Guiding neuronal development with *in situ* microfabrication

Bryan Kaehr*, Richard Allen*, David J. Javier, John Currie, and Jason B. Shear†

Department of Chemistry and Biochemistry and The Institute for Cellular and Molecular Biology, University of Texas, 1 University Station A5300, Austin, TX 78712

Communicated by Norman Hackerman, The Robert A. Welch Foundation, Houston, TX, September 29, 2004 (received for review June 1, 2004)

We report the ability to modify microscopic 3D topographies within dissociated cultures, providing a means to alter the development of neurons as they extend neurites and establish interconnections. In this approach, multiphoton excitation is used to focally excite noncytotoxic photosensitizers that promote protein crosslinking, such as BSA, into matrices having feature sizes ≥250 nm. Barriers, growth lanes, and pinning structures comprised of crosslinked proteins are fabricated under conditions that do not compromise the viability of neurons both on short time scales and over periods of days. In addition, the ability to fabricate functional microstructures from crosslinked avidin enables submicrometer localization of controllable quantities of biotinylated ligands, such as indicators and biological effectors. Feasibility is demonstrated for using *in situ* microfabrication to guide the contact position of cortical neurons with micrometer accuracy, opening the possibility for engineering well defined sets of synaptic interactions.

biofabrication | multiphoton cell patterning | growth cone

Studies of neuronal function increasingly rely on methods for precisely manipulating cellular properties. Innovations in electrophysiology, photolytic release of effectors, and inducible knockout technologies (1–3) have made it possible to explore cellular phenomena at levels of reduction few anticipated a quarter-century ago. Despite this technological revolution, approaches for influencing neuronal morphology, motility, and interconnectivity remain relatively primitive, a limitation of considerable importance to fundamental and applied neuroscience. An ability to prescribe the exact location at which an extending neurite makes contact with a target cell, or to constrain neuronal migration at a specific time point in development, would be of great value to studies of signal transduction and integration within individual cells and neural networks.

Neurite orientation and growth can be modified in real time by various stimuli, including diffusible neurotrophin gradients (4), electric fields (5), and near-IR light (6), but these approaches exert relatively coarse influences over neurite pathfinding and have not been used to accurately guide cellular interactions. Finer delimitation of neurite development can be achieved by using patterned surfaces and topologies microfabricated in silicon and other materials (7–9); such structures, however, must be prepared before cells are introduced for culture when the detailed features of neurite arborization cannot be known.

Multiphoton excitation provides an alternative approach for constructing 3D defined microscopic materials that, in principle, could be fabricated within cellular environments. Used extensively in 3D fluorescence imaging, multiphoton excitation also has proved useful for promoting photochemical reactions with high spatial and temporal control (10–13). Application of this strategy to ''direct-write'' material fabrication can be achieved by focusing light from a pulsed femtosecond laser to submicrometer dimensions, an approach that limits nonlinear excitation to subfemtoliter focal volumes. By translating this laser focus within a reagent solution containing an appropriate photoreactive chromophore, 3D plastic forms can be constructed rapidly (14, 15).

Although such chemistries are useful for creating exquisitely detailed structures, including microscopic farm animals (16), photopolymerization of synthetic materials has required use of nonaqueous solvents and strong radical initiators. In contrast, biological materials offer the possibility for aqueous-based photofabrication, a requisite for maintaining cell viability. Proteins are known to undergo crosslinking *in vivo* via numerous enzymatic and photonic pathways, processes that result in materials such as bone, connective tissue, and cataracts (17–19). Protein photocrosslinking, generally promoted by excitation of a photosensitizer by using UV or visible light, has been shown to proceed through both type I (free radical) and type II (singlet oxygen) mechanisms, depending on the excitation wavelength, photosensitizer, and accessible protein surface residues (20–22). Multiphoton fabrication of protein-based 3D microstructures in aqueous solution has been reported by Campagnola and coworkers (23), but has relied on the use of rose bengal, benzophenone, and other cytotoxic sensitizers. In this article, we describe the development and application of nontoxic multiphoton direct-write procedures, allowing the microscopic topographies of neuronal environments to be modified on the fly, as they develop. We demonstrate the feasibility for using this approach to guide contact positions of cortical neurons with micrometer accuracy.

Materials and Methods

Cell Culture. Neuroblastoma-glioma (NG108-15) cells were cultured in DMEM supplemented with 10% FBS, penicillin (100 mg/liter), and streptomycin (100 kilounits/liter). Flasks were maintained at 37°C in a 10% $C0_2$ atmosphere with saturated H_2O. For all experiments, cells were seeded on 0.01% poly-L-lysine-coated glass coverslips and incubated for 1–3 days in a low-serum (1% FBS) growth medium. In the periods immediately before and after microfabrication, cells were maintained in a supplemented pH 7.4 Hepes buffer (10 mM sodium Hepes/140 mM NaCl/5 mM KCl/1 mM $MgCl_2$/1 mM $CaCl_2$/10 mM D-glucose). This solution was further supplemented with 1 mM dibutyrl cyclic adenosine monophosphate immediately after the microfabrication process.

Rat brain cortical cells (embryonic days 18–19) were harvested by QBM Cell Science (Ottawa) and cultured according to standard procedures. Briefly, cryopreserved neurons were transferred to poly-L-lysine or uncoated flame-treated coverslips and incubated in neurobasal medium (Invitrogen) with L-glutamine, 1 unit/ml penicillin-streptomycin, and 2% B27 serum-free culture supplement. Microfabrication experiments were performed 1–3 days after plating.

Scanning Electron Microscopy Preparation. Samples were fixed in 4% gluteraldehyde/Hepes (vol/vol) solution for 30 min, dehydrated by using 10-min sequential washes [2:1 EtOH/H_2O; 2× 100% EtOH; 1:1 EtOH/hexamethyldisilazane (HMDS); 100%

*B.K. and R.A. contributed equally to this work.

†To whom correspondence should be addressed. E-mail: jshear@mail.utexas.edu.

www.pnas.org/cgi/doi/10.1073/pnas.0407204101

HMDS; all solutions vol/vol], allowed to air dry, and sputter-coated nominally to 12 nm with Au/Pd.

Matrix Fabrication. Two-photon excitation of photosensitizers provides 3D control over the photochemical reactions that result in protein crosslinking. In this approach, high peak power laser light is focused to submicrometer dimensions by using a high numerical aperture microscope objective; the nonlinear dependence of photosensitizer excitation on laser intensity restricts the reaction both radially (i.e., in the focal plane) and axially (i.e., along the optical axis), resulting in a protein crosslinking "voxel" (volume element) of <1 fl (1 μm^3). By translating the relative position of the voxel within crosslinking solution, a continuous matrix can be fabricated in the form of lines, platforms, walls, arcs, etc.

Fabrication of crosslinked BSA (Equitech-Bio, Kerrville, TX) structures was performed in Hepes buffer (pH 7.4) containing 1–4 mM FAD (Sigma, F-6625) and 50–200 mg/ml BSA. Cell exposure to this solution was ≈1 min or less, but longer periods do not appear to be detrimental to NG108-15 cells or cortical neurons. Protein lines were written on a Zeiss Axiovert microscope with a femtosecond titanium/sapphire laser (Coherent Mira, Santa Clara, CA); typically, the laser was tuned to 790 nm, but in some cases wavelengths as short as 740 nm were used. The laser output was adjusted to approximately fill the back aperture of a high-power objective (Zeiss ×100 Fluar, 1.3 numerical aperture, oil immersion); laser powers entering the microscope were 30–60 mW. Lines were written by scanning the position of the sample with a motorized (XY) stage (scan speed, ≈5 μm/s) and rectangular platforms were created by raster scanning the focused laser beam within the focal plane with a confocal scanner (BioRad MRC600) then manually altering the position of the coverslip relative to the focal point to extend the structures along the dimension of the optical axis. Microstructures comprised of proteins other than BSA were fabricated by using similar procedures. In some experiments that did not involve cells, methylene blue (250 μM) was used as a photosensitizer in place of FAD. Biotin binding of avidin matrices was assessed by applying fluorescein-biotin (B1370, Molecular Probes) to structures post-fabrication at a concentration of 1.2 μM, with labeled structures subjected to 10–15 rinses with Hepes (pH 7.4) to minimize nonspecific binding.

Results and Discussion

Multiphoton photocrosslinking can be used to create protein structures that have a broad range of chemical and morphological features (Fig. 1). It is straightforward, for example, to fabricate avidin matrices that display variable binding capacities (Fig. 1*a*), offering a means to localize tunable densities of biotinylated compounds, including indicators, enzymes, and recognition peptides. This ability to functionalize protein microstructures may prove valuable in a variety of cell biology applications, from chemical sensing to the creation of defined chemical gradients that could affect growth cone extension (24) or collapse (25). From a topographical standpoint, protein matrices may be fabricated that range dramatically in form and scale. Vertical cables <1 μm in diameter can be made that extend between surfaces separated by at least 150 μm (data not shown), and functional microscopic "banjo" strings comprised of crosslinked BSA (Fig. 1*b*) can be constructed by forming arcs that loop from a single surface. Far more complex structures, similar to those fabricated from synthetic polymers (16), should be feasible by using programmable scanning routines.

Because even subtle topographical cues can exert influence over cellular development via contact guidance, it was of particular interest to evaluate whether the simplest crosslinked protein matrices (low-profile lines) could be used to effect neurite elaboration and pathfinding. Studies with various materials demonstrate that chemically inert topographical features can have dramatic effects on neuronal outgrowth. Grooved quartz, for example, has been shown to effect the directionality of neurite outgrowth for grooves as shallow as 14 nm (26). In the

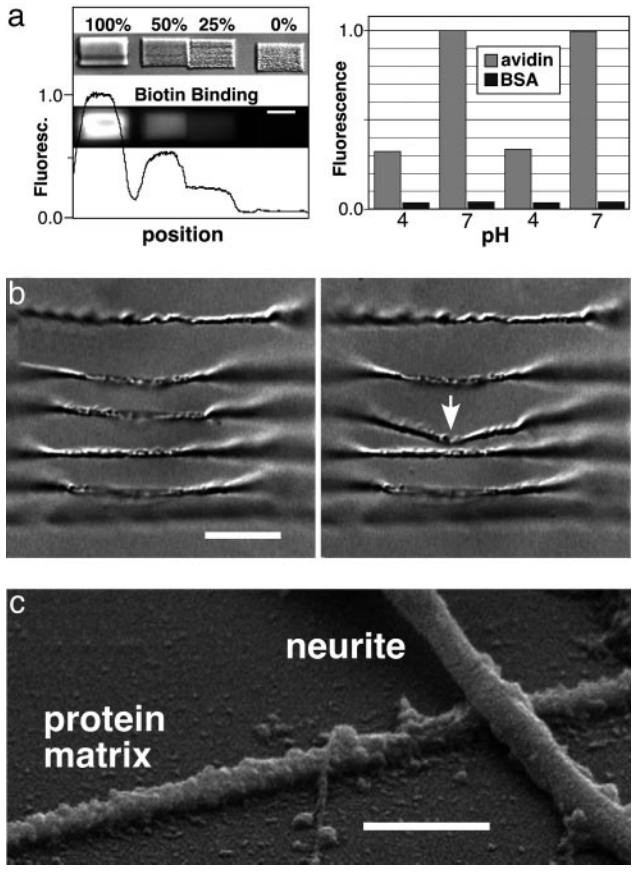

Fig. 1. Chemical and topographical features of microfabricated protein matrices. (*a*) Functional avidin microstructures. (*Left*) Biotin-binding capacity can be tuned by altering the composition of photocrosslinking solutions. The top strip shows differential interference contrast images of 3D structures produced by using solutions containing methylene blue and either 200 mg/ml avidin (i.e., 100% avidin), 100 mg/ml avidin plus 100 mg/ml BSA (50% avidin), 50 mg/ml avidin plus 150 mg/ml BSA (25% avidin), or 200 mg/ml BSA (0% avidin). After the structures were incubated in a fluorescein-biotin solution (1.2 μM) and rinsed, fluorescence imaging shows that photocrosslinked avidin retains the capacity to capture biotin (lower strip) with a binding capacity that scales essentially as the ratio of avidin in the fabrication solution. Plotted data represents signal along a horizontal line drawn through the four structures. (Scale bar: 10 μm.) (*Right*) Probing solution pH by using avidin microstructures. The matrices shown (*Left*) were subjected to cycling between acidic (pH 4 acetate) and neutral (pH 7 Hepes) solutions, causing reversible and reproducible modulation in the fluorescence intensity of fluorescein. Bar plots represent normalized signal from the 100% and 0% avidin structures, with signal averaged from (50 × 100 pixel) regions within the centers of the microstructures. From experiments in which two-photon fluorescence was used to probe labeled avidin lines (data not shown), biotin-binding capacities were estimated to be in the low- to mid-micromolar range (≈10^3 molecules per μm^2 of surface). (*b*) 3D protein lines created by multiphoton crosslinking of BSA. (*Left*) To construct functional banjo strings, the laser focus was first scanned along the surface of a coverslip (out of focus in the image), then into solution several micrometers (out-of-focus middle regions on the highest strings), and finally back to the coverslip to obtain a second attachment region. (*Right*) The third string is "plucked" by using a continuous-wave titanium/sapphire laser focus as an optical tweezer (arrow). (Scale bar: 10 μm.) (*c*) Scanning electron micrograph of a low-profile protein line fabricated underneath an NG108-15 neurite that is descending from the cell body (out of view, above image) to the glass substrate. (Scale bar: 1 μm.)

current studies, excitation of photosensitizers using a tightly focused titanium/sapphire laser beam promotes photochemical reactions, most likely through a combination of reactive oxygen and atom abstraction mechanisms (20). The nonlinear nature of the absorption and photochemical processes involved in multiphoton photopolymerization chemistries (16) makes it possible to construct surface-adherent features substantially smaller than the wavelength of the laser light: scanning electron micrographs show that crosslinked albumin structures with dimensions as small as ≈250 nm can be fabricated by using an excitation wavelength of 790 nm (Fig. 1*c*). For the studies reported here, we estimate feature heights to range from this minimum dimension to somewhat <1 μm.

With the goal of identifying conditions useful for fabricating interactive microstructures in the presence of developing neurons, we evaluated biological compounds as sensitizers for multiphoton photocrosslinking of proteins. Various biologic species have long been known to efficiently photosensitize UV protein crosslinking (27, 28) and generally have low cytotoxicity. In our studies, flavins, pyridine nucleotides, and the neurotransmitter serotonin all proved useful for creating 3D defined structures of crosslinked proteins under biologically compatible conditions. Because FAD offered relatively large two-photon cross sections at accessible titanium/sapphire wavelengths and could be readily obtained at high purity, it was adopted as the principal photosensitizer for these studies. We have found that FAD can effectively sensitize the multiphoton crosslinking of various proteins, including BSA, cytochrome *c*, glutamate dehydrogenase, and (neutr)avidin.

Previous work has shown that photoexcitation of flavins can produce a number of reactive compounds, including superoxide, singlet oxygen, and flavin radicals (20, 29). In principle, such compounds have the capacity to disrupt cellular processes via damage to membranes, proteins, and nucleic acids, leading to cell damage or death through a variety of mechanisms. To assess whether photocrosslinking reactions in the vicinity of a cell compromise membrane integrity (30), cytosolic calcium-ion levels were probed by using the fluorescent calcium indicator, x-Rhod-1. Tests on primary cortical cells show no detectable transient (i.e., within the resolution of a 2.4-Hz frame capture rate) or long-lasting ($>$10 s) disruption of calcium homeostasis when FAD-promoted BSA crosslinking is performed at distances as close as ≈1.5 μm from the plasma membrane of neurites and soma. It is important to note that, because the fabrication of guidance structures requires no direct irradiation of developing neurons, issues related to direct multiphoton photoexcitation of cellular material were not of concern. Combined with our observations that neurons can thrive and develop at normal rates for multiple days after these procedures, cells do not appear to be sensitive to the photocrosslinking solution and fabrication procedure.

Fig. 2*a* demonstrates that neurite pathfinding can be redirected by low-profile protein lines for NG108-15 cells undergoing neuronal differentiation. As a result of a topographical barrier (a BSA protein "corral") fabricated in the pathway of an extending process, the neuritic architecture forms a set of elaborate self-interactions within several hours. In general, neuritic development of these cells was not strongly constrained by the lowest profile protein lines that could be fabricated, a result that is not unexpected given the rapid and erratic outgrowth generally observed with neuronally derived cancer cells.

Processes extending from rat cortical neurons also can be influenced by protein microstructures, appearing more responsive to low-profile lines than are NG108-15 cells. Not surprisingly, barriers of different heights are needed to constrain and redirect different neuritic features. Although fine filopodia may transiently lift from the coverslip substrate as they investigate their environment, they typically do not direct outgrowth of processes over low-profile BSA lines; in contrast, broad growth cones, particularly at the termini of large-diameter neurites, are less likely to be deterred by very low profile protein structures. As in developmental studies on other microfabricated substrates, such as grooved quartz (26), the scale of photofabricated protein matrices required for redirecting neurite growth is expected to depend on cell type and maturity, as well as microstructure dimensions; fortunately, *in situ* photofabrication provides a high degree of 3D control, allowing near-vertical barriers to be constructed with profiles as low as ≈250 nm or, by scanning a beam vertically, to heights of several micrometers or more.

Interestingly, developing neurites that are crossed in the process of protein line fabrication can be pinned for extended periods, provided that matrix continuity is not broken at the crossing point. In such cases, processes become joined to the glass substrate, constraining cell migration and morphology at specified coordinates during growth and elaboration. Formation of structures in integral contact with cells is likely more disruptive than fabrication of materials that are micrometers or more from the membrane; further investigation is needed to assess the precise damage caused by photosensitizer excitation in such cases. Nevertheless, immobilization of NG108-15 neurites followed by continued viability can be achieved regularly as long as irradiation of cytosolic regions is minimized. Beyond the spatial confinement achieved with this technique, the possibility of performing reactions within such structures [e.g., in features comprised of crosslinked enzymes (31) or electroactive proteins] may enable cells to be stimulated at multiple, precisely defined positions with high temporal control.

We have constructed low-profile structures to guide the specific interaction site of developing cortical neurons in culture. Although it should be feasible to construct complex microfabricated patterns to guide extensive sets of neuronal interactions, Fig. 2*b* demonstrates that it is possible to use a simple approach, involving construction of only a single barrier, to influence how and where cells make contact. Here, the photofabrication path of a narrow protein line is passed over an existing process (cell 1), causing partial retraction of the neurite. As shown in the time sequence, reextension of the neurite is guided toward a desired contact point on target cell 2 through interactions of filopodia with the protein barrier. It should be noted that guidance of neurite extension does not ensure that an active synapse will be formed; as in all dynamic culture environments, any individual contact between cells may prove stable or highly transitory. In this experiment, one of the contacts between cells 1 and 2 (Fig. 2*b Right*, arrow) forms within ≈30 min of photofabrication and persists with no observable movement for the duration of the experiment (≈100 additional min). A second contact between cells 1 and 2 in Fig. 2*b Right* (slightly to the left) moves several micrometers along the cell 2 neurite over a similar time frame.

These studies provide a description of a strategy for creating physically and chemically interactive microstructures within cell cultures, a technique that offers capabilities for interfacing with neurons during development in fundamentally new ways. Multiphoton photocrosslinking of proteins is rapid, minimally intrusive to cells, and, given the availability of multiphoton imaging facilities, should be rapidly adopted within the cellular neuroscience community. Although the current study focuses on the use of these methods only with cells of neural origin, biocompatible microfabrication will likely have important applications in the study of other cell types that undergo directional growth or motility (e.g., keratocytes, cancer cells, and stem cells).

Capabilities for restricting the developmental options of neurons in highly controlled fashions should have a substantial impact on studies of neuronal signaling and plasticity, and, ultimately, on efforts in neurocomputation and neuromedicine. The results demonstrated here represent an important landmark toward such goals. Higher levels of sophistication, in which neuronal interactions are guided, not only along a planar cov-

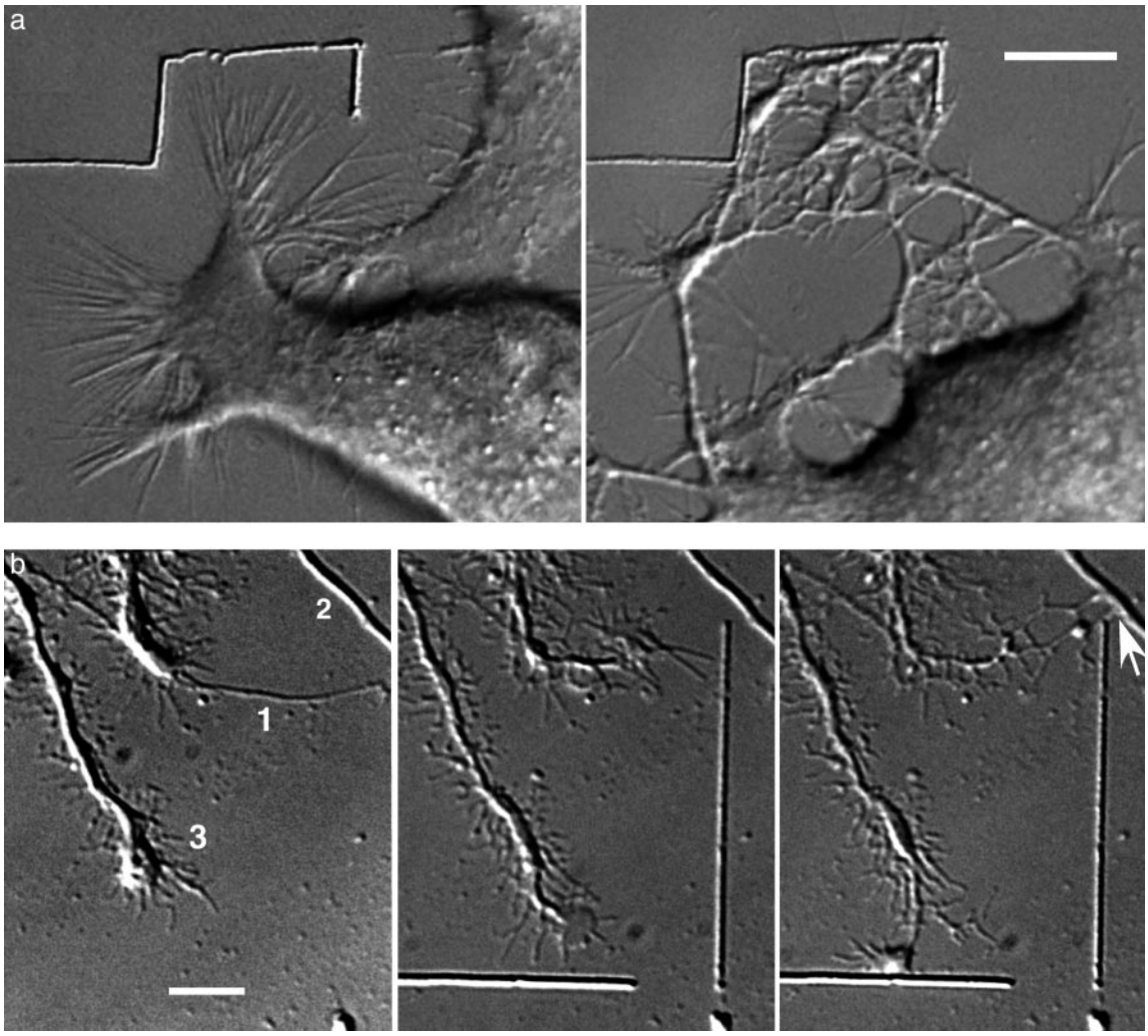

Fig. 2. Constraint and redirection of cellular processes. (*a*) Confinement of neurite growth by a corral fabricated *in situ* by photocrosslinking BSA immediately before acquisition of the image in *Left*. After 3.5 h (*Right*), the corral has exerted strong confinement on the elaboration of the NG108-15 cell, having guided formation of numerous neuritic interaction sites. (Scale bar: 10 μm.) (*b*) Time sequence demonstrating use of *in situ* photofabrication to guide neuron interaction. (*Left*) Neurites from three separate rat cortical neurons (labeled 1–3) before photofabrication. The horizontal (BSA) and vertical (BSA/laminin) lines (*Center* and *Right*) were fabricated 5 min and 42 min, respectively, after acquisition of the first image. (*Center*) Revealed are filopodia from cell 1 interrogating the vertical line as the neurite undergoes reextension (15 min postfabrication). Within another 12 min, one of the filopodia had successfully navigated this barrier (data not shown), forming a contact with cell 2 just beyond the terminus of the line. This contact site, identified by an arrow in *Right* (acquired 61 min postfabrication), persisted for at least tens of minutes. (Scale bar: 5 μm.)

erslip, but in three dimensions and using structures comprised of gradients of biospecific materials, will afford substantially greater abilities to construct complex sets of cellular interactions.

We thank D. Romanovicz, E. Anslyn, D. Vanden Bout, and A. Ellington for advice and experimental assistance and the Welch Foundation (Grant F-1331), the Army Research Office Multidisciplinary University Research Initiative, and the National Science Foundation (Grant 0317032) for financial support. B.K. is a predoctoral fellow in the National Science Foundation Integrative Graduate Education and Research Traineeship Program in Biomedical Optics. J.B.S. is a Fellow at the Institute for Cellular and Molecular Biology.

1. Wang, H., Shimizu, E., Tang, Y.-P., Cho, M., Kyin, M., Zuo, W., Robinson, D. A., Alaimo, P. J., Zhang, C., Morimoto, H., *et al.* (2003) *Proc. Natl. Acad. Sci. USA* **100,** 4287–4292.
2. Chang, H. Y., Takei, K., Sydor, A. M., Born, F. R. & Jay, D. G. (1995) *Nature* **376,** 686–690.
3. Shimizu, E., Tang, Y. P., Rampon, C. & Tsien, J. Z. (2000) *Science* **290,** 1170–1174.
4. Song, H. J., Ming, G. L. & Poo, M. M. (1997) *Nature* **388,** 275–279.
5. Patel, N. B. & Poo, M.-M. (1982) *J. Neurosci.* **2,** 483–496.
6. Ehrlicher, A., Betz, T., Stuhrmann, B., Koch, D., Milner, V., Raizen, M. G. & Käs, J. (2002) *Proc. Natl. Acad. Sci. USA* **99,** 16024–16028.
7. Jung, D. R., Kapur, R., Adams, T., Giuliano, K. A., Mrksich, M., Craighead, H. G. & Taylor, D. L. (2001) *Crit. Rev. Biotechnol.* **21,** 111–154.
8. Zeck, G. & Fromherz, P. (2001) *Proc. Natl. Acad. Sci. USA* **98,** 10457–10462.
9. Maher, M. P., Pine, J., Wright, J. & Tai, Y.-C. (1999) *J. Neurosci. Methods* **87,** 45–56.
10. Denk, W., Strickler, W. & Webb, W. W. (1990) *Science* **248,** 73–76.
11. Denk, W. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 6629–6633.
12. Pettit, D. L., Wang, S. S.-H., Gee, K. R. & Augustine, G. J. (1997) *Neuron* **19,** 465–471.
13. Plenert, M. L. & Shear, J. B. (2003) *Proc. Natl. Acad. Sci. USA* **100,** 3853–3857.
14. Cumpston, B. H., Ananthavel, S. P., Barlow, S., Dyer, D. L., Ehrlich, J. E., Erskine, L. L., Heikal, A. A., Kuebler, S. M., Lee, I.-Y. S., McCord-Maughon, S., *et al.* (1999) *Nature* **398,** 51–54.
15. Kuebler, S. M., Braun, K. L., Zhou, W., Cammack, J. K., Yu, T., Ober, C. K., Marder, S. R. & Perry, J. W. (2003) *J. Photochem. Photobiol. A* **158,** 163–170.
16. Kawata, S., Sun, H.-B., Tanaka, T. & Takada, K. (2001) *Nature* **412,** 697–698.
17. Knott, L. & Bailey, A. J. (1998) *Bone* **22,** 181–187.

18. Duance, V. C., Crean, J. K., Sims, T. J., Avery, N., Smith, S., Menage, J., Eisenstein, S. M. & Roberts, S. (1998) *Spine* **23,** 2545–2551.
19. Aquilina, J. A., Carver, J. A. & Truscott, R. J. (1999) *Biochemistry* **38,** 11455–11464.
20. Spikes, J. D., Shen, H.-R., Kopeceková, P. & Kopecek, J. (1999) *Photochem. Photobiol.* **70,** 130–137.
21. Verweij, H. & Van Steveninck, J. (1982) *Photochem. Photobiol.* **35,** 265–267.
22. Shen, H.-R., Spikes, J. D., Kopeceková, P. & Kopecek, J. (1996) *J. Photochem. Photobiol. B* **34,** 203–210.
23. Pitts, J. D., Howell, A. R., Taboada, R., Banerjee, I., Wang, J., Goodman, S. L. & Campagnola, J. (2002) *Photochem. Photobiol.* **76,** 135–144.
24. Tashiro, K., Sephel, G. C., Weeks, B., Sasaki, M., Martin, G. R., Kleinman, H. K. & Yamada, Y. (1989) *J. Biol. Chem.* **264,** 16174–16182.
25. Hess, D. T., Patterson, S. I., Smith, D. S. & Skene, J. H. (1993) *Nature* **366,** 562–565.
26. Rajnicek, A., Britland, S. & McCaig, C. (1997) *J. Cell Sci.* **110,** 2905–2913.
27. Dubbelman, T., De Goeij, A. & Van Steveninck, J. (1978) *Biochim. Biophys. Acta* **511,** 141–151.
28. Kato, Y., Uchida, K. & Kawakishi, S. (1994) *Photochem. Photobiol.* **59,** 343–349.
29. Egorov, S. Y., Krasnovsky, A. A., Jr., Bashtanov, M. Y., Mironov, E. A., Ludnikova, T. A. & Kritsky, M. S. (1999) *Biochemistry Mosc.* **64,** 1117–1121.
30. Valenzeno, D. P. & Tarr, M. (2001) *Photochem. Photobiol.* **74,** 605–610.
31. Pitts, J. D., Campagnola, P. J., Epling, G. A. & Goodman, S. L. (2000) *Macromolecules* **33,** 1514–1523.