents like acetic acid, as opposed to methanol. The dishes are ready for use 24–48 h after the silicone has been applied, and are generally stored in 80% ethanol/dH₂O to maintain sterility. The assembled dish with a lid can be seen in Fig. 3, left panel.

We also constructed Teflon tops for these dishes in both open (with metal ports for chemical or gas exchange as shown in Fig. 3, left panel) and closed (no ports, for sealed dishes) configurations (dish, right panel). For gas exchange, we connect the open version top to humidified prebottled 5% CO₂ in synthetic air (Air Liquide GmbH, Cologne, Germany) to maintain the same atmospheric control afforded by

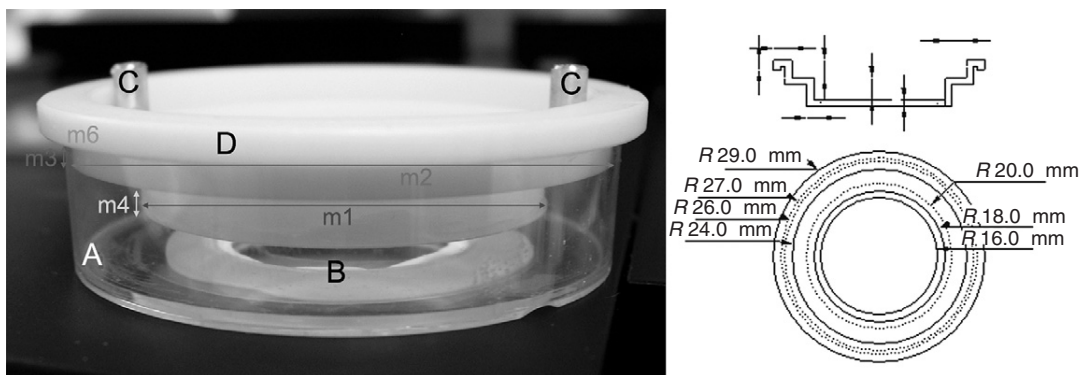


Fig. 3 Design of the experimental dish (left). A 26-mm-diameter hole is cut from the center of a standard 2-in. polystyrene Petri dish (A). The hole is then covered by gluing from the outside a glass coverslip of 40-mm diameter, 170- μm thickness (VWR Germany, Cat No. 631-0177), with silicone (General Electric, RTV 108) (B). Gas exchange is conducted through metal connectors (C), which are 3.5 mm in outer diameter, with a 5.5-mm collar to prevent it from falling into the holes (3.6 mm) where they plug into the Teflon top (D). The dimensions are: m1 = 40.0 mm, m2 = 52.7 mm, m3 = 3.0 mm, and m4 = 4.9 mm. Detailed design of the Teflon top, with the top and cutaway views and dimensions is shown in the right panel.

the incubator. The tops have the same glass coverslip surface as the bottom, providing a window in the center to allow transmitted light illumination.

Before use, the dishes are cleaned again with 80% ethanol and rinsed with sterile phosphate buffered saline (PBS) in a sterile culture hood. Typically, neurons adhere rather poorly to glass, and a surface coating of matrix proteins is often used to promote adhesion. Laminin (Sigma L2020) can be adsorbed at a concentration of 40 $\mu\text{g}/\text{ml}$ in PBS, using a volume large enough to cover the glass surface ($\sim 50 \mu\text{l}$). After 1–3 h at 37 °C (or room temperature overnight), the surface is gently rinsed twice with sterile PBS, never allowing the adsorbed laminin layer to dry out. Poly-L-lysine (Sigma P8920) may be used in place of laminin as described above by applying 0.5 ml of a 0.1 mg/ml solution to coat 25 cm², although it may be allowed to dry out over about 24 h. After rinsing three times with PBS, a laminin layer may be applied as described above, or the cell suspension may be directly added. Cells plated directly onto poly-L-lysine appear less mobile than those on laminin, probably because adhesion to cationic polylysine is electrostatic rather than specific to adhesion receptors. Typically, PC12 cells show substantial extended neurites after about 24–48 h of exposure to NGF at 25–50 ng/ml, whereas NG108-15 cells are often ready for experimentation in as little as 4 h after plating without exposure to NGF.

For short-term experiments of 2 h or less, the dishes are often filled with HEPES-buffered medium (including NGF in the case of PC12 cells), allowed to equilibrate under the appropriate CO₂, and then sealed with the closed top and some parafilm or vacuum grease. Thus, only temperature needs to be controlled, as other conditions do not vary appreciably over this short time period.

Temperature control can be accomplished using a dish heater to keep the bulk medium warm, and (most importantly) an objective heater for oil immersion objectives, which otherwise behave as a heat sink that lowers the temperature of the medium in the observation field. The temperature must not be driven above 37 °C, as cells have very little tolerance for overheating. Underheating is typically far less serious a problem than overheating; while cells at 30 °C may not be optimal, they are unlikely to be damaged as is often seen at temperatures above 40 °C.

The dish heater consists of an aluminum cup, which is fitted to hold the Petri dish snugly, and has the center bottom cut out similarly to the dishes, to allow the passage of light (Fig. 4, right panel). The dish is thermally insulated from the microscope in our case, by having only three small steel-bearing “feet” as the support contacts between heater and stage. Additionally, the cup is held on the stage with a Teflon mount to reduce thermal conduction. The aluminum cup is warmed by two PT100 elements (Conrad Electronics, item 171778-62, Heraeus GmbH) in parallel, one on each side of the cup, and a third PT100 element, which functions as the temperature sensor. The PT100 sensor is connected to a homebuilt electronic controller (detailed schematic available on request), which monitors the temperature relative to the set point and sends a proportionally regulated voltage to heat the dish. One could also connect the PT100 chips to a DC power supply without feedback, and calibrate an appropriate voltage based on dish temperature

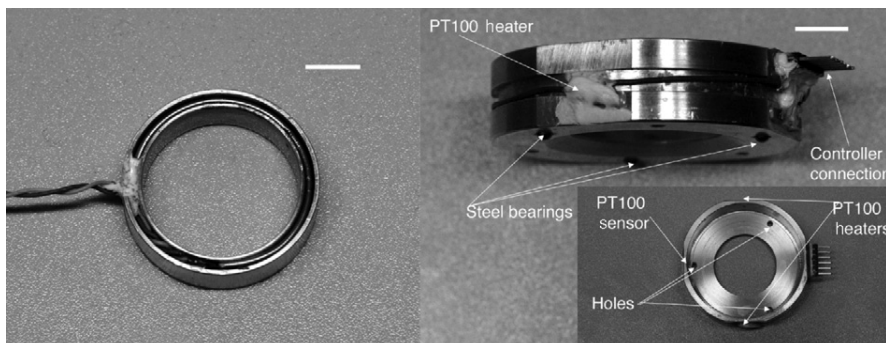


Fig. 4 Photographs of homebuilt heater systems. The left panel depicts the objective heater, which is necessary for use with oil immersion objectives. Thermocoax cable is wound three times within the machined aluminum, glued into place, and then connected via wires to an external power supply. The inner diameter is 29.0 mm and the outer is 36.5 mm, but the dimensions will obviously vary with different objectives. The right panel shows a side view of the Petri dish heater, which has an outer diameter of 60.0 mm and an inner diameter of 54.2 mm. The inner diameter should be small enough to ensure good thermal contact with the Petri dish, but not tight enough to prevent easy removal of the Petri dishes. The PT100 heater and thermometer elements are indicated as are the steel-bearing feet that help to isolate the heater thermally from the stage. The right panel inset shows a view from above of the dish heater; here one can see the PT100 elements, and holes which aid in removing the Petri dish from the heater. Scale bars are 10 mm.

measurements. The voltage output required typically ranges from 0 to 10 V to heat the dish cup to $\sim 40^\circ\text{C}$ (the dish cup is heated marginally above 37°C , as some heat is lost to the environment).

The microscope objective also requires a heater due to the strong thermal contact of oil immersion objectives with the sample. As a heating element, we use thermocoaxial cable (item number 2NcNcAc15, Thermocoax SAS, Cedex, France) wound into a coil and encased in an aluminum cylinder that snugly fits the objective, as shown in the left panel of Fig. 4. Since different objectives vary slightly in diameter, we constructed separate heaters for each objective, keeping all of the objectives constantly warmed so as not to potentially damage internal lens alignments with stresses from frequent heating and cooling. We use a typical DC power supply (Conrad Electronics, Voltcraft PS 303 Pro) to power the objective heater and calibrate the temperature against the voltage with a digital thermometer. However, one could use the same feedback approach as described above for the dish cup heater.

E. Adapting Existing Systems for Optical Guidance

As mentioned earlier, an optical guidance system may be adapted from existing optical tweezers, using a range of near-IR wavelengths with or without the AOD or galvanometer beam-steering optics as described above. A minimal system may consist simply of a focused near-IR laser beam as a static optical trap. Many

biological science laboratories will likely already have access to on-stage cell culture systems, confocal laser-scanning microscopes (CLSMs), or optical tweezers, and these systems can be modified to allow optical neuronal guidance experiments without needing to build an entirely new system.

Static optical traps can be used for automated optical guidance in two possible scenarios. With the help of an automated microscope stage, one can move the sample instead of the laser beam. Alternatively, the static trap may be turned into a movable trap by the use of an inexpensive mechanical actuator that controls the x - y position of the telescope lens, at a fraction of the cost compared to using precise, automated microscope stages (Svoboda and Block, 1994; Visscher *et al.*, 1996). Beam and sample positioning represent equally feasible options. While these simple approaches may have a limited range, an accessible area of $\sim 10 \times 10 \mu\text{m}^2$ is already sufficient for growth cones growing at $1\text{--}2 \mu\text{m}/\text{min}$ over 10 min.

As a further simplification, one can control the position of the laser spot by moving the sample or the laser spot by hand. While computer control certainly eases the burden on the researcher, it is not strictly necessary. In fact, our original experiments (Ehrlicher *et al.*, 2002) were performed with a CLSM by manually adjusting the position of the laser spot every few seconds. The Ti:Sapphire laser, as often found in multiphoton CLSMs, was used in CW mode as opposed to the typical pulsed mode required for multiphoton microscopy, and the CCD images were simply recorded with a VCR.

III. Experiments

We have observed optical guidance in PC12 cells (Ehrlicher *et al.*, 2002), both with the standard cell line (Greene and Tischler, 1976) and a gelsolin-overexpressing version (Furnish *et al.*, 2001). PC12 cells grow in a very regular fashion with well-defined shapes. NG108-15 cells, which appear to be a more robust cell line that tolerates higher deviations from ideal conditions outside incubators, have also been successfully used in optical guidance experiments. Furthermore, for transfections with green fluorescent protein (GFP) constructs, the efficiency with NG108-15 cells is higher than that with PC12 cells using lipofectamin (invitrogen) or nanofectin (PAA).

A. Turns

The general procedure we have used to optically guide cells is as follows. First, one must identify an actively extending cell that displays dynamic filopodia and lamellipodia. This is critical, as optical guidance only presumes to guide the extension of active cells kept under optimal chemical and temperature control. Next, a direction for biased extension is chosen. For establishing the effectiveness of the technique, the chosen guidance direction should be significantly different from the native direction of growth in order to obtain an unambiguous guidance

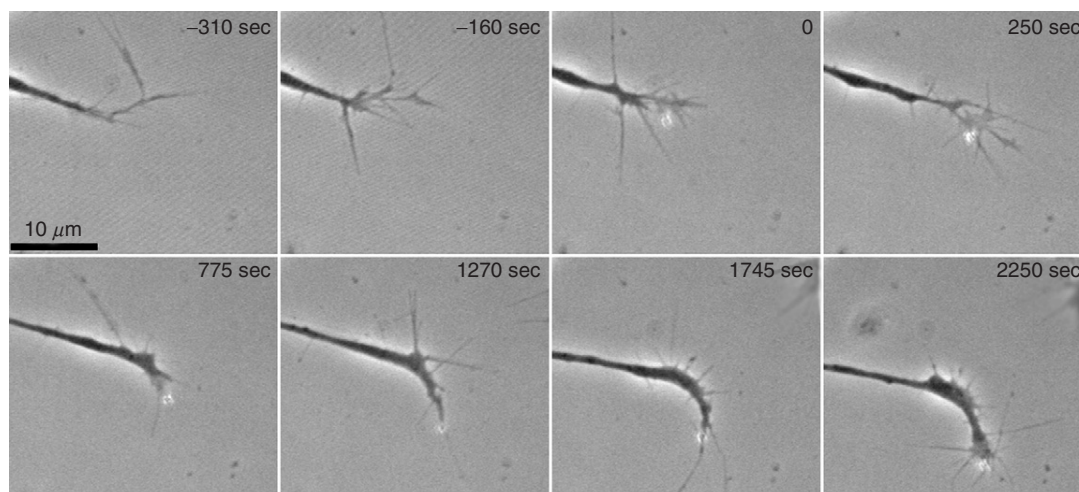


Fig. 5 Optically induced turn of a PC12 neurite. In this example, a 150-mW laser beam, seen as a small white spot, is applied to the lower edge of a small extending PC12 neurite. The neurite subsequently grows toward the laser beam and continues to extend and turn over ~37 min.

response; however, actual direction selections will of course vary with the desired application. The growth is first recorded for ~5–10 min to establish the native growth behavior, after which the laser is introduced at the edge of lamellipodium in the direction of desired biased growth. Optimal results are obtained when approximately a third to a half of the beam spot overlaps the lamellipodium and some filopodia.

Sometimes neurites will stop growing, or even retract, after the experiment has begun; however, it is rare for a neuron to continue to grow along the original direction and away from the beam. Laser powers ranging from ~20 to 200 mW are effective, with most experiments performed in the 100- to 200-mW range. However, there have been no systematic studies on the dependence of the power on guidance effectiveness. [Stevenson *et al.* \(2006\)](#) showed that powers of 9–25 mW using wavelengths of both 780 and 1064 were effective in optically guiding turns.

[Figures 5 and 6](#) show typical examples of optical guidance. In [Fig. 5](#), the laser is positioned at time zero at the right edge of the growth cone. The growth cone asymmetrically extends into the laser and proceeds to grow toward the bottom of the image. In [Fig. 6](#), the laser is introduced on the right side of the growth cone, and again the neurite extends into the focus and grows toward the left side of the image.

B. Accelerated Growth

Both in asymmetric optical guidance and when the laser has been placed directly in front of the extending growth cone, we have often seen a rapid increase in the growth cone's translocation speed. In the left panel of [Fig. 7](#), one can see a direct

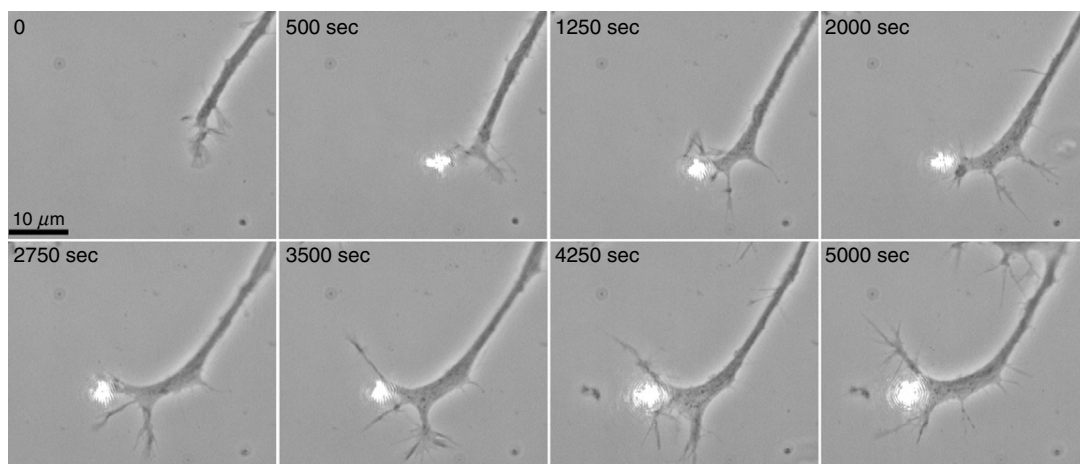


Fig. 6 Optically induced turn of a PC12 neurite. In this example, a 160-mW laser beam is applied to the left edge of a large extending PC12 neurite, causing the neurite to grow toward the laser beam. The neurite extends and turns, pulling the axonal stump behind it, over ~83 min.

comparison between two side-by-side PC12 growth cones, one of which is optically guided. The optically guided one increases in speed from $7 \pm 5 \mu\text{m/h}$ to $37.5 \pm 22.3 \mu\text{m/h}$.

C. Bifurcations

We have more rarely observed other optically guided phenomena such as splitting of a growth cone or halting extension, as shown in Fig. 7. Splitting or bifurcating of a growth cone seems to occur where it is easier for the growth cone to follow the laser by forming a new branch rather than turning the entire extending neurite, as when the laser is placed further back along the edge of the stump of a neurite, attempting to induce a more radical turn (Fig. 7, center panel). This process may have interesting applications as a method to split neurites. To stop a growth cone, we have placed the laser well within the lamellipodium near the center of the growth cone and observed that local extension ceases, as seen in the right panel of Fig. 7. The extension typically resumes on the removal of the laser beam.

D. Other Observations

Optical guidance is not an indefinitely prolonged effect, and after 10–15 min the growth rate typically decreases, which may be attributable a shortage of cytoskeletal components among other possible causes. In Fig. 8, one observes a sizable extension between panels A ($t = 0$) and B ($t = 500$ sec), followed by a pause until panel E ($t = 1500$ sec), whereupon a wave of external lamellipodium activity has flowed to the growth cone allowed the growth cone to resume its advance.

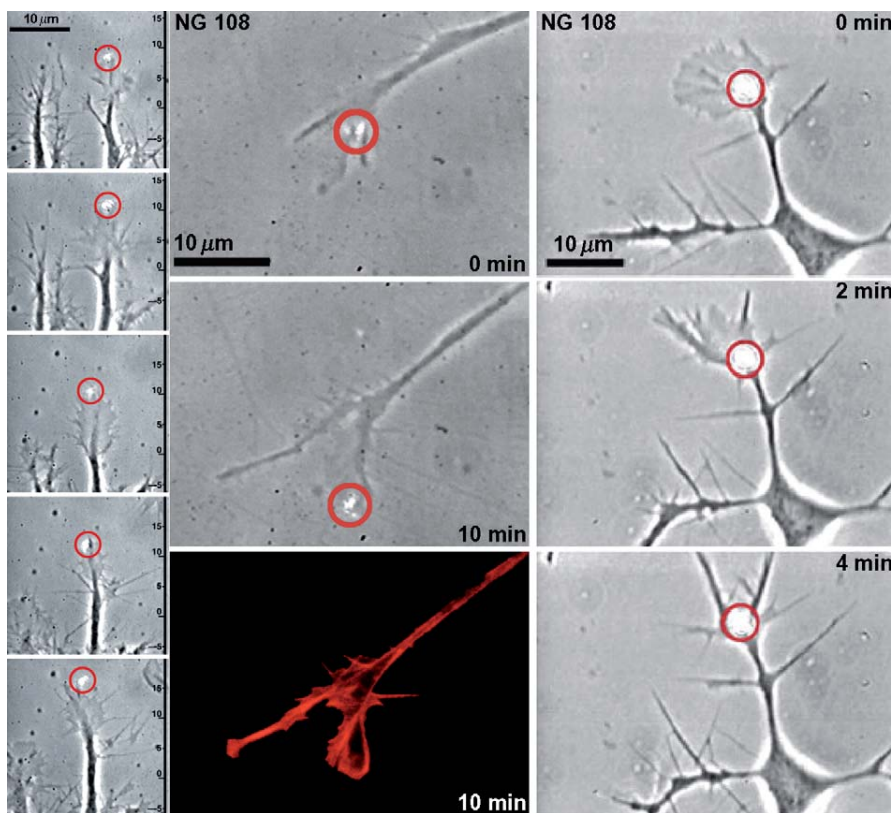


Fig. 7 Enhanced extension, bifurcation, and optically induced halting in growth cones. Position of the laser beam is indicated by red circles. In the left panel, two side by side PC12 growth cones are observed. A 20-mW laser (circled in red) is placed at the leading edge of the growth cone on the right, resulting in an increase in speed from $7 \pm 3 \mu\text{m/h}$ to $37.5 \pm 22.5 \mu\text{m/h}$. The time interval between frames is 10 min, and the reference marks to the right are in micrometer. In the center panel, an optically induced bifurcation of a growth cone is shown. A growth cone, which is growing toward the lower left, sprouts off an extension to the lower right under the influence of the laser. The last picture displays the distribution of actin filaments by rhodamine-phalloidin staining. Actin filaments have clearly accumulated at the areas of lamellipodia extension. In the right panel, a laser positioned within the lamellipodium causes the growth cone to halt without causing damage, as the growth cone resumed growth after removing the laser.

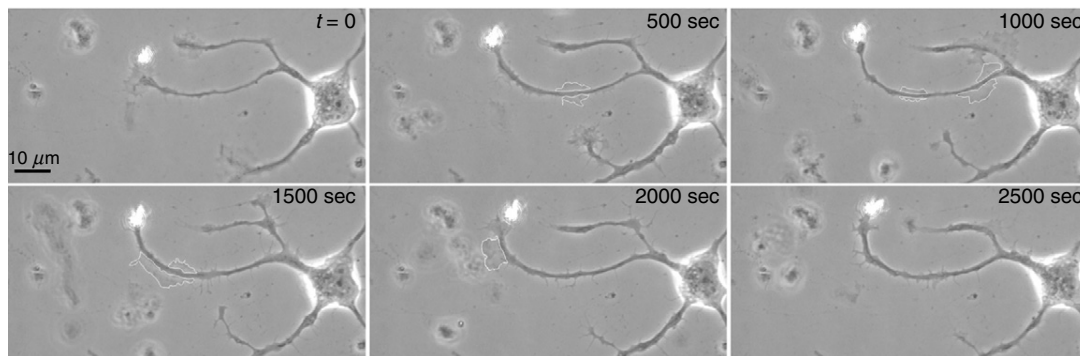


Fig. 8 Time series of an optically guided growth cone in a primary neonatal mouse cortical neuron. This time series shows neurite extension in response to the application of a 90-mW laser beam. In the first period of activity between 0 and 500 sec, the neurite increases in area by $\sim 19.8 \mu\text{m}^2$. The translocation pauses after about 600 sec, with little growth in the neurite until ~ 1840 sec, when a second surge of material was seen flowing up to the growth cone. The resulting temporary increase in translocation velocity leads to an increase in area by $\sim 19.1 \mu\text{m}^2$ between 1500 and 2500 sec. Material surges are outlined in white for clarity. The observations suggest that the duration of guidance is likely to be limited by available materials, without necessarily involving a more complicated mechanism of biochemical or genetic “tolerance.”

Unguided neurites also tend to grow discontinuously in phases of extension, retraction, and rest, which make unambiguous guidance difficult. We have found that these phases can be stabilized with mood-altering psychoactive drugs such as valproic acid (VPA), lithium, or carbamazepine (CBZ). These drugs have been shown to inhibit the collapse of growth cones and to increase the growth cone area in sensory neurons (Williams *et al.*, 2002). With NG108-15 and PC12 cells, these drugs inhibit proliferation while promoting neurite extension. VPA stood out from the other drugs in its dose-dependent effectiveness, as shown in Fig. 9.

IV. Plausible Mechanisms of Optical Guidance

We reasoned originally that the optical trap may be able to locally bias the diffusion and concentration of actin monomers and oligomers, which may locally increase the rate of actin polymerization and enhance the polymerization-driven extension. While this concept of laser-enhanced actin concentration/polymerization has been detailed previously (Ehrlicher *et al.*, 2002) and remains a possibility, we have also proposed several other possible mechanisms.

A. Filopodial Asymmetries

As discussed in Section I, filopodia are critically important in two- and three-dimensional guidance of neurons. These filopodia control the distribution of exploratory microtubules, which are necessary and sufficient to turn a neurite.

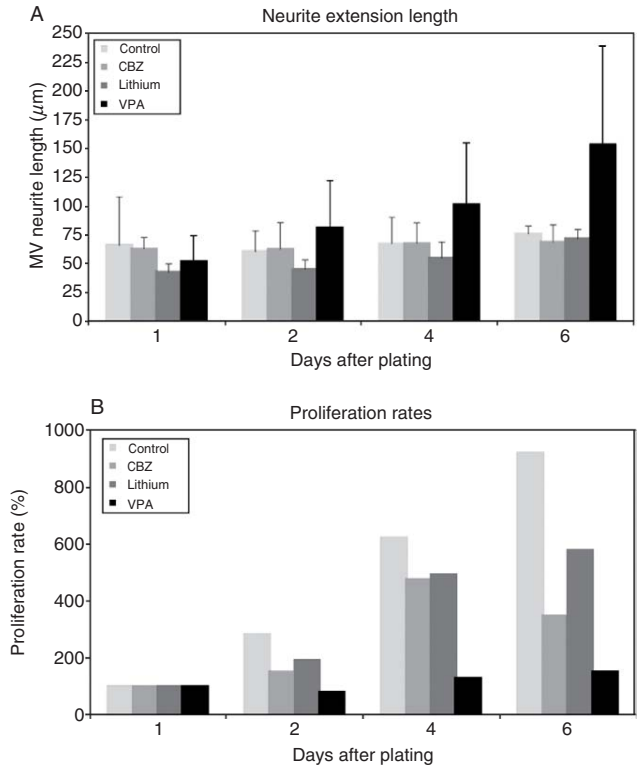


Fig. 9 NG108-15 neurite extension and proliferation under the influence of pharmacological substances. Valproic acid (VPA) at 1 mM, shown in black, causes significantly higher average neurite extension as compared to 100- μM carbamazepine (CBZ), 1-mM lithium, or control (A). VPA also inhibits proliferation of new cells (B). This inverse relationship between extension rate and cell proliferation is not surprising, since cell division and neurite elongation appear to be mutually exclusive events. All observations were done in serum-free DMEM (Sigma D6429) supplemented with 100-U/ml penicillin/100- $\mu\text{g/ml}$ streptomycin and 10-mM HEPES.

The laser may be able to introduce an asymmetry in filopodia distribution around the growth cone, and additionally provide a positive force feedback by holding the filopodia in place.

In considering the potential mechanism for optical guidance, a significant observation is that the method does not appear to work for migrating nonneuronal systems such as keratocytes (unpublished data). These other cells show both centripetal actin flux and retrograde flow but lack the prominent filopodia found in growth cones, implicating filopodia as the primary structures responsible for the detection and/or response to the optical forces.

B. Retrograde Flow

In growth cones, the actin network moves radially inward with respect to the leading edge, as discussed in [Section I \(Schaefer *et al.*, 2002\)](#). In the presence of optical guidance, the applied forces may pull the denser actin structures within the cytosol forward and hinder the retrograde flow, generating an additional traction-like resistance for lamellipodia extension, and/or aiding microtubule extension. Furthermore, the laser might trigger additional connections between the cell and the substrate, thus reducing the rate of retrograde flow and increasing the net rate of extension as explained earlier.

C. Actin Polymerization via Membrane Tweezing

Another possibility is that the laser trap may pull on the membrane, enhancing actin polymerization and protrusion as was described in the thermal ratchet mechanism in the introduction. Pulling the membrane forward with the laser would increase the probability of actin monomers attaching to the extending filament, and thus increase the polymerization rate of actin filaments that push the membrane forward.

[Figure 10](#) shows ~ 20 nm of movement of a living NG108-15's membrane with 40 mW of applied laser power. This movement indicates that the hypothesis of enhanced actin polymerization due to membrane tweezing is plausible and deserves consideration.

D. Laser-Induced Heating

One possibility is that a high-power focused laser beam can cause localized heating, which might influence reactions in the immediate area and bias growth cone extension. However, this hypothesis does not seem very probable since temperature increases should be very small. There is a strong asymptotic dependence of heating on the distance to the surface of the substrate. As an optical trap comes closer to the surface, the induced heating rapidly declines ([Peterman *et al.*, 2003](#)). For 500-nm beads suspended far away (at least $10\text{ }\mu\text{m}$) from the surface in glycerol, this heating is estimated to be $41.1 \pm 0.7\text{ kW}$, but in water there is far less localized heating due to lower absorbance, as well as a greater thermal conductivity. Thus, one would expect only $7.7 \pm 1.2\text{ kW}$ in water under the same conditions. For spherical particles near the glycerol-glass interface, about 10-kW heating is expected. Considering the lower absorption of water, $\sim 1.9\text{ kW}$ is expected at a water-glass interface ([Peterman *et al.*, 2003](#)). Given the lower laser powers used in optical guidance ($<200\text{ mW}$ and typically around 80 mW), a spherical particle would experience 0.15- to 0.40-kW heating. The heating of a growth cone is expected to be even lower since growth cones are only a few micrometers in thickness with a fairly circular extended shape $\sim 10\text{ }\mu\text{m}$ in diameter. With such a large aspect ratio, the growth cone is completely thermally coupled to the substrate, which would decrease heating to only a fraction of that for a sphere. Therefore, we

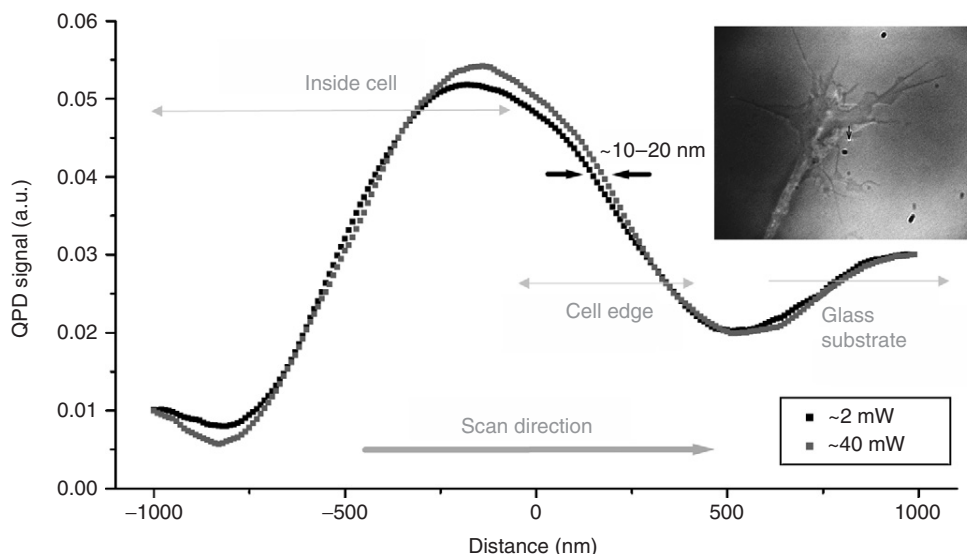


Fig. 10 Effect of membrane tweezing on a living NG108-15 neuron. The laser is scanned with 10-nm steps over a path of 2000 nm perpendicular to the cell's edge (black arrow in inset image indicates end point and the length of the arrow represents the scanned path). Black data points show the scan with an applied laser power of ~2 mW. Gray data points show the same scan with ~40-mW laser power. The laser exerts a pulling force on the edge of the cell, resulting in a small but clear difference of ~20 nm between the curves. This demonstrates that the laser does indeed pull on the membrane.

believe that heating is minimal even from an intense laser beam, and is unlikely to contribute to the guidance effect. [Stevenson *et al.* \(2006\)](#) have also shown identical guidance results from a 780- to 1064-nm laser, suggesting no influence of heating on optical guidance, as the 1064-nm laser should heat the sample more due to higher absorption at that wavelength.

V. Summary

Optical guidance of neurites allows a degree of flexibility in guidance path not available from other methods. In its most minimal form, the apparatus only requires optical tweezers, a microscope, and some method to stabilize the cells for the duration of the experiment. There are numerous future possible applications for this technology. One wishful direction might be in the field of regenerative medicine. It is conceivable that the technique could be used with an optical fiber to guide neurons *in vivo*, possibly to repair connections severed through trauma. Another possibility would be to promote the assembly of specific complex neuronal networks, which would be ideal for preclinical pharmaceutical studies. Finally,

a great deal of work has gone into neuron–semiconductor interfaces (Merz and Fromherz, 2002), and the ability to connect the interfaced neurons in a specific fashion would be a considerable advantage.

Acknowledgments

The authors acknowledge generous financial support from the Deutsche Forschungsgemeinschaft (DFG KA 1116/3-2) and Ms. Marianne Duda.

References

- Bershadsky, A. D., Ballestrem, C., Carramusa, L., Zilberman, Y., Gilquin, B., Khochbin, S., Alexandrova, A. Y., Verkhovsky, A. B., Shemesh, T., and Kozlov, M. M. (2006). Assembly and mechanosensory function of focal adhesions: Experiments and models. *Eur. J. Cell Biol.* **85**, 165–173.
- Betz, T., Lim, K., and Käs, J. (2006). Neuronal growth: A bistable stochastic process. *Phys. Rev. Lett.* **96**, 0981031–0981034.
- Blawas, A. S., and Reichert, W. M. (1998). Protein patterning. *Biomaterials* **19**, 595–609.
- Bohanon, T., Elender, G., Knoll, W., Koberle, P., Lee, J. S., Offenhausser, A., Ringsdorf, H., Sackmann, E., Simon, J., Tovar, G., and Winnik, F. M. (1996). Neural cell pattern formation on glass and oxidized silicon surfaces modified with poly(N-isopropylacrylamide). *J. Biomater. Sci. Polym. Ed.* **8**, 19–39.
- Branch, D. W., Wheeler, B. C., Brewer, G. J., and Leckband, D. E. (2000). Long-term maintenance of patterns of hippocampal pyramidal cells on substrates of polyethylene glycol and microstamped polylysine. *IEEE Trans. Biomed. Eng.* **47**, 290–300.
- Brown, M. E., and Bridgman, P. C. (2003). Retrograde flow rate is increased in growth cones from myosin IIB knockout mice. *J. Cell Sci.* **116**(Pt. 6), 1087–1094.
- Buck, K. B., and Zheng, J. Q. (2002). Growth cone turning induced by direct local modification of microtubule dynamics. *J. Neurosci.* **22**, 9358–9367.
- Challacombe, J. F., Snow, D. M., and Letourneau, P. C. (1996). Actin filament bundles are required for microtubule reorientation during growth cone turning to avoid an inhibitory guidance cue. *J. Cell Sci.* **109**, 2031–2040.
- Chen, X. L., Zhong, Z. G., Yokoyama, S., Bark, C., Meister, B., Berggren, P. O., Roder, J., Higashida, H., and Jeromin, A. (2001). Overexpression of rat neuronal calcium sensor-1 in rodent NG108–15 cells enhances synapse formation and transmission. *J. Physiol.* **532**, 649–659.
- Chien, C. B., Rosenthal, D. E., Harris, W. A., and Holt, C. E. (1993). Navigational errors made by growth cones without filopodia in the embryonic *Xenopus* brain. *Neuron* **11**, 237–251.
- Chierzi, S., Ratto, G. M., Verma, P., and Fawcett, J. W. (2005). The ability of axons to regenerate their growth cones depends on axonal type and age, and is regulated by calcium, camp and ERK. *Eur. J. Neurosci.* **21**, 2051–2062.
- Clark, P., Britland, S., and Connolly, P. (1993). Growth cone guidance and neuron morphology on micropatterned laminin surfaces. *J. Cell Sci.* **105**, 203–212.
- Dent, E. W., and Kalil, K. (2001). Axon branching requires interactions between dynamic microtubules and actin filaments. *J. Neurosci.* **21**, 9757–9769.
- Dewez, J. L., Lhoest, J. B., Detrait, E., Berger, V., Dupont-Gillain, C. C., Vincent, L. M., Schneider, Y. J., Bertrand, P., and Rouxhet, P. G. (1998). Adhesion of mammalian cells to polymer surfaces: From physical chemistry of surfaces to selective adhesion of defined patterns. *Biomaterials* **19**, 1441–1445.
- Diefenbach, T. J., Latham, V. M., Yimlamai, D., Liu, C. A., Herman, I. M., and Jay, D. G. (2002). Myosin 1c and myosin IIB serve opposing roles in lamellipodial dynamics of the neuronal growth cone. *J. Cell Biol.* **158**, 1207–1217.

- Ehrlicher, A., Betz, T., Stuhrmann, B., Koch, D., Milner, V., Raizen, M. G., and Kas, J. (2002). Guiding neuronal growth with light. *Proc. Natl. Acad. Sci. USA* **99**, 16024–16028.
- Forscher, P., and Smith, S. J. (1988). Actions of cytochalasins on the organization of actin filaments and microtubules in the growth cone. *J. Cell Biol.* **107**, 1505–1516.
- Franze, K., Grosche, J., Skatchkov, S. N., Schinkinger, S., Schild, D., Uckermann, O., Travis, K., Reichenbach, A., and Guck, J. (2007). Spotlight on glial cells: Living optical fibers in the vertebrate retina. *Proc. Natl. Acad. Sci. USA* (in press).
- Fromherz, P., Schaden, H., and Vetter, T. (1991). Guided outgrowth of leech neurons in culture. *Neurosci. Lett.* **129**, 77–80.
- Furnish, E. J., Zhou, W., Cunningham, C. C., Kas, J. A., and Schmidt, C. E. (2001). Gelsolin overexpression enhances neurite outgrowth in PC12 cells. *FEBS Lett.* **508**, 282–286.
- Gordon-Weeks, P. R. (1991). Evidence for microtubule capture by filopodial actin filaments in growth cones. *Neuroreport* **2**, 573–576.
- Greene, L. A., and Tischler, A. S. (1976). Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proc. Natl. Acad. Sci. USA* **73**, 2424–2428.
- Grier, D. G., and Roichman, Y. (2006). Holographic optical trapping. *Appl. Opt.* **45**, 880–887.
- Hamprecht, B., Glaser, T., Reiser, G., Bayer, E., and Propst, F. (1985). Culture and characteristics of hormone responsive neuroblastoma X glioma hybrid cells. *Methods Enzymol.* **109**, 316–341.
- Heidemann, S. R., Lamoureux, P., and Buxbaum, R. E. (1990). Growth cone behavior and production of traction force. *J. Cell Biol.* **111**, 1949–1957.
- Herbert, C. B., McLernon, T. L., Hypolite, C. L., Adams, D. N., Pikus, L., Huang, C. C., Fields, G. B., Letourneau, P. C., Distefano, M. D., and Hu, W. S. (1997). Micropatterning gradients and controlling surface densities of photoactivatable biomolecules on self-assembled monolayers of oligo(ethylene glycol) alkanethiolates. *Chem. Biol.* **4**, 731–737.
- Ingvar, S. (1920). Reactions of cells the galvanic current in tissue culture. *Proc. Soc. Exp. Biol. Med.* **17**, 198–199.
- Jay, D. G. (2000). The clutch hypothesis revisited: Ascribing the roles of actin-associated proteins in filopodial protrusion in the nerve growth cone. *J. Neurobiol.* **44**, 114–125.
- Jurado, C., Haserick, J. R., and Lee, J. (2005). Slipping or gripping? Fluorescent speckle microscopy in fish keratocytes reveal two different mechanisms for generating a retrograde flow of actin. *Mol. Biol. Cell* **16**, 507–518.
- Kabir, N., Schaefer, A. W., Nakhost, A., Sossin, W. S., and Forscher, P. (2001). Protein kinase C activation promotes microtubule advance in neuronal growth cones by increasing average microtubule growth lifetimes. *J. Cell Biol.* **152**, 1033–1044.
- Kaverina, I., Krylyshkina, O., and Small, J. V. (2002). Regulation of substrate adhesion dynamics during cell motility. *Intl. J. Biochem. Cell Biol.* **34**, 746–761.
- Livesey, F. J. (1999). Netrins and netrins receptors. *Cell Mol. Life Sci.* **56**, 62–68.
- Lu, Y. B., Franze, K., Seifert, G., Steinhauser, C., Kirchhoff, F., Wolburg, H., Guck, J., Janmey, P., Wei, E. Q., Kas, J., and Reichenbach, A. (2006). Viscoelastic properties of individual glial cells and neurons in the CNS. *Proc. Natl. Acad. Sci. USA* **103**, 17759–17764.
- Martenson, C., Stone, K., Reedy, M., and Sheetz, M. (1993). Fast axonal transport is required for growth cone advance. *Nature* **366**, 66–69.
- McCaig, C. D., and Rajinick, A. M. (1991). Electrical fields, nerve growth and nerve regeneration. *Exp. Physiol.* **76**, 473–494.
- Medeiros, N. A., Burnette, D. T., and Forscher, P. (2006). Myosin II functions in actin-bundle turnover in neuronal growth cones. *Nat. Cell Biol.* **8**, 215–216.
- Merz, M., and Fromherz, P. (2002). Polyester microstructures for topographical control of outgrowth and synapse formation of snail neurons. *Adv. Mater.* **14**, 141–144.
- Mohanty, S. K., Sharma, M., Panicker, M. M., and Gupta, P. K. (2005). Controlled induction, enhancement, and guidance of neuronal growth cones by use of line optical tweezers. *Opt. Lett.* **30**, 2596–2598.

- Mogilner, A., and Oster, G. (2003). Force generation by actin polymerization II: The elastic ratchet and tethered filaments. *Biophys. J.* **84**, 1591–1605.
- Patel, N., Padera, R., Sanders, G. H., Cannizzaro, S. M., Davies, M. C., Langer, R., Roberts, C. J., Tendler, S. J., Williams, P. M., and Shakesheff, K. M. (1998). Spatially controlled cell engineering on biodegradable polymer surfaces. *FASEB J.* **12**, 1447–1454.
- Patel, N., and Poo, M. M. (1982). Orientation of neurite growth by extracellular fields. *J. Neurosci.* **2**, 483–496.
- Peterman, E. J., Gittes, F., and Schmidt, C. F. (2003). Laser-induced heating in optical traps. *Biophys. J.* **84**(2 Pt 1), 1308–1316.
- Ponti, A., Machacek, M., Gupton, S. L., Waterman-Storer, C. M., and Danuser, G. (2004). Two distinct actin networks drive the protrusion of migrating cells. *Science* **305**, 1782–1786.
- Rivers, T. J., Hudson, T. W., and Schmidt, C. E. (2002). Synthesis of a novel, biodegradable electrically conducting polymer for biomedical applications. *Adv. Funct. Mater.* **12**, 33–37.
- Rosoff, W. J., Urbach, J. S., Esrick, M. A., McAllister, R. G., Richards, L. J., and Goodhill, G. J. (2004). A new chemotaxis assay shows the extreme sensitivity of axons to molecular gradients. *Nat. Neurosci.* **7**, 678–682.
- Rudolph, T., Zimmer, K., and Betz, T. (2006). Microstructuring of UV-transparent functionalized films on glass by excimer laser irradiation. *Mater. Sci. Eng. C* **26**(5–7), 1131–1135.
- Schaefer, A. W., Kabir, N., and Forscher, P. (2002). Filopodia and actin arcs guide the assembly and transport of two populations of microtubules with unique dynamic parameters in neuronal growth cones. *J. Cell Biol.* **158**, 139–152.
- Schmidt, C. E., Shastri, V. R., Vacanti, J. P., and Langer, R. (1997). Stimulation of neurite outgrowth using an electrically conducting polymers. *Proc. Natl. Acad. Sci. USA* **94**, 8948–8953.
- Stevenson, D. J., Lake, T. K., Agate, B., Garces-Chavez, V., Dholakia, K., and Gunn-Moore, F. (2006). Optically guided growth at near infrared wavelengths. *Opt. Express* **14**, 9786–9793.
- Stuhrmann, B., Goegler, M., Betz, T., Ehrlicher, A., Koch, D., and Kas, J. (2005). Automated tracking and laser micromanipulation of cells. *Rev. Sci. Instr.* **76**, 0351051–8.
- Stuhrmann, B., Jahnke, H.-G., Schmidt, M., Jähn, K., Betz, T., Müller, K., Rothermel, A., Käs, J., and Robitzki, A. A. (2006). A versatile optical manipulation system for inspection, laser processing, and isolation of individual living cells. *Rev. Sci. Instrum.* **77**, 0631161–11.
- Svoboda, K., and Block, S. (1994). Biological applications of optical forces. *Annu. Rev. Biophys. Biomol. Struct.* **23**, 247–285.
- Takezawa, T., Mori, Y., and Yoshizato, K. (1990). Cell culture on a thermo-responsive polymer surface. *Biotechnology* **8**, 854–856.
- Theriot, J. A. (2000). The polymerization motor. *Traffic* **1**, 19–28.
- Valentini, R. F. (1995). “The Biomedical Engineering Handbook.” CRC, Boca Raton, FL.
- Visscher, K., Gross, S. P., and Block, S. M. (1996). Construction of multiple-beam optical traps with nanometer-resolution position sensing. *IEEE J. Select. Topics Quantum Electron.* **2**, 1066–1076.
- Vogt, A. K., Lauer, L., Knoll, W., and Offenhausser, A. (2003). Micropatterned substrates for the growth of functional neuronal networks of defined geometry. *Biotechnol. Prog.* **19**, 1562–1568.
- Wakatsuki, T., Schwab, B., Thompson, N. C., and Elson, E. L. (2001). Effects of cytochalasin D and latrunculin B on mechanical properties of cells. *J. Cell Sci.* **114**, 1025–1036.
- Walsh, F. S., Meiri, K., and Doherty, P. (1997). Cell signaling and CAM-mediated neurite outgrowth. *Soc. Gen. Physiol. Ser.* **52**, 221–226.
- Walter, J., Henke-Fahle, S., and Bonhoeffer, F. (1987). Avoidance of posterior tectal membranes by temporal retinal axons. *Development* **101**, 909–913.
- Wang, F. S., Liu, C. W., Diefenbach, T. J., and Jay, D. G. (2003). Modeling the role of myosin 1c in neuronal growth cone turning. *Biophys. J.* **85**, 3319–3328.
- Williams, R. S., Cheng, L., Mudge, A. W., and Harwood, A. J. (2002). A common mechanism of action for three mood-stabilizing drugs. *Nature* **417**, 292–295.

- Williamson, T., Gordon-Weeks, P. R., Schachnet, M., and Taylor, J. (1996). Microtubule reorganization is obligatory for growth cone turning. *Proc. Natl. Acad. Sci. USA* **93**, 15221–15226.
- Yamada, N., Okano, T., Sakai, H., Karikusa, F., and Sawasaki, Y. (1990). Thermo-responsive polymeric surfaces: Control of attachment and detachment of cultured cells. *Makromol. Chem. Rapid. Commun.* **11**, 571–576.
- Zheng, J., Lamoureux, P., Santiago, V., Dennerl, T., Buxbaum, R. E., and Heidemann, S. R. (1991). Tensile regulation of axonal elongation initiation. *J. Neurosci.* **11**, 1117–1125.