Two-Photon Fluorescent Microlithography for Live-Cell Imaging

SANTIAGO COSTANTINO,[1]* KATRIN G. HEINZE,[1] OSCAR E. MARTÍNEZ,[2]
PAUL DE KONINCK,[3] AND PAUL W. WISEMAN[1,4]
[1]*Department of Physics, McGill University, Montréal, Québec, Canada*
[2]*Laboratorio de Electrónica Cuántica, Departamento de Física, FCEyN, Universidad de Buenos Aires, Buenos Aires, Argentina*
[3]*Département de Biochimie et de Microbiologie, Université Laval, Centre de Recherche Université Laval Robert-Giffard, Québec, Canada*
[4]*Department of Chemistry, McGill University, Montréal, Québec, Canada*

KEY WORDS fluorescent lithography; fluorescent fiduciary marks; microcontact printing; nonlinear optical lithography

ABSTRACT Fluorescent dyes added to UV-cure resins allow the rapid fabrication of fluorescent micropatterns on standard glass coverslips by two-photon optical lithography. We use this lithographic method to tailor fiduciary markers, focal references, and calibration tools, for fluorescence and laser scanning microscopy. Fluorescent microlithography provides spatial landmarks to quantify molecular transport, cell growth and migration, and to compensate for focal drift during time-lapse imaging. We show that the fluorescent patterned microstructures are biocompatible with cultures of mammalian cell lines and hippocampal neurons. Furthermore, the high-relief topology of the lithographed substrates is utilized as a mold for poly(dimethylsiloxane) stamps to create protein patterns by microcontact printing, representing an alternative to the current etching techniques. We present two different applications of such protein patterns for localizing cell adhesion and guidance of neurite outgrowth. *Microsc. Res. Tech. 68:272–276, 2005.*

INTRODUCTION

The rapid development of organic and genetically encoded fluorescent dyes, combined with technical innovations in photonics and microscopy, has revolutionized the field of cellular imaging. These tools allow the monitoring of cell growth and migration, subcellular structures and organelles, proteins, DNA, RNA, and even ions, in a dynamic fashion. Surprisingly, however, methods to make fiduciary markers that can be used during fluorescence imaging have been lacking. For example, accurate measurements of motility (Ridley et al., 2003), cytoskeletal dynamics, and vesicular transport in nerve cells over the short and long term (Shah and Cleveland, 2002) are often hampered by the lack of suitable fluorescent references in the field of view and in the *z*-plane, during focal drift. Some studies have relied on randomly locating fluorescent spots, which appeared immobile throughout the duration of the time-lapse imaging, to act as fiduciary references. To produce fixed fluorescent markers with custom dimensions, we extend a two-photon-based lithography approach (Maruo et al., 1997; Sun et al., 2001) using a femtosecond laser, microscope optics, and light-curing adhesive mixed with different fluorescent dyes.

The use of photopolymerizable resins has been proposed to create microoptical and micromechanical structures, including photonic bandgap-type materials, optical storage devices, and cantilever arrays (Cumpston et al., 1999). Furthermore, it has been shown that such adhesives are an accessible alternative in the formation of complex micromachines for laser tweezers manipulation (Galajda and Ormos, 2001). In this study, we create fluorescent structures on standard glass coverslips by moving a diffraction-limited laser spot along a glass surface, covered with a commercially available UV-cure resin. As a consequence of two-photon nonlinear photopolymerization, the material is only solidified in the direct vicinity of the focal spot with micrometer resolution.

We show that the polymerized structures containing a fluorescent dye of choice can be used in combination with adherent cells and serve as fluorescent fiduciary markers directly on the glass substrate. To our knowledge, we present the first biological application of this type of fabricated structures, including its use in primary neuronal cell culture.

Moreover, the high-relief topology of the lithographed substrates can be used as molds for poly(dimethylsiloxane) (PDMS) stamps to spatially pattern proteins. Custom protein distributions created by microcontact printing (μCP) (Chen et al., 1997; Singhvi et al., 1994) have been shown to be useful for studying the mechanisms of cell signaling, adhesion, migration, and neurite outgrowth (Lehnert et al., 2004; Thomas et al., 2002). In μCP, these stamps are first "inked" with a solution of molecules (often proteins or thiols), and

*Correspondence to: Santiago Costantino, Department of Physics, McGill University, 3600 University Street, Montréal, Québec, Canada H3A2T8.
E-mail: santiago.costantino@mcgill.ca

Received 10 August 2005; accepted in revised form 23 August 2005

Contract grant sponsors: Natural Sciences and Engineering Research Council of Canada, The Canadian Foundation for Innovation, The Canadian Institutes of Health Research, and The International Human Frontiers Science Program.

S.C. and K.G.H. contributed equally to this work.

DOI 10.1002/jemt.20247

Published online in Wiley InterScience (www.interscience.wiley.com).

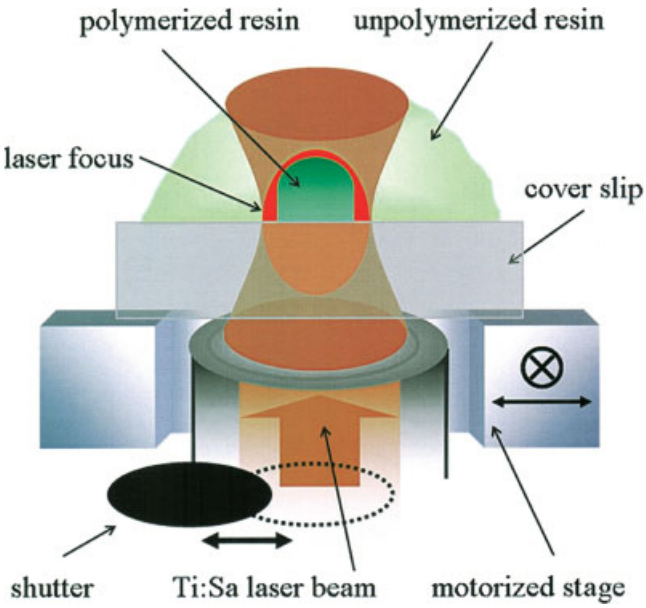

Fig. 1. Schematic of the setup. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

then brought into contact with a substrate. The fabrication of the stamps is usually performed using etched silicon wafers as masters. In this work, we create stamps using masters made by two-photon lithography, which simplifies and accelerates the production.

The applications that we present, comprising fluorescent spatial references as well as masters for μCP, illustrate the range and versatility of this lithographic technique for quantitative live-cell microscopy studies.

MATERIALS AND METHODS

Experimental Setup and Microlithographic Procedure

The experimental setup is based on a Ti:Sa laser, a high-NA objective lens and a movable stage. The lithographic process involves a simple three-step procedure. First, on a glass coverslip, we place a drop of a mixture of a UV-curing adhesive NOA60 (Norland Products, Cranbury, NJ) and a fluorescent dye of choice dissolved in ethanol. Second, to induce polymerization by two-photon absorption, a Ti:Sa laser (100 fs pulses, 750 nm peak wavelength) is focused through a 1.2 NA objective lens (UPlanAPO/IR 60×, NA 1.2, water immersion, Olympus) at the interface between the coverslip and the liquid adhesive/dye mixture as depicted in Figure 1. Moving the sample with respect to the laser focus using two motorized translation stages creates a tailor-made two-dimensional pattern of polymerized material on the coverslip surface. Finally, rinsing with acetone and ethanol solutions removes the unpolymerized adhesive, while leaving the cured glass-like fluorescent pattern attached to the cover glass.

Precise positioning of the laser focus is accomplished by monitoring the intensity of the back-reflection of the cover glass in a confocal setup. A mechanical shutter (Newport, Mountain View, CA) provides the flexibility to pattern intricate designs. Motorized translation stages (Thorlabs, Newton, NJ) were set up, to move the coverslip with respect to the stationary laser and provide accurate control of the translation velocity and position. The motors and the shutter were controlled using a PC, with custom-written LabVIEW 7.0 (National Instruments, Austin, TX) programs and drivers.

Cell Culture

Hippocampal cultures from neonatal rats were prepared as described earlier (Hudmon et al., 2005). The hippocampal cells were grown at high density (300–600 cells/mm^2) for the experiments on fluorescent grids, and at low density (50–100 cells/mm^2) for the experiments of neurite outgrowth (Fig. 4). The grid coverslips were coated with poly-D-lysine (250 μg/mL; BD Biosciences). The growth medium consisted of a neurobasal medium, supplemented with B27, Glutamax (500 mM, Gibco, Carlsbad, CA), penicillin/streptomycin (50 U mL^{-1}/50 mg mL^{-1}, Gibco), and cytosine arabinoside (5 mM, from day 2 to 5, Gibco).

CHO K1 eGFP-α5 integrin were prepared as previously described (Knight et al., 2000) and provided by A.F. Horwitz (University of Virginia). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% FBS, 4 mM L-glutamine, 100 U/mL penicillin, 0.1 mg/mL streptomycin, 0.1 mM nonessential amino acids, and 0.5 mg/mL G418, to maintain transfection (Gibco). Cells were detached by trypsin, and dissociated cells were washed in DMEM for 3 min, centrifuged, and seeded on the patterned substrata in DMEM (ca. 100 cells/mm^2). For the adhesion experiments on the fibronectin patterns, cells were cultured for 1 h on the substrate, and then fixed with a solution of 4% paraformaldehyde in phosphate buffer, before imaging.

Both cell types were maintained in a humidified (5% CO_2) atmosphere at 37°C.

Transfection

Ten-day old neuronal cultures were transfected as described (Hudmon et al., 2005), with eGFP (BD Biosciences), using lipofectamine 2000 (Invitrogen, Carlsbad, CA) and maintained for 2 more days in culture. Cells were then fixed for 10 min with a solution of 4% paraformaldehyde, 0.1 M PB (pH 7.4), and 16% sucrose. Cells on glass coverslips were mounted on glass slides in ProLong Gold antifade reagent (Molecular Probes, Carlsbad, CA). Fluorescence images were acquired using an Olympus FV300 laser scanning microscope (LSM) and a 60× (1.4 NA) oil-immersion objective (Olympus, Melville, NY). Two excitation wavelengths were used: 488 nm-Argon and 633 nm-He-Ne and proper emission and excitation filters.

Immunostaining

Hippocampal cells were fixed as described earlier, washed three times, for 5 min, with phosphate buffered saline (PBS) containing 0.1% Triton X-100, and incubated with mouse anti MAP-2 (microtubule-associated protein-2) antibody (1:10,000, HM-2 Sigma, Oakville, ON) for 1 h at room temperature, or overnight at 4°C. Cells were then washed three times for 5 min with PBS and incubated for 1 h at room temperature with goat anti-mouse Alexa 488 (1:1,000; Molecular Probes). Cells were mounted for imaging using a LSM (FV300, Olympus) as described earlier.

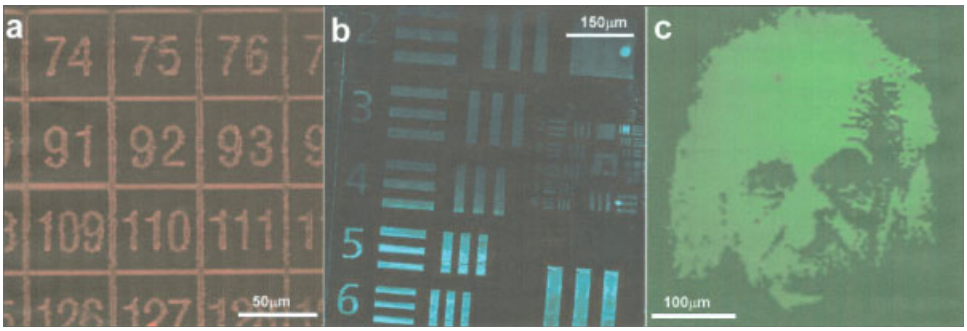

Fig. 2. (**a**) A fluorescent numbered square grid for cell counting. (**b**) Fluorescent reproduction of a USAF resolution test target. (**c**) A 1-bit color depth fluorescent portrait of A. Einstein. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Microcontact Printing

Masters for μCP were fabricated by two-photon lithography and silicone stamps were then cast. The complementary topology is transferred from the lithographed master to the PDMS stamp, and the molds can be reused to generate a large number of stamps. In this routine, 2–3 mL of viscous elastomer kit (Sylgard 184 (Dow Corning)) with a mixing ratio of 10:1 of component A/B (A, silicone prepolymer; B, curing agent) were poured onto the mold and heated for 30 min at 120°C. Afterward, the hardened elastomer was peeled-off the master cover glass. Detailed protocols for achieving the desired mechanical properties of the elastomer can be found elsewhere (Bernard et al., 2000).

Protein solutions of 20 mg/mL fibronectin (Gibco) in PBS for CHO cells, and 250 μg/mL poly-D-lysine for the hippocampal neurons were applied on the PDMS stamps and incubated for 1 h at 4°C. The protein solutions were mixed with fluorescent markers for visualization of the pattern to be printed: Alexa647-fibrinogen (Molecular Probes) was mixed with the fibronectin, and GFP with the poly-D-lysine. After incubation, the stamps were rinsed with PBS, blown dry, and printed onto clean glass coverslips. For the CHO cells, the coverslips were additionally blocked with 1% bovine serum albumin (Sigma) in DMEM (Gibco), before plating.

RESULTS

Fiduciary Marks and Focal References for Fluorescence Microscopy

The lithographic technique allows us to create fluorescent grid structures, or any arbitrary design on standard glass coverslips, using ultrafast laser tools and microscope optics. The procedure typically takes between 2 and 20 min, depending on the complexity of the pattern. While the nonlinear absorption in combination with the high NA of the objective lens confines the polymerization to a femtoliter volume, the minimum size of the structures generated is a function of the laser power and the translation velocity of the coverslip relative to the laser focus. Calibration measurements on various sample patterns show that a constant translation velocity of 300 μm/s and ~1.5 MW/cm^2 laser-power density in the focal spot (corresponding to 20 mW of mean laser power before the objective lens in our experimental setup) are required to achieve dimensions of ~2 μm in diameter and height.

Optimally, the fluorophore has an excitation peak between the laser line and its second harmonic, as this minimizes photobleaching effects during the polymerization of the adhesive. A large majority of the commercially available fluorescent dyes fulfills this requirement. We have successfully embedded fluorescein (Fluka), ADS675MT (American Dye Source), rhodamine 6G chloride (Molecular Probes) and 10-nm-radius fluorescent beads (Molecular Probes) in resins NOA60 and NOA61 (Norland) and generated robust patterns with high fluorescence yields.

The two-dimensional pattern is custom-designed, and the maximum size of the pattern on the coverslip is only limited by the total travel range of the motorized translation system. To create accurate patterns, the direct current motors we used have built-in optical encoders, with a large number of counts per revolution to ensure submicron positioning. In practice, there is a laser intensity window between the power required to initiate the polymerization process and a damage intensity threshold (typically between 0.5 and 10 MW/cm^2), which depends on the dye concentration in the resin.

In Figure 2, three examples of fluorescent patterns made with dye ADS675MT are shown in false color. The chosen designs represent fluorescent reproductions of patterns typically etched in glass or made by metallic deposition. The numbered grid (Fig. 2a) provides a way of counting cells, using fluorescence microscopy that is complementary to conventional phase contrast methods, but additionally permits quantification of fluorescence transfection efficiency. A portrait of Albert Einstein (Fig. 2c) on a micron scale demonstrates the versatility of the method.

Similar fabricated fluorescent microstructures can then be used as fiduciary or calibration markers for live-cell imaging. To test the biocompatibility of these patterned dye–polymer mixtures, we generated red fluorescent grids on coverslips, and used them as substrates for cultures of CHO-K1 cells stably transfected with EGFP-α5 integrin fusion protein (Fig. 3a), as well as primary cultures of rat hippocampal neurons (Figs. 3b–3c). Cover glasses for neuronal cultures were additionally coated with poly-D-lysine and UV-light-sterilized after the lithographic procedure. Neither the CHO cells (2 days in vitro (DIV)), nor the neurons (21 DIV) showed any visible difference in their viability and morphology compared to those plated on coverslips without fluorescent patterns. The neurons extended their processes to the same degree as they normally do on untreated coverslips and could also be transfected with eGFP (Fig. 3c). These results show that various cell types grow normally on the fluorescent lithographed coverslips.

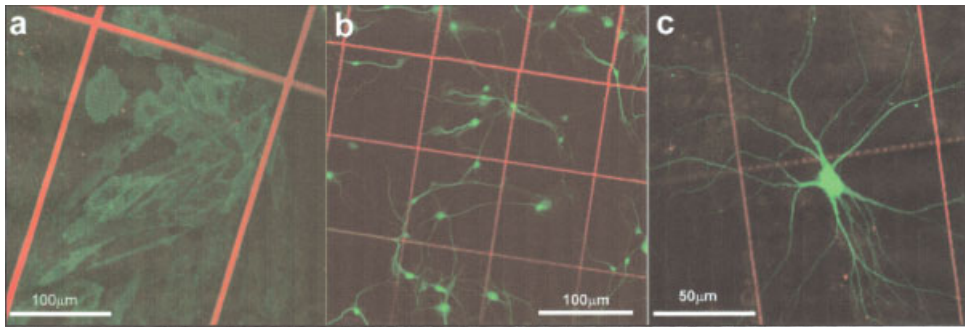

Fig. 3. Two-color LSM images of cells on red fluorescent grids, using ADS675MT as dye. (**a**) CHO cells expressing eEGFP-α5 integrin. (**b**) Rat hippocampal neurons (10DIV) immunostained for MAP-2 to reveal somato-dendritic compartments. (**c**) Rat hippocampal neuron (12 DIV) transfected with eGFP. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

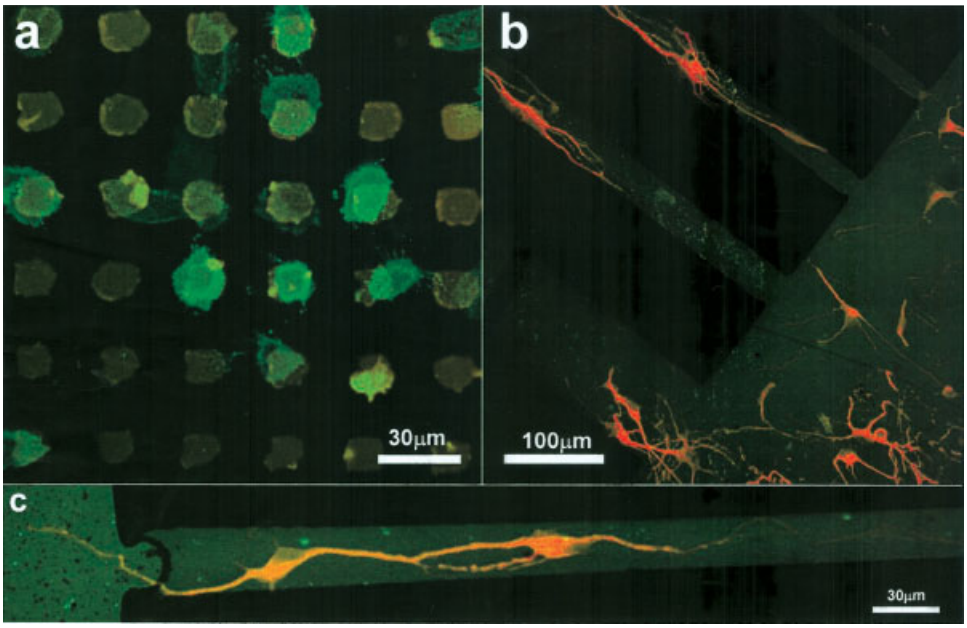

Fig. 4. Dual-color confocal images. (**a**) CHO cells expressing eGFP-α5 integrin plated on a patterned substrate with a mixture of fibronectin and Alexa633-human fibrinogen. (**b**) Pattern of poly-D-lysine+eGFP on a glass substrate, allowing rat hippocampal neurons (immunostained for MAP-2) to grow their neurites in specific corridors. (**c**) Higher resolution image of two neurons. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Thus, these substrates can serve as external coordinate frames of reference for time-lapse studies. They provide suitable fluorescent fiduciary marks in the field of view so as to make proper distance quantifications. In addition, these types of fluorescent structures may serve as references to correct for the focal drift during image-time-series acquisition.

Molds for μCP

An entirely different application of the high-relief topology of the lithographed substrates is as molds for μCP. For this application, the inclusion of fluorophores in the resin is not necessary, but allows easy visual testing of the master samples under a fluorescence microscope.

We generated PDMS stamps with two different designs by using our lithographed patterns on cover glasses as masters. The protein-patterned cover glasses were used as substrates in cell culture as is described in the Materials and Methods. Figure 4a shows patterns of fibronectin mixed with Alexa633-fibrinogen, acting as small islands of adhesive substrate for individual CHO cells. Figure 4b depicts patterns of poly-D-lysine (mixed with GFP for visualization), which guide and confine neurite outgrowth from neonatal hippocampal neurons (4 DIV) in the defined corridors. This approach can be used for single-cell selection to generate clonal cell lines for neuronal microcultures, or to probe the role of extracellular matrix proteins for nerve regeneration studies and cell migration.

CONCLUSIONS

We have created fluorescent patterns with micrometer dimensions on standard glass coverslips. We showed that these substrates are compatible with long-term cell culture, and can be used as passive markers for fluorescence microscopy or as active devices for cell guidance or containment.

For fluorescence microscopy, this approach nicely complements conventional etching on glass coverslips that is used to make calibration standards for phase and differential interference contrast light microscopy. Furthermore, these fluorescent markers of defined shape, size, and emission wavelength on coverslips provide spatial references to correct for focal drift during

image-time-series acquisition. The fluorescence and topology of these markers should also prove useful for calibrating hybrid systems that combine scanning probe microscopy and fluorescence detection and for near-field scanning optical microscopy.

The flexibility of the technique is such that it can be implemented on commercial two-photon microscopes, with only ad hoc modifications to the galvanometric scanning system control software. Moreover, the material cost per sample is negligible.

The use of our microlithographed substrates in μCP provides a new way to achieve protein patterns. The combination of rapid fabrication and minimal cost should broaden the access of μCP to researchers for a wide range of applications.

Microlithography to tailor structures with defined fluorescence and topology has not yet been exploited for biophysical applications. Our study shows the proof of principle and demonstrates the power of the technique.

ACKNOWLEDGMENTS

We thank Dr. T. Kennedy and S. Rodrigues (Montréal Neurological Institute) for contributing to preliminary tests, F. Nault (Université Laval) for neuronal culture support, and Dr. R. Horwitz (University of Virginia) for providing the CHO cell line.

P.D.K. was supported by a Career Award from the Fond de la Recherche en Santé du Québec. S.C. was supported by the Neurophysics CIHR Strategic Training Program Grant. K.G.H. was supported by the Max Planck Society and a CIHR NET grant.

REFERENCES

Bernard A, Renault JP, Michel B, Bosshard HR, Delamarche E. 2000. Microcontact printing of proteins. Adv Mater 12:1067–1070.

Chen CS, Mrksich M, Huang S, Whitesides GM, Ingber DE. 1997. Geometric control of cell life and death. Science 276: 1425–1428.

Cumpston BH, Ananthavel SP, Barlow S, Dyer DL, Ehrlich JE, Erskine LL, Heikal AA, Kuebler SM, Lee IYS, McCord-Maughon D, Qin J, Röckel H, Rumi M, Wu XL, Marder SR, Perry JW. 1999. Two-photon polymerization initiators for three-dimensional optical data storage and microfabrication. Nature 398:51–54.

Galajda P, Ormos P. 2001. Complex micromachines produced and driven by light. Appl Phys Lett 78:249–251.

Hudmon A, LeBel E, Roy H, Schulman H, Waxham N, De Koninck P. 2005. A mechanism for Ca^{2+}/calmodulin-dependent protein kinase II clustering at synaptic and non-synaptic sites based on self-association. J. Neurosci 25:6971–6983.

Knight E, Laukitisa C, Akhtarb N, Hotchinb NA, Edlunde M, Horwitz AR. 2000. Visualizing muscle cell migration in situ. Curr Biol 10: 576–585.

Lehnert D, Wehrle-Haller B, David C, Weiland U, Ballestrem C, Imhof BA, Bastmeyer M. 2004. Cell behaviour on micropatterned substrata: limits of extracellular matrix geometry for spreading and adhesion. J Cell Sci 117:41–52.

Maruo SO, Nakamura O, Kawata K. 1997. Three-dimensional microfabrication with two-photon absorbed photopolymerization. Opt Lett 22:132–134.

Ridley AJ, Schwartz MA, Burridge K, Firtel RA, Ginsberg MH, Borisy G, Parsons JT, Horwitz AR. 2003. Cell migration: integrating signals from front to back. Science 302:1704–1709.

Shah SV, Cleveland DW. 2002. Slow axonal transport: fast motors in the slow lane. Curr Opin Cell Biol 14:58–62.

Singhvi R, Kumar A, Lopez GP, Stephanopoulos GN, Wang DI, Whitesides GM, Ingber DE. 1994. Engineering cell shape and function. Science 264:696–698.

Sun HB, Tanaka T, Takada K, Kawata S. 2001. Two-photon photopolymerization and diagnosis of three-dimensional microstructures containing fluorescent dyes. Appl Phys Lett 79:1411–1413.

Thomas CH, Collier JH, Sfeir CS, Healy KE. 2002. Engineering gene expression and protein synthesis by modulation of nuclear shape. Proc Natl Acad Sci USA 99:1972–1977.