

A fast flexible ink-jet printing method for patterning dissociated neurons in culture

Neville E. Sanjana^{a,1}, Sawyer B. Fuller^{b,*,1}

^a Department of Brain and Cognitive Sciences, Massachusetts Institute of Technology (MIT), Cambridge, MA, USA

^b Department of Mechanical Engineering and Center for Biomedical Engineering, E25-425, MIT, 77 Massachusetts Avenue, Cambridge, MA 02139, USA

Received 17 October 2003; received in revised form 9 January 2004; accepted 9 January 2004

Abstract

We present a new technique that uses a custom-built ink-jet printer to fabricate precise micropatterns of cell adhesion materials for neural cell culture. Other work in neural cell patterning has employed photolithography or “soft lithographic” techniques such as micro-stamping, but such approaches are limited by their use of an un-alterable master pattern such as a mask or stamp master and can be resource-intensive. In contrast, ink-jet printing, used in low-cost desktop printers, patterns material by depositing microscopic droplets under robotic control in a programmable and inexpensive manner. We report the use of ink-jet printing to fabricate neuron-adhesive patterns such as islands and other shapes using poly(ethylene) glycol as the cell-repulsive material and a collagen/poly-D-lysine (PDL) mixture as the cell-adhesive material. We show that dissociated rat hippocampal neurons and glia grown at low densities on such patterns retain strong pattern adherence for over 25 days. The patterned neurons are comparable to control, un-patterned cells in electrophysiological properties and in immunocytochemical measurements of synaptic density and inhibitory cell distributions. We suggest that an inexpensive desktop printer may be an accessible tool for making micro-island cultures and other basic patterns. We also suggest that ink-jet printing may be extended to a range of developmental neuroscience studies, given its ability to more easily layer materials, build substrate-bound gradients, construct out-of-plane structure, and deposit sources of diffusible factors.

© 2004 Elsevier B.V. All rights reserved.

Keywords: Patterned neural networks; Ink-jet printing; Surface chemistry; Cell attachment; Cell growth control; Dissociated cell culture; Glia patterning; Hippocampus

1. Introduction

Our understanding of the cellular basis of learning and memory has been aided by the use of in vitro neural cell culture—the growth of neurons in a controlled environment outside of the living organism. Dissociated cell culture contrasts with explant culture, brain-slice, and in vivo preparations in that the physical structure of the tissue is lost during enzymatic and mechanical digestion. Once the dissociated neurons settle they may adhere to the culture surface (the “substrate”), where they re-grow processes and form synapses with other neurons. The growth of these

processes, the migration of cell bodies, and other in vitro phenomena may be influenced by fabricating patterns of chemicals and/or physical structure bound to the substrate. Patterned cell culture may be used for studying smaller isolated networks, developmental cues, or single-cell properties. A long-term goal is to utilize substrate-bound patterns of biomaterials as a simulation environment and test-bed for analyzing biological signals encountered by neurons undergoing development.

Early work in patterned neuron cultures employed small islands of adhesive protein or cells arranged over an inhibitory background such as agarose gel so that each island contained a small number of neurons. Such cultures simplified studies of synapses between neurons and cardiac myocytes by limiting connections in several of the myocytes islands to a single neuron (Furshpan et al., 1976). Though the technique relied on a relatively simple airbrush

* Corresponding author. Tel.: +1-617-452-2691; fax: +1-617-452-2913.

E-mail address: minster@mit.edu (S.B. Fuller).

¹ Both authors contributed equally to this work.

procedure to generate the tiny droplets, the sizes and locations of the islands were arbitrary, and only round islands could be produced. Newer, more controlled patterning techniques have included contact masks (Westermarck, 1978), photolithography using photoresists (Kleinfeld et al., 1988; Wyart et al., 2002) and photochemistry (Hickman et al., 1994), microstamping (Branch et al., 2000; Scholl et al., 2000; Wheeler et al., 1999), and microfluidic channels (Martinoia et al., 1999). However, these techniques share a limiting characteristic in that they rely on the existence of an un-alterable master pattern and so limit experimentation with different patterns. To change the pattern master to fit changing experimental needs may require multiple days or even weeks, which can be especially problematic when iterative refinement is necessary or when under an experimental deadline.

To overcome this and other limitations, we introduce ink-jet printing as a method for fabricating patterns of materials to influence neuron growth. In contrast to desktop ink-jet printers which print dyes or pigments onto paper, we print biologically-active materials onto coverslips. Ink-jet printing has the advantage that the pattern is stored entirely in digital form and can be altered and re-printed in minutes. This flexibility is absent from the master pattern-based approaches. In addition, and perhaps more importantly, because it is fundamentally different than the above techniques, it has unique advantages for neuroscience research: Compared to master pattern-based approaches, ink-jet printing makes it much easier to deposit multiple layers of different materials, build out-of-plane structure, deposit large amounts of material, and fabricate substrate-bound gradients, as well as being relatively inexpensive.

Though the resolution of ink-jet printing is low (tens of microns) relative to the lithographic techniques described above (sub-micron), we feel there is a need for patterning in a co-culture environment with glia. In addition, co-culture of neurons with glia provides a more biologically plausible environment *in vitro*. Glia are more difficult to confine to patterns with micron-sized features (St John et al., 1997; Wyart et al., 2002), and thus the resolution of the printer we present is closer to the resolution limit for glia co-culture. In addition, we will show that it is possible to create features significantly smaller than the size of a single droplet by printing negative relief, in which spaces between droplets become the features instead of the droplets themselves.

In this work, we demonstrate one of the first uses of ink-jet printing for influencing the behavior of neural cells in culture by using it to print improved micro-island culture patterns (Roth et al., *in press*; Turcu et al., 2003). We describe a flexible surface chemistry procedure and a dissociated hippocampal cell culture protocol suitable for patterning cell adhesion biomolecules on a cell-repulsive background. We present results for these protocols that demonstrate the patterned growth of healthy neurons for periods as long as 3 weeks. We quantitatively assess neuron compliance with printed patterns from 5 to 25 days in culture. In addition,

using intracellular recording and immunocytochemistry we show that patterned cultures are similar to un-patterned cultures in passive membrane properties, resting potentials, synaptic density, and inhibitory cell density. Lastly, we suggest that such patterns may be fabricated using a much less complex desktop printer, and that custom hardware such as is reported here could be used for neural development studies.

2. Methods

2.1. Ink-jet printer design

Ink-jet printing consists of depositing single, tightly-controlled microscopic droplets 10–100 μm in diameter onto a substrate. While continuous-jet printers expel a continuous pressurized stream that breaks up into small droplets, drop-on-demand (DOD) printers, such as the one reported here, eject single droplets in response to a pressure impulse in the ink chamber (Heinzl and Herz, 1985). The pressure impulse is generated by a piezo crystal that deforms in response to a voltage pulse generated under computer control. The droplet takes a 1–10 mm ballistic trajectory to the substrate, where it lands and adheres. The liquid evaporates, leaving a round deposit of solid material. The print head is moved robotically over the substrate as droplets are ejected, leaving a pattern of round dots (Fig. 1).

Ink-jet printing has recently been employed in a range of fabrication applications, such as three-dimensional object fabrication (Z Corporation, Cambridge, MA; 3D Systems, Valencia, CA), printed electronics and micro-mechanical structures out of nanoparticle-based inks (Fuller and Jacobson, 2000; Fuller et al., 2002), and organic light-emitting diode (OLED) displays (Cambridge Display Technology, Cambridge, UK). In the biosciences, ink-jet printing has been used to print patterns of fibronectin for cell micropositioning (Klebe, 1988), growth factors for myoblast cells (Watanabe et al., 2003), three-dimensional cell scaffolding for liver cells (Griffith et al., 1997), cast collagen gels into ink-jet printed wax molds for cell culture (Sachlos et al., 2003), and whole mammalian cells (Wilson and Boland, 2003). Here, we apply the technology to arranging chemical patterns for controlling and assessing the properties of neurons in culture.

For this particular purpose, we developed both a custom-designed printer and MS Windows-based computer aided design (CAD) software environment (Figs. 2 and 3). The printer was designed to enable a wider range of studies than would be possible with off-the-shelf desktop printer hardware. For instance, a custom printer nozzle with a single orifice was used so that print head priming volumes were smaller than 1 ml, allowing us to print biomaterials for which only small quantities were available. Drawings were specified in a vector-based format consisting of points and lines of droplets, which was a simple yet sufficiently capable system for the work described here. In addition to requiring

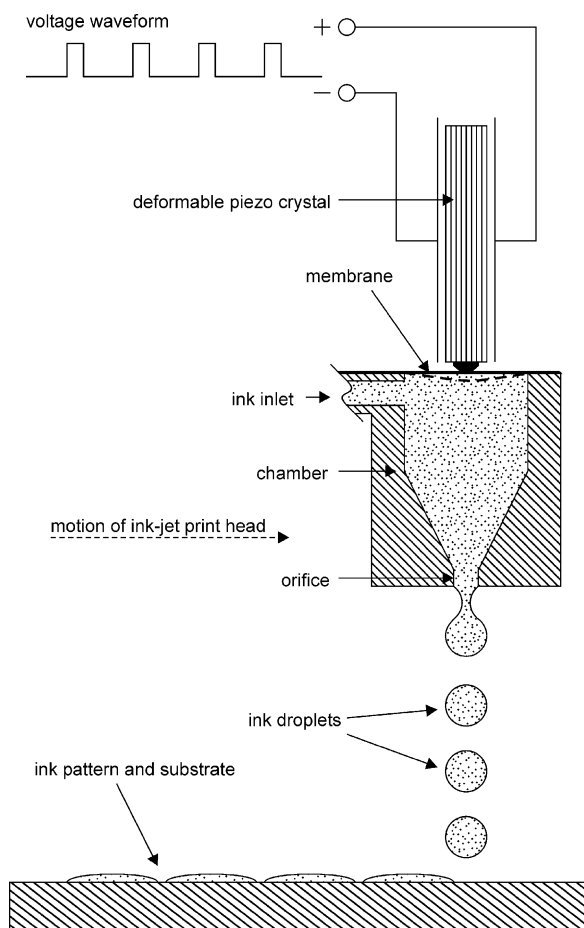


Fig. 1. Diagram of the operation of a piezo drop-on-demand ink-jet print head. Microscopic ink droplets are ejected individually through an orifice by means of a pressure impulse delivered by a piezo crystal. Each droplet, 10–100 μm in diameter, takes a ballistic trajectory a small distance (~ 1 mm) to the glass substrate. The print head is moved robotically in two-dimensions above the substrate as droplets are ejected, leaving a pattern of round dots on the substrate.

less liquid for priming, using a single nozzle required less software and hardware, was less prone to failure when attempting to use new, experimental “inks,” and could position individual droplets with more precision than a rasterizing printer. The entire printer was made as small as possible and was controlled by a laptop computer so it could be moved easily, such as into a sterile laminar flow hood or a cold room.

The nozzle was procured from Microfab Technologies, a manufacturer specializing in ink-jet printing research (Fig. 3, inset). The nozzle used an annular piezo crystal that “squeezed” a glass tube to eject droplets. The tip was drawn to an orifice diameter of 30 μm (model MJ-AB-030). Inks could be water- or solvent-based with a solids density less than or equal to 2.5 mg/ml. The ink feed for the print head consisted of a 20 cm length of small-diameter Tygon tubing connecting the barbed fitting of the print nozzle to a dulled syringe needle with a luer fitting to a 5 ml syringe. Manual manipulations of syringe pressure were used to generate pressure differentials at the print-head. Voltage pulses to the

head to eject droplets were generated by custom-designed high-voltage hardware with a proprietary serial RS-232 command set (Microfab Technologies, model JetDrive III). The voltage impulse to eject a droplet of water-based “ink” was 28 V for 20 μs . Droplet ejection frequencies ranged from 1 to 500 Hz.

The print head was fixed over a moving substrate support riding on roller bearings and actuated by two perpendicular leadscrew-driven servomotors (Newport Corp. model 850-G-HS) driving the X- and Y-axes. A three-axis closed-loop stage controller (Newport Corp. model ESP-300) coordinated the motion of the two motors. Motion commands and jet pulse commands were sequentially generated and orchestrated by the custom-written CAD software environment based on drawing data. The printing surface was horizontal, allowing the substrate, such as a round glass coverslip, to be positioned on top. The temperature of the substrate was optionally controlled using a closed-loop controller (Watlow, series 965) and a temperature-detecting thermocouple. The controller used a power relay in pulse-width modulated mode driving a peltier device (TE Technology) in either positive bias (cooling) or negative bias (heating). The peltier device was bonded with a thermal adhesive to a PC microprocessor heatsink of sufficient thermal capacity.

2.2. Ink-jet printing procedure

Prior to printing, all surfaces in contact with the substrate were sterilized with a 70% ethanol solution, including the printing surface upon which the substrate was placed, the ink feed, and the nozzle orifice itself. The nozzle was back-flushed for 30 s with 70% ethanol in water to sterilize and then back-flushed for 30 s with Millipore water. Back-flushing and ink loading were achieved by generating negative pressure in the syringe to pull liquid in through the small tip of the nozzle, effectively filtering it to the nozzle size. The nozzle was then loaded with 200–500 μl of ink. Once loaded, the ink was forcibly ejected from the orifice for a short time to insure that no bubbles remained and that the orifice was clear of debris. Pressure in the ink feed was then equalized to atmospheric pressure by opening a valve connected to the syringe. A lint-free cloth was used to remove excess liquid from the tip of the head and then the head driver box was commanded to send pulses at 1 kHz, generating a barely-visible stream of droplets that could be judged for quality. If droplets were ejecting diagonally, or not at all, more ink was forcibly ejected to clear any nozzle debris and the pressure was again equalized. Once the stream was satisfactory, the print head driver hardware was set to eject droplets at 10 Hz continuously to insure that the nozzle did not dry out and clog during periods of inactivity.

Drawing files usually included a ring of large islands printed around the periphery. The islands were visible to the naked eye, providing visual feedback during printing that the nozzle was still ejecting droplets and had not dried out or clogged. In addition, islands on the perimeter served to

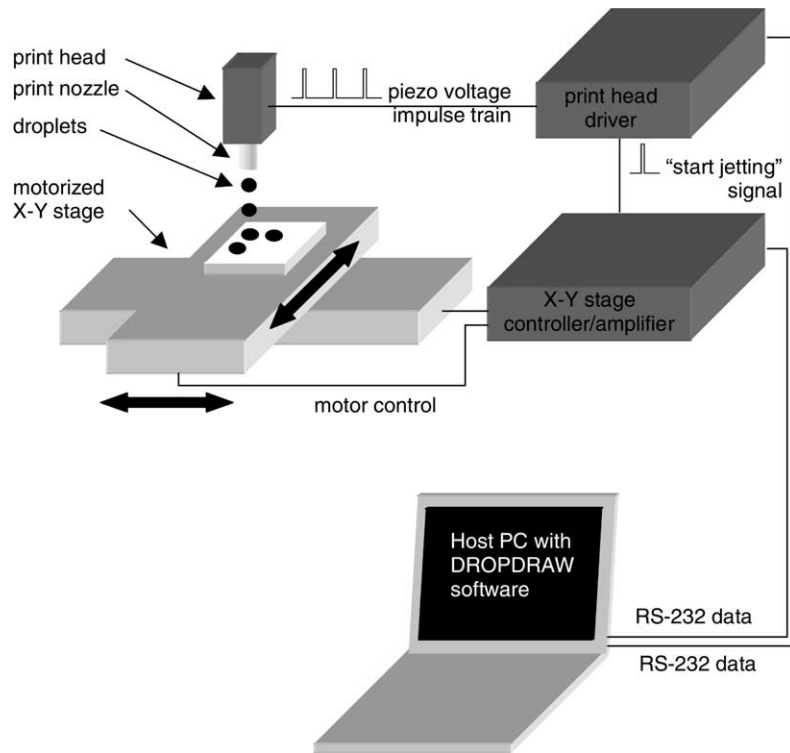


Fig. 2. Schematic of the elements of the custom-built ink-jet printer used in this work. A PC with a custom-written software environment sends data through a serial port to command an X–Y stage servomotor controller to move according to a drawing stored in the software. When a commanded position is reached, the stage controller sends a signal to the print head driver to generate a particular waveform specified by serial commands from the PC. This signal causes one or more droplets to be ejected from the nozzle orifice at the desired position.

spread out cultured neurons so that a disproportionate number of them were not adhering to the outer edge of the pattern of interest. After printing, the coverslips were sterilized by ultraviolet radiation for 15 min.

2.3. Pattern chemistry

A surface chemistry was needed that provided a strong contrast between cell-adhesive and cell-repulsive areas to constrain the locations of cell bodies and neurites for extended periods of time. To optimize versatility, we wanted a chemistry protocol that allowed the “ink” to be either cell-adhesive or cell-repulsive *without requiring any change in materials*. That is, either the cell-adhesive or cell-repulsive biomolecule could be used as a background coating the coverslip or as the printed foreground. For simplicity, in this work, we demonstrate only results in positive relief (cell-adhesive material patterned onto a cell-repulsive background).

The positive relief patterning process required two major components: (1) a repulsive background coating the slide entirely and uniformly that did not allow cell adhesion and (2) an adhesive molecule that remained in place on top of the repulsive background, that cells adhered to, and that could be ink-jet printed. The printed cell-adhesive material was a collagen/poly-D-lysine (PDL) mixture and the repul-

sive background was poly(ethylene) glycol (PEG) covalently bonded to the glass surface. A diagram summarizing the process is shown in Fig. 4.

The first major component, the repulsive background, had unique requirements. To achieve precise and reproducible results with ink-jet printing, the background being printed upon needed to be smooth and uniform. As mentioned, we also wanted the repulsive molecule to have the additional flexibility that it itself could be printed. For example, agarose is not easy to print by ink-jet because it dissolves in water only at high temperatures. In general, for reliable printing, the “ink” needed to be non-reactive with the print head and to be below 0.5% weight in water. For instance, other candidate inhibitory materials, such as silanes, were eliminated because they could react with the glass of the print head and change its wetting characteristics. In the future, this limitation might be circumvented with the advent of all-stainless steel, small-volume print heads.

Thus, in this work, we chose poly(ethylene) glycol as the inhibitory background material. PEG has been shown to provide long-term inhibition of cell adhesion in several previous studies of patterned neural cell culture (Branch et al., 2000; Wheeler et al., 1999). It is non-reactive with the glass print head, is non-toxic to cells when rinsed properly (see Section 2.4), and can be linked to the coverslip to form a uniform layer. The PEG background application procedure

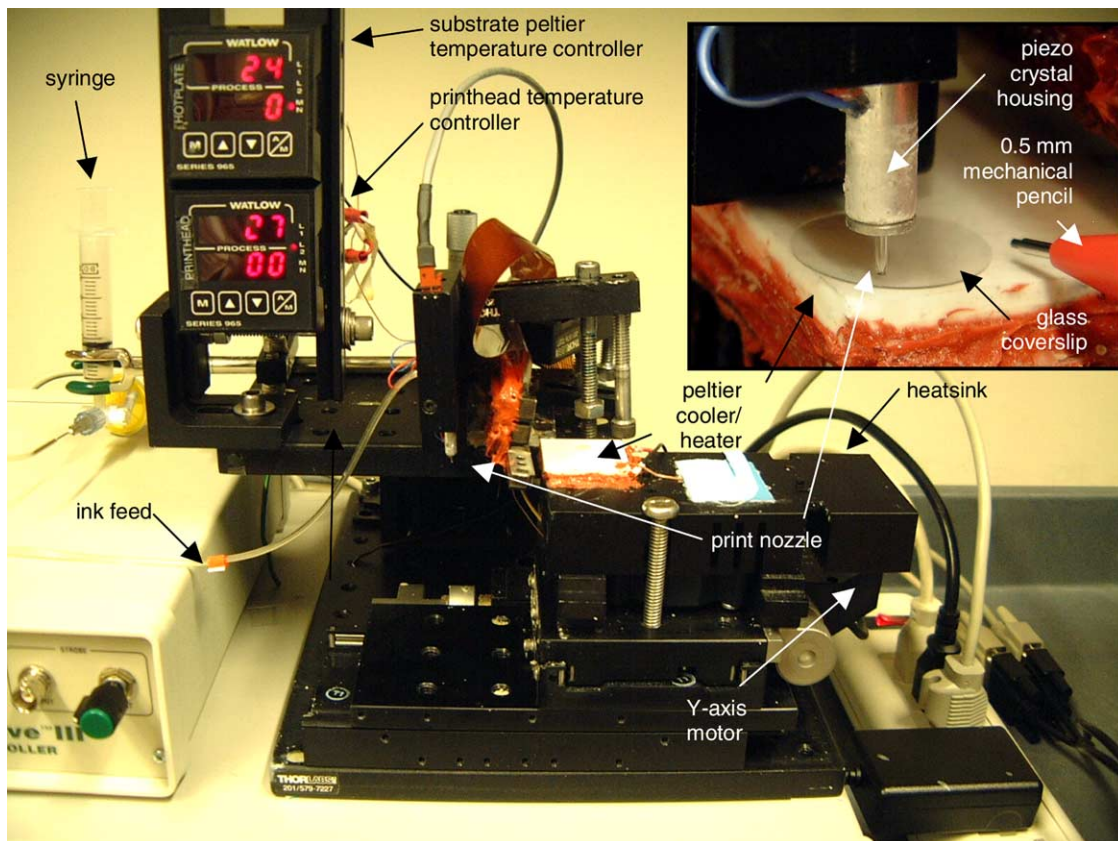


Fig. 3. Photograph of the ink-jet printer with inset detail of print nozzle. Components include X–Y servomotors, a rollerbearing stage, a peltier cooler/heater for controlling the substrate temperature, the piezo print head, ink feed, and digital closed-loop temperature controllers. The tip of a 0.5 mm mechanical pencil is included in the print nozzle detail for scale.

was adapted from a protocol in Cass and Ligler (1998) for immobilizing biomolecules containing an amino group to glass substrates.

Round coverslips (German glass, 12 mm diameter, Electron Microscopy Sciences 72196-12) were cleaned overnight in a 10% KOH solution to remove contaminants, and then soaked in a 2.5% (by volume) aqueous

solution of aminopropyltriethoxysilane (Sigma A3648) to expose reactive amino groups for 30 min and then rinsed in water and ethanol before being dried in a vacuum oven overnight at 80 °C. Coverslips were then soaked in the homobifunctional crosslinker glutaraldehyde (Sigma G7651) in a 0.1M aqueous solution of sodium carbonate (Na_2CO_3) for 2 hours, exposing an aldehyde group that is reactive with amino groups. For the final step, coverslips were soaked in a 0.5% (by weight) aqueous solution of amino-terminated poly(ethylene) glycol (MW 5000, Shearwater Corp. 2M2V0H01) for 1–2 days prior to printing.

The second major component, the adhesive molecule, needed to fulfill three requirements. First, it needed to stay in place on top of the repulsive background. For example, we found that a synthetic extracellular matrix peptide with a RADA16-motif (arginine–alanine–aspartate–alanine) delaminated from the PEG surface despite good neuron adherence to the peptide (Holmes et al., 2000; Zhang et al., 1993, 1995, 1999). Secondly, cells needed to adhere to the molecule. Common cell adhesion substrates include collagen, poly-D-lysine, laminin, and MatriGel (Banker and Goslin, 1998). Thirdly, it must be able to be ink-jet printed reliably by meeting the previously mentioned constraints.

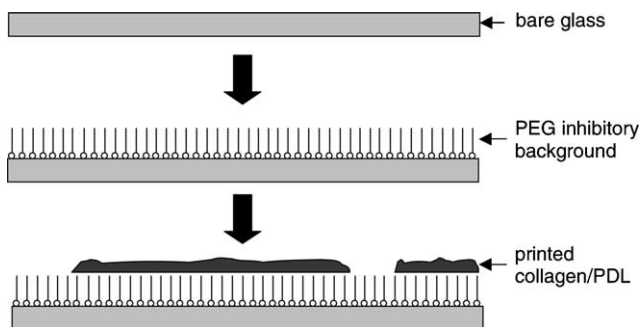


Fig. 4. Diagram of the surface patterning process. The bare glass was uniformly coated with a PEG inhibitory background. The amino-terminated PEG was covalently bonded to the glass surface via a three-step chemical process that used a glass-bonding aminosilane and the crosslinker glutaraldehyde. The adhesive collagen/PDL mixture was printed by ink-jet on top of the PEG background, leaving a pattern of cell-adhesive material that persisted for weeks in culture.

For the adhesive molecule, we chose to use a 25:1 mixture of collagen (4 mg/ml, Biosciences, BD 354236) and PDL (1 mg/ml, Sigma P0899). This mixture was diluted in water so that it was approximately 0.05% by weight. We found that this mixture was able to meet all three requirements described in the previous paragraph. In patterned culture, this mixture was ink-jet printed onto PEG-coated slides.

2.4. Cell culture

Cells from P1 rat hippocampi were obtained using a protocol similar to that which has been described elsewhere (Banker and Goslin, 1998). The dissection solution was HBSS (Sigma H2387) supplemented with 4 mM NaHCO₃ and 5 mM HEPES. Hippocampal tissue was proteolyzed for 40 min at 37 °C, followed by trituration and washing. The digestion solution consisted of dissection solution plus 20 U/ml Papain (Worthington 3126), 0.5 mM EDTA, 1.5 mM CaCl₂, 1 mM L-cysteine, and 1 μg/ml DNase. 0.5 ml of the dissociated cell suspension was plated at 40,000–50,000 cells/ml onto a 12 mm coverslip inside a 24-well plate. The plating medium consisted of BME (Invitrogen 21010-046), 10 mM HEPES (pH 7.35), 1 mM Na-pyruvate, 6 mg/ml glucose, 10% FBS (Hyclone SH30071.02), Mito serum extender (Biosciences, BD 355006), and 2% B27 (Invitrogen 17504-044).

For the patterned cell population: Prior to plating, patterned coverslips were incubated in the culture medium overnight, which was aspirated before plating. We found this pre-plating incubation significantly increased our yield of healthy cells, perhaps because some residual toxicity from the PEG chemistry was reduced through this rinse. Another possible explanation is that the serum facilitated greater protein adsorption onto the printed collagen/PDL (Schaffner et al., 1995), but we did not investigate such mechanisms further. Four hours after plating, the plating medium was gently aspirated and replaced by a serum-free Neurobasal medium (Gibco/Invitrogen 12348-017) with 2% B27 and 0.25% glutamine (Brewer et al., 1993). The same serum-free medium was used for feeding (50% replacement every 4 days in vitro).

This replacement of standard culture medium with serum-free Neurobasal was intended to preserve pattern adherence; serum-containing culture medium is incompatible with this goal since glial growth is excessive when serum is present (Banker and Goslin, 1998; Wyart et al., 2002). We found that this culture protocol limited glial growth, as has been previously reported with the use of Neurobasal, while still preserving some glia in the culture (Brewer et al., 1993). Glia, which previously were thought of as passive support cells for neurons, have been found to actively control synaptogenesis, synapse number, and synaptic plasticity (see Haydon (2001) for a recent review). Thus, using Neurobasal, the population of glia was kept relatively low but extant. This improved pattern adherence and maintained the

neurons on-pattern in a more biologically-realistic manner than glia-free cultures.

We separately prepared low-density control cultures which did not include the cell-repulsive background or ink-jet printed cell adhesion molecules of the patterned cultures. In the low-density control cultures, neither overnight incubation in the culture medium prior to plating nor post-plating replacement of the culture medium was done. Since control cultures did not have patterns requiring adherence, they were plated on coverslips covered with an established astrocyte layer to increase neuron survivability. Control cultures were fed with culture medium containing serum (50% replacement every 4 days in vitro). All cells were maintained in an incubator with a 5% CO₂ atmosphere at 37 °C.

2.5. Electrophysiology

We used the patch-clamp technique to measure several electrophysiological properties of the different cultured cells. For all cells, we continuously calculated and recorded access resistance, membrane resistance, and membrane capacitance. Patch recordings were made using a HEKA EPC 8 amplifier with a 5 kHz (eight pole Bessel) low-pass filter and custom software. All recordings were made between 10 and 12 days in vitro. During the recordings, the culture medium was replaced by an extracellular bath solution containing NaCl (145 mM), HEPES (10 mM), glucose (8 mM), KCl (3 mM), CaCl₂ (3 mM), and MgCl₂ (2 mM) with pH adjusted to 7.3. Pipettes made of borosilicate glass were pulled on a Flaming/Brown puller (Sutter Instruments) such that pipette resistance in the bath solution was kept between 3 and 7 MΩ. Pipettes contained κ-gluconate (130 mM), KCL (10 mM), MgCl₂ (5 mM), EGTA (0.6 mM), HEPES (5 mM), CaCl₂ (0.06 mM), Mg-ATP (2 mM), GTP (0.2 mM), leupeptine (0.2 mM), phosphocreatine (20 mM), creatine phosphokinase (50 U/ml) (Arancio et al., 1995). Seals were always above 1 GΩ resistance and most often were several GΩ. Average access resistance was kept below 20 MΩ for all recordings.

2.6. Immunocytochemistry

Antibody staining was done using mouse monoclonal anti-GABA (1:200, Sigma A0310) and rabbit polyclonal anti-synapsin I (1:750, Chemicon AB1543) antibodies. Secondary antibodies from Molecular Probes were goat IgG anti-rabbit (Alexa Fluor 350, A-11046) and anti-mouse (Alexa Fluor 488, A-11001); both used at 1:200 dilution. Cells were first fixed for 20 min in a 4% formaldehyde solution, rinsed three times in PBS, and then permeabilized in a 0.25% Triton X solution. After a single rinse and then blocking in a 10% goat serum solution, cells were incubated overnight at 4 °C with the primary antibody at the indicated concentration. Cells were then rinsed three times and incubated with secondary antibodies for 1 h. Again, cells were rinsed three times and stored at 4 °C until analysis. As a

control, cells were incubated without the primary antibodies. Images were acquired using a CoolSnap HQ (Roper Scientific) CCD camera. Analysis was done using custom software. For GABAergic cell detection, we set an appropriate brightness threshold that was used for all images from a single plate and a minimum cell size to automate the process. Only when cells overlapped did we adjust cell counts accordingly. For synapsin I puncta detection, we applied a radial tophat filter to reduce distortion from light scattering and then manually counted puncta.

3. Results

All results reported are mean \pm standard deviation, unless indicated otherwise.

3.1. Island size scales with the number of droplets deposited

To design a pattern to print out as expected, it was necessary to know how many droplets were needed to print a certain island size. At a given X – Y location for the print head, an island was made by depositing multiple droplets in quick

succession (500 Hz) so that all droplets were deposited before any significant drying had occurred. The resulting hemisphere of liquid on the substrate, much larger than a single droplet, slowly dried until a circle of material remained. The size of a micro-island depended on the number of droplets deposited. Also, due to variation in the hydrophobicity of different substrates, the contact angle of the water with the substrate—and hence the resulting dot diameter—depended on the substrate being printed onto. Previous studies of dot morphology by atomic force microscopy (AFM) have indicated that more material is deposited around the edges of the droplet than in the middle (Fuller et al., 2002).

Micro-islands consisting of varying numbers of droplets of the collagen/PDL mixture were printed onto bare glass and onto PEG-coated glass to determine their sizes. Dried islands had enough solid material that they were visible in phase contrast microscopy and were measured using an eyepiece micrometer. Islands ranged in size from $65 \pm 5 \mu\text{m}$ in diameter for a single droplet of collagen/PDL mixture on PEG to $460 \pm 5 \mu\text{m}$ for 512 droplets (Fig. 5, $n = 8$). Results indicated that the dot diameter varied as the cube root of the number of droplets deposited, as expected. As shown, micro-islands are larger on glass, most likely due to the substrate's relatively higher hydrophilicity, and thus,

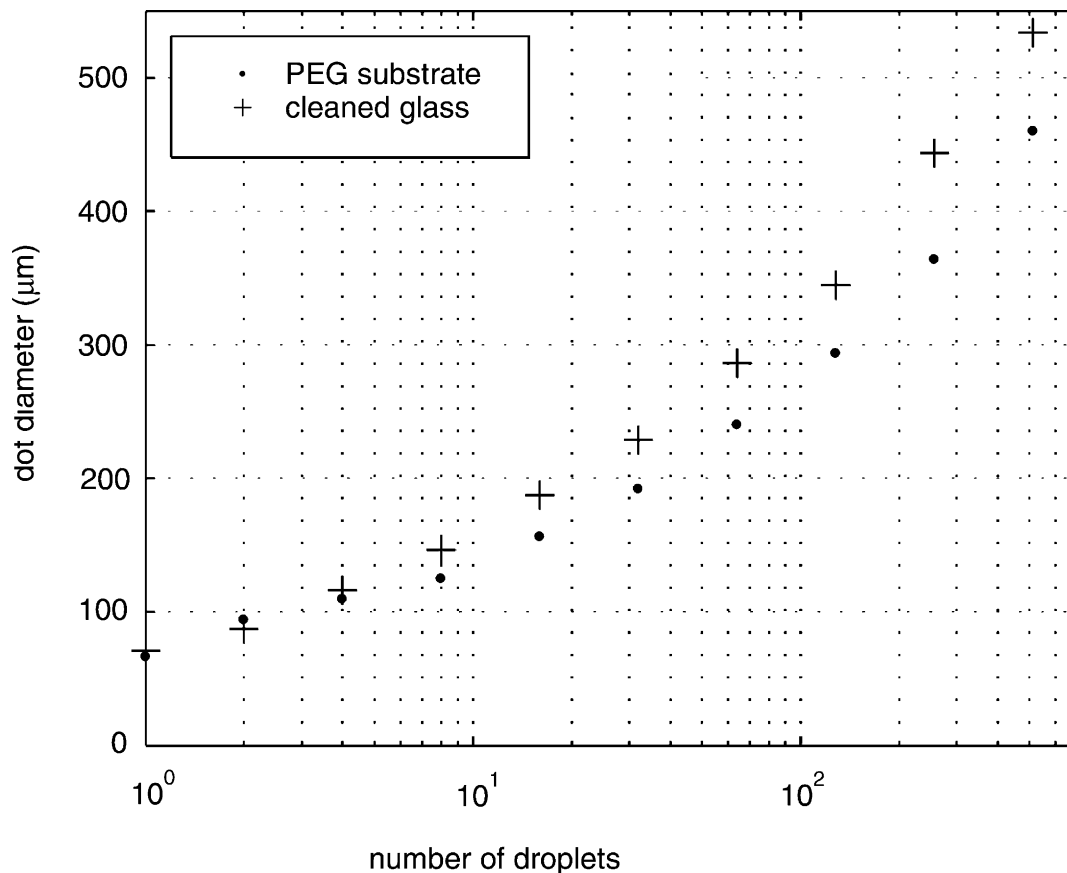


Fig. 5. Micro-island size scales with droplets deposited. For each data point, the ink-jet print head was placed at a single X – Y location and multiple droplets were deposited in quick succession (500 Hz) so that they were all deposited before any significant drying had occurred. The diameter of a micro-island grew slowly as the number of droplets deposited increased and also depended on the composition of the substrate being printed onto ($n = 8$).

lower droplet contact angles. We found that heating the substrate had little effect on the size of micro-islands (data not shown).

3.2. Arbitrary patterns can be printed

Lines of droplets were fabricated by depositing droplets sequentially at a sufficiently low speed such that each droplet had fully evaporated before the subsequent droplet was deposited. To expedite this process, the substrate was heated to 34 °C and droplets were ejected at the rate of 1 Hz. Line-type printing structures were useful for making shapes other than micro-islands. When connecting a larger micro-island to

a line of droplets, it was important to deposit the island first, and then subsequently the line, to ensure that the relatively large volume of liquid in the micro-island was not wicked away into an oblong shape by a previously existing line of printed material. Patterns such as micro-islands, connected micro-islands, box shapes, and alphanumeric text were printed with the collagen/PDL mixture onto the repulsive PEG background (Fig. 6, left column).

An ink-jet printed pair of neighboring lines of droplets was printed with a gap of $8 \pm 2 \mu\text{m}$ between them and maintained for a distance of over a millimeter (data not shown). This small feature size suggests the ability of ink-jet printing to fabricate certain patterns much smaller

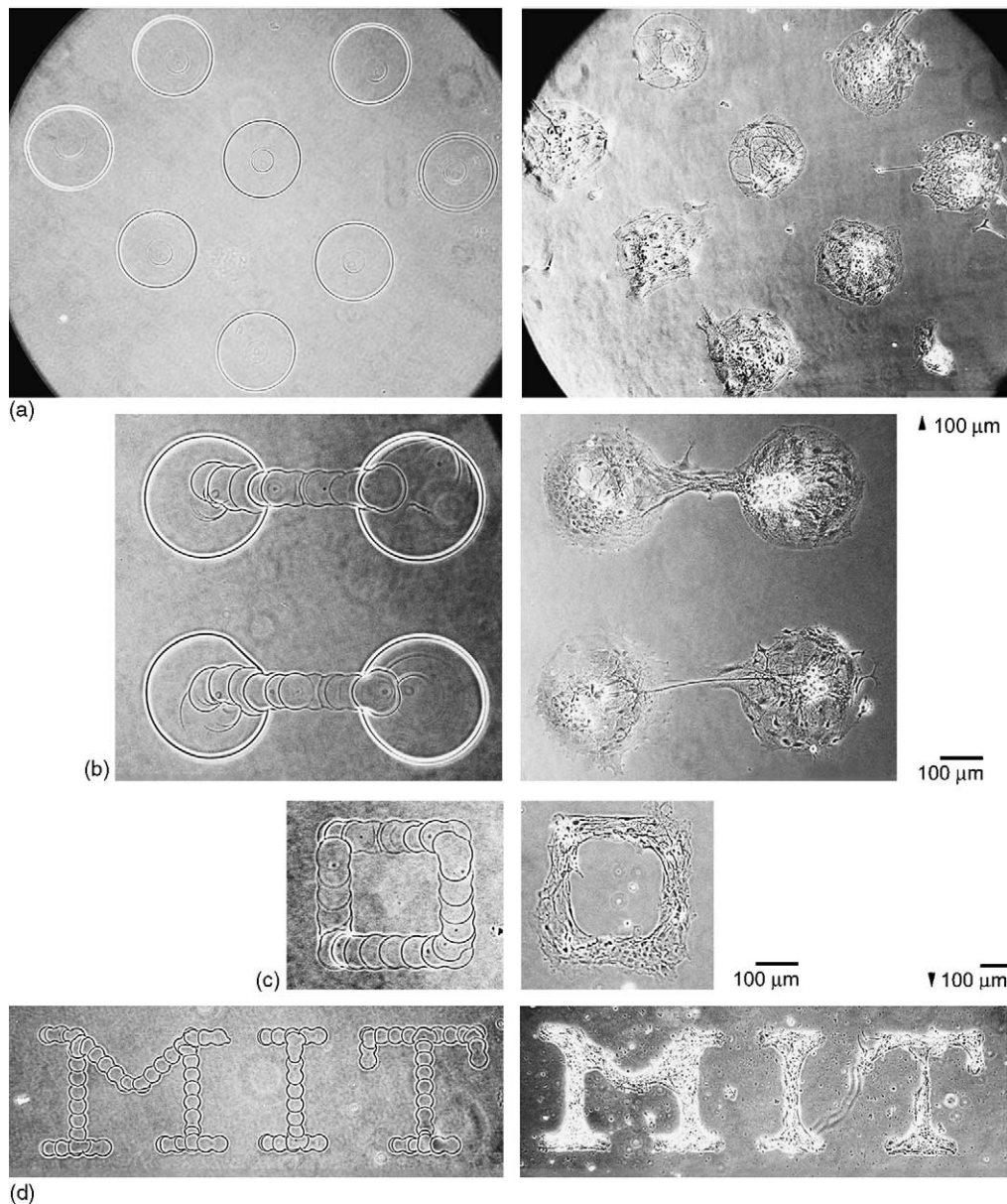


Fig. 6. Cells adhere to printed patterns. Left: collagen/PDL mixture printed onto a uniform PEG inhibitory background; right: cells adhered to patterns after 8–10 days in culture. Patterns include (a) an array of micro-islands 350 μm in diameter, (b) micro-islands connected by narrow channels 100 μm across, (c) a square, and (d) text.

than the diameter of a single droplet (65 μm for our print head and printing parameters) by printing in reverse. If the printed material were cell-repulsive, such as PEG, and if the unprinted areas were adhesive, then such small channels could be sufficiently small to only allow processes, not cell bodies, to grow inside the channels. This type of pattern was aided by the use of our custom hardware, which had a position repeatability of $\pm 1 \mu\text{m}$.

3.3. Cells retain pattern adhesion through 25 days *in vitro*

Neurons and glia plated onto the collagen/PDL on PEG patterns showed strong compliance to the printed patterns (Fig. 6, right column) after 8–10 days in culture and longer. In Fig. 7, we show some smaller islands with only one or two neurons on each island.

Confinement of plated neurons to micro-islands was quantified by phase-contrast imaging of cultures with 350 μm

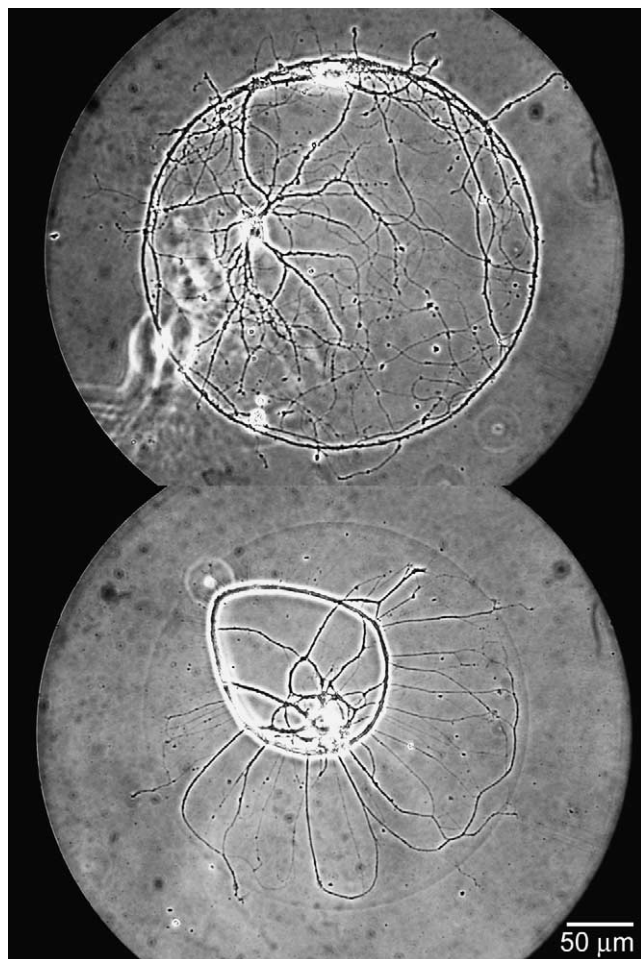


Fig. 7. Close-up of small numbers of neurons on printed micro-islands after 20 days *in vitro*. Top: a pair of neurons with processes encircling the border of the micro-island; bottom: a single neuron on an island. Its processes appear to have turned inward upon reaching the boundary between the collagen/PDL island and the PEG background.

diameter printed micro-islands (250 droplets) at days 5, 8, 10, 15, 20, and 25 *in vitro*. An intact micro-island had no neurons or processes making contact with other islands. The percentage of intact micro-islands was 75% after 5 days *in vitro* and stabilized to approximately 50% after 20 days. (Fig. 8).

3.4. Patterned cultures have normal passive membrane properties and resting potentials

A prerequisite for further studies with patterned culture is for the patterned neurons to display normal, healthy electrophysiological properties. To investigate this, we compared passive membrane properties and resting potentials of the cells grown on patterned substrates to those of the low-density, un-patterned cultures. We measured these properties in whole-cell mode of the patch-clamp amplifier and the results are given in Table 1.

Resting potentials were measured immediately after rupture. As shown in the table, patterned and control cultures had very similar mean resting potentials. Membrane capacitance and membrane resistance were measured using a 20 ms test pulse of 3 mV magnitude, repeated twice each second for a 5 min period to establish a stable value. Membrane resistance was somewhat higher in control cultures ($402 \pm 163 \text{ M}\Omega$) than in patterned cultures ($277 \pm 125 \text{ M}\Omega$) but the difference was not significant (independent *t*-test; $t(22) = 2.12$; $P > 0.01$). In both types of culture, membrane resistance varied greatly from cell to cell but always remained in the 100 $\text{M}\Omega$ to 1 $\text{G}\Omega$ range, as mentioned in previous reports (Evans et al., 1998; Wyart et al., 2002). We found that membrane capacitance was slightly higher in patterned cultures ($80 \pm 29 \text{ pF}$) than in control cultures ($57 \pm 20 \text{ pF}$) but also not significantly different ($t(22) = -2.15$; $P > 0.01$). Despite the small differences in mean membrane resistance and capacitance, membrane time constants, which are the product of membrane resistance and capacitance, were very similar in both patterned cultures ($19 \pm 6 \text{ ms}$) and control cultures ($21 \pm 5 \text{ ms}$) and the difference between the conditions was even less significant ($t(22) = 0.63$; $P > 0.05$).

3.5. Patterned cultures have similar distributions of GABAergic cells and synapsin I puncta as control cultures

We also wanted to compare the relative percentage of inhibitory and excitatory cells in our patterned cultures with classical, un-patterned culture. Since a majority of inhibitory cells in the hippocampus are GABAergic cells (Segal, 1991), immunoreactivity for GABA, which stains entire cell bodies and axons, is a good indicator of inhibitory cells. As seen in Fig. 9A, patterned cultures contain both GABAergic cells and non-GABAergic (most likely glutamatergic) cells. For our purposes, total cell counts were established in brightfield using differential interference contrast (DIC) microscopy and then GABAergic cells were counted by immunoreactivity for GABA, similar to previous studies (Benson et al., 1994). We found that 25% of neurons were GABAergic cells

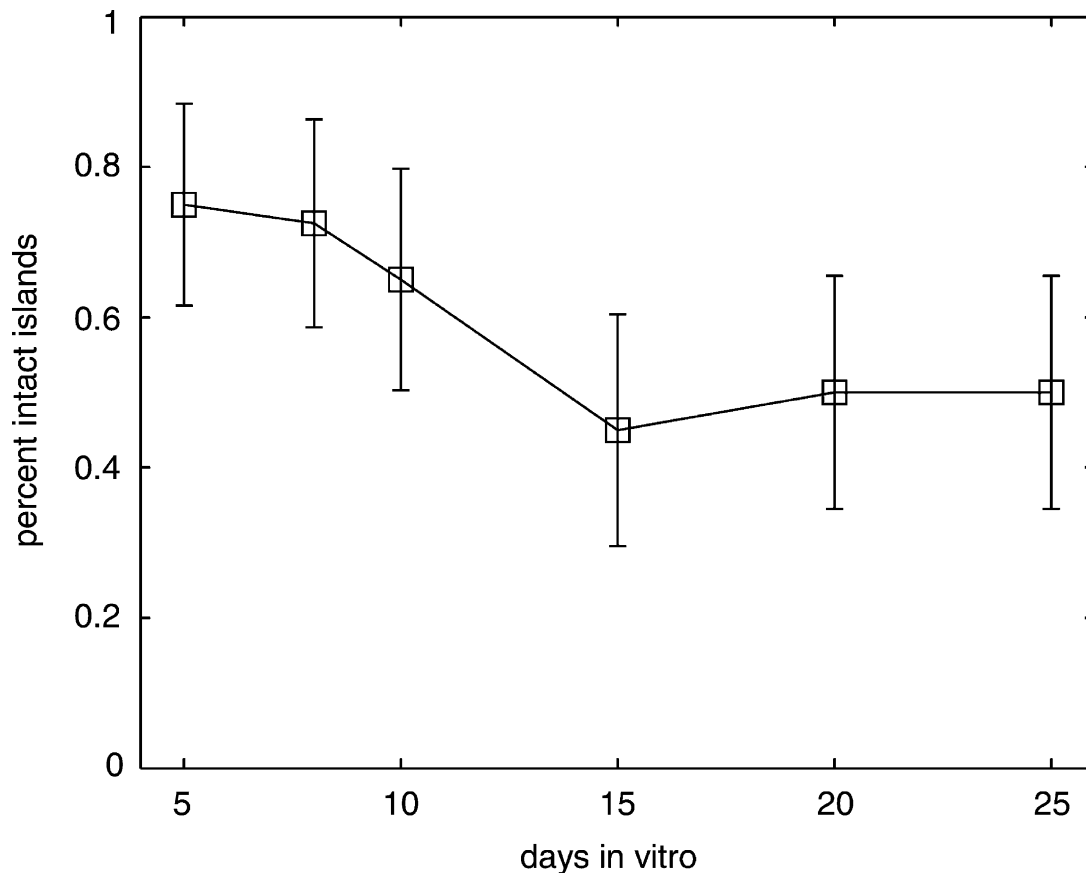


Fig. 8. Percentage of intact micro-islands. Islands were intact if no cells on the island made contact to other islands and not intact otherwise; 75% of islands were intact after 5 days in vitro and 50% were intact after 20 days ($n = 40$). Error bars denote 95% confidence intervals.

Table 1
Passive membrane properties of patterned cells and un-patterned control cells

	Resting potential (mV)	Membrane resistance ($M\Omega$)	Membrane capacitance (pF)	Membrane time constant (ms)
Control ($n = 12$)	-55.3 (8.1)	402 (163)	58 (20)	21 (5)
Pattern ($n = 12$)	-54.7 (6.6)	277 (125)	80 (29)	19 (6)

Resting potentials were measured immediately after rupture. The membrane time constant is a product of membrane resistance and capacitance. Patterned and control cultures had very similar mean resting potentials and membrane time constants. *Format*: mean (standard deviation).

in patterned cultures ($n = 308$ cells), in comparison to 33% GABAergic cells in control cultures ($n = 378$) (Fig. 9B). These percentages are not significantly different (chi-squares test, d.f. = 2; $P > 0.01$) and are comparable to previous results with hippocampal cell culture (Legido et al., 1990; Walker and Peacock, 1981).

We also compared the mean density of synapsin I puncta along axons in patterned and control cultures. Synapsins are abundant phosphoproteins found in virtually all presynaptic terminals (Greengard et al., 1993). Fig. 9C shows a sample synapsin I immunostaining of a single printed micro-island. In both pattern and control cultures, synapsin I immunoreactivity is distributed in distinct clusters, corresponding most likely to synapses in the cultures. Patterned cultures ($n = 253$ puncta) had an average of 2.95 puncta/ $20 \mu\text{m}^2$ and control cultures ($n = 276$) had

an average of 2.29 puncta/ $20 \mu\text{m}^2$ (Fig. 9D). These values are not significantly different (independent t -test; $t(46) = -2.46$; $P > 0.01$) and similar synaptic density results have been found in high-density, un-patterned hippocampal cultures (Renger et al., 2001).

4. Discussion

We have presented the design and operation of a novel ink-jet printer, pattern chemistry, and culture protocol that yields patterned neurons and glia. This is one of the first known applications of ink-jet printing to fabricating chemical patterns for neurons in culture (Roth et al., in press; Turcu et al., 2003). The method has advantages over other patterning techniques in that it is both programmable and relatively

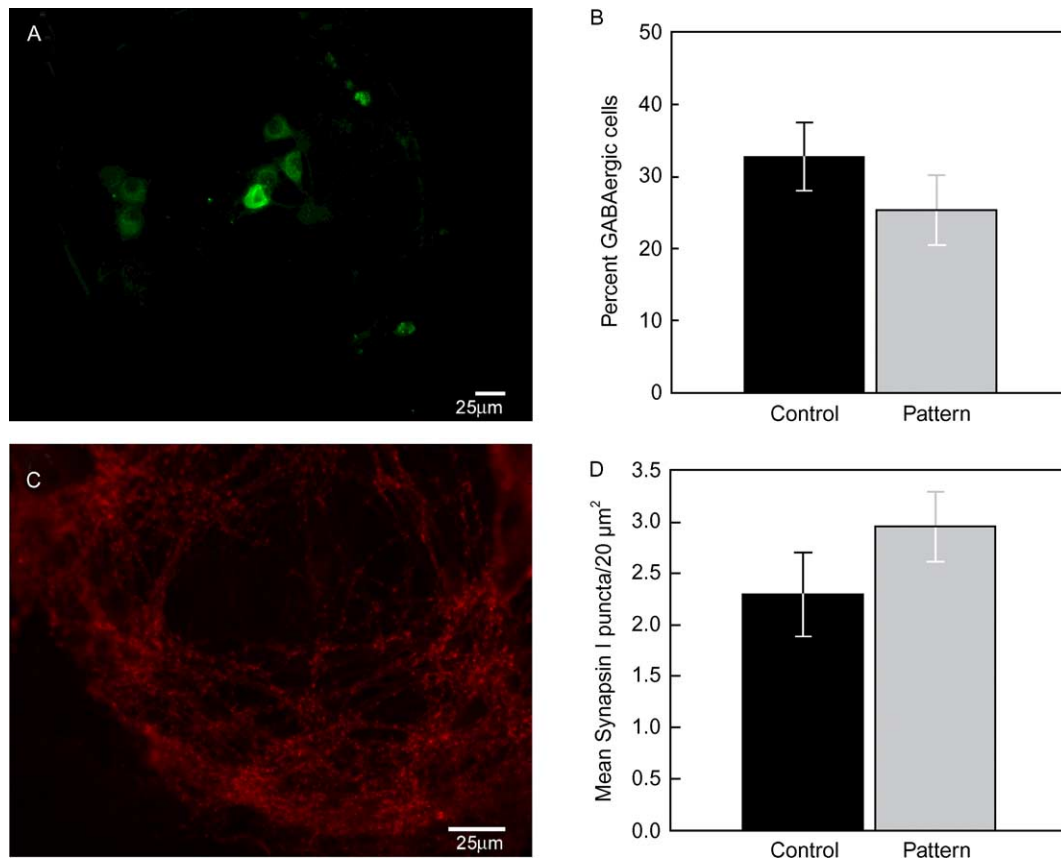


Fig. 9. Immunostaining for GABA and synapsin I. (A) A sample micro-island stained for GABA. (B) Comparison of the mean percentage of GABAergic cells in patterned ($n = 308$ cells) and un-patterned ($n = 378$) cultures. Percentages were estimated by counting cells with GABA immunoreactivity and counting all cells visible under brightfield DIC microscopy. (C) A sample micro-island stained for synapsin I. (D) Comparison of the mean density of synapsin I puncta per $20 \mu\text{m}^2$ in patterned ($n = 253$ puncta from 24 processes) and un-patterned ($n = 276$ puncta from 24 processes) cultures. In both graphs, error bars denoting 95% confidence intervals indicate that the difference between pattern and control culture populations is not significant.

inexpensive. In addition, ink-jet printing has other potential advantages for neuroscience including the ability to fabricate gradients, layered patterns, and non-planar structures much more easily than master pattern-based approaches. We expect dot size for the best, commercially-available ink-jet printing technology, which has been steadily decreasing but is fundamentally limited by fluid mechanics, to be roughly $30 \mu\text{m}$, which is lower than the $65 \mu\text{m}$ resolution demonstrated here. Newer technologies in drop generation based on print heads different than the kind used in this work, such as acoustic droplet generators, may push this result even lower in the future but printing reliability (e.g. frequency of clogging) will have to be addressed.

The surface chemistry presented uses a uniform, covalently-bonded layer of PEG as the inhibitory background and a collagen/PDL mixture printed on top as the cell-adhesive foreground. In principle, this chemistry also has the flexibility to be reversed, such that the PEG is printed atop a reactive surface of aldehyde groups and then is washed briefly with the adhesive collagen/PDL. Patterns for neurons included controlled micro-islands, lines of dots as narrow as $65 \mu\text{m}$, gaps as small as $8 \pm 2 \mu\text{m}$, and arbitrary shapes. Cell adhesion to micro-islands was com-

parable to previous techniques (Branch et al., 2000) and our electrophysiological and immunocytochemical investigations indicated that patterned cultures also displayed similar properties to classical, un-patterned low-density cultures. These results demonstrate that ink-jet printing can be used to fabricate chemical patterns that are neuron-compatible, and we suggest that the technology may prove to be useful for a range of studies on neurons in vitro.

Our recommendations are the following. For making simple, relatively low-resolution patterns, we can recommend the use of an inexpensive desktop printer and a cartridge refill kit filled with the desired biomolecule to print onto coverslips taped to sheets of regular paper (Klebe, 1988; Roda et al., 2000). We expect resolution to be lower than that demonstrated here because of less control over driver software and over the positioning precision of the head. However, such an approach would leverage sophisticated drawing software for the PC and would be sufficient to do the PDL/collagen printing on PEG reported here. A more high-end solution could use relatively expensive research-grade hardware that has been recently released for such applications (e.g. Jetlab™ II, MicroFab Technologies), or a custom-built solution such as is reported here.

Lastly, there are certain tasks which might be better suited to patterning techniques that are based on a single master pattern, but we believe these are limited because the need to change patterns is likely to arise often during experimental work. The relatively slow printing speed employed in this work for the smallest lines (one drop per second, to ensure complete drying between adjoining droplets) resulted in printing times of about 5 min per coverslip for most of the patterns used in this work, making the hardware less suitable for complex or repeating patterns. Such patterning might be better suited to photolithography or stamping.

Lastly, we believe there are many other applications for ink-jet patterning with neural cell culture. The effects of many extracellular matrix proteins and other non-endogenous signaling molecules could be tested in a combinatoric fashion using ink-jet patterning. It may be possible to print actual neurons through the print head, but problems with drying out of the neurons or changing osmolarity with evaporation during printing would have to be addressed. We have already used our printer to create three-dimensional structures, such as walls and tunnels from hot-melt wax, and substrate-bound gradients of laminin (unpublished data). We believe these preliminary results represent the future of the body of work reported here. Printed patterns and gradients of neurotrophic factors could facilitate *in vitro* studies of axon guidance, target selection and cell maturation during neural development. The potential ease of printing substrate-bound gradients—by printing varying numbers of layers of very low-concentration ink—connected to other arbitrary patterns could be a major advantage of ink-jet patterning, since many neurotrophic factors and their receptors have already been shown to use such gradients (Bonhoeffer and Huf, 1982; Dertinger et al., 2002; Houchmandzadeh et al., 2002; Sperry, 1963).

Acknowledgements

We are grateful to Professor H. Sebastian Seung (MIT Department of Brain and Cognitive Science), Dr. Shuguang Zhang (MIT Center for Biomedical Engineering), and Professor Joseph Jacobson (MIT Media Laboratory) for guidance, resources, and Lab space. We are indebted to Jung Choi for his dedication and outstanding results with neuron culture. We are grateful to Emily Hueske for multiple, insightful discussions about immunocytochemistry, to Shuwang An for assistance with surface chemistry procedures, to Thomas Griffiths for statistical advice, and to Guosong Liu, Bing Li, and Yuki Goda for guidance with culture techniques. Adrien Desjardins, David Kleinfeld, Venkatesh Murthy, Bayle Shanks, Nathan Wilson and an anonymous reviewer provided valuable comments on various drafts of the manuscript. NES is supported by a HHMI Predoctoral Fellowship. SBF is supported by a NSF Graduate Fellowship. This work was funded by the Defense Advanced Project Research Agency/BioComp, NSF

CCR-0122419 to MIT's Media Laboratory Center for Bits and Atoms for the printer, and a Whitaker Foundation grant to Shuguang Zhang. H. Sebastian Seung is funded by the David and Lucille Packard Foundation and the Howard Hughes Medical Institute.

References

- Arancio O, Kandel ER, Hawkins RD. Activity-dependent long-term enhancement of transmitter release by presynaptic 3', 5'-cyclic GMP in cultured hippocampal neurons. *Nature* 1995;376:74–80.
- Banker G, Goslin K. *Culturing nerve cells*. 2nd ed. Cambridge, Mass: MIT Press, 1998.
- Benson DL, Watkins FH, Steward O, Banker G. Characterization of GABAergic neurons in hippocampal cell cultures. *J Neurocytol* 1994;23:279–95.
- Bonhoeffer F, Huf J. *In vitro* experiments on axon guidance demonstrating an anterior-posterior gradient on the tectum. *Embo J* 1982;1:427–31.
- Branch DW, Wheeler BC, Brewer GJ, Leckband DE. Long-term maintenance of patterns of hippocampal pyramidal cells on substrates of polyethylene glycol and microstamped polylysine. *IEEE Trans Biomed Eng* 2000;47:290–300.
- Brewer GJ, Torricelli JR, Evege EK, Price PJ. Optimized survival of hippocampal neurons in B27-supplemented Neurobasal, a new serum-free medium combination. *J Neurosci Res* 1993;35:567–76.
- Cass T, Ligler FS. *Immobilized biomolecules in analysis: a practical approach*. Oxford: Oxford University Press, 1998.
- Dertinger SK, Jiang X, Li Z, Murthy VN, Whitesides GM. Gradients of substrate-bound laminin orient axonal specification of neurons. *Proc Natl Acad Sci USA* 2002;99:12542–7.
- Evans MS, Collings MA, Brewer GJ. Electrophysiology of embryonic, adult and aged rat hippocampal neurons in serum-free culture. *J Neurosci Methods* 1998;79:37–46.
- Fuller SB, Jacobson JM. Ink-jet fabricated nanoparticle micro-electromechanical systems. In: *Proceedings of the IEEE conference on micro-electromechanical systems*, 2000; pp. 138–41.
- Fuller SB, Wilhelm EJ, Jacobson JA. Ink-jet printed nanoparticle microelectromechanical systems. *J Microelectromech Sys* 2002;11:54–60.
- Furshpan EJ, MacLeish PR, O'Lague PH, Potter DD. Chemical transmission between rat sympathetic neurons and cardiac myocytes developing in microcultures: evidence for cholinergic, adrenergic, and dual-function neurons. *Proc Natl Acad Sci USA* 1976;73:4225–9.
- Greengard P, Valtorta F, Czernik AJ, Benfenati F. Synaptic vesicle phosphoproteins and regulation of synaptic function. *Science* 1993;259:780–5.
- Griffith LG, Wu B, Cima MJ, Powers MJ, Chagnaud B, Vacanti JP. *In vitro* organogenesis of liver tissue. *Ann NY Acad Sci* 1997;831:382–97.
- Haydon PG. GLIA: listening and talking to the synapse. *Nat Rev Neurosci* 2001;2:185–93.
- Heinzl J, Herz CH. Ink-jet printing. *Adv Electron Electron Phys* 1985;65:91–171.
- Hickman JJ, Bhatia SK, Quong JN, Shoen P, Stenger DA, Pike CJ, Cotman CW. Rational pattern design for *in-vitro* cellular networks using surface photochemistry. *J Vac Sci Technol Vac Surf Films* 1994;12:607–16.
- Holmes TC, de Lacalle S, Su X, Liu G, Rich A, Zhang S. Extensive neurite outgrowth and active synapse formation on self-assembling peptide scaffolds. *Proc Natl Acad Sci USA* 2000;97:6728–33.
- Houchmandzadeh B, Wieschaus E, Leibler S. Establishment of developmental precision and proportions in the early *Drosophila* embryo. *Nature* 2002;415:798–802.
- Klebe RJ. Cytoscribing: a method for micropositioning cells and the construction of two- and three-dimensional synthetic tissues. *Exp Cell Res* 1988;179:362–73.

- Kleinfeld D, Kahler KH, Hockberger PE. Controlled outgrowth of dissociated neurons on patterned substrates. *J Neurosci* 1988;8:4098–120.
- Legido A, Reichlin S, Dichter MA, Buchhalter J. Expression of somatostatin and GABA immunoreactivity in cultures of rat hippocampus. *Peptides* 1990;11:103–9.
- Martinoia S, Bove M, Tedesco M, Margesin B, Grattarola M. A simple microfluidic system for patterning populations of neurons on silicon micromachined substrates. *J Neurosci Methods* 1999;87:35–44.
- Renger JJ, Egles C, Liu G. A developmental switch in neurotransmitter flux enhances synaptic efficacy by affecting AMPA receptor activation. *Neuron* 2001;29:469–84.
- Roda A, Guardigli M, Russo C, Pasini P, Baraldini M. Protein microdeposition using a conventional ink-jet printer. *Biotechniques* 2000;28:492–6.
- Roth EA, Xu T, Das M, Gregory C, Hickman JJ, Boland T. Inkjet printing for high-throughput cell patterning. *Biomaterials*, in press.
- Sachlos E, Reis N, Ainsley C, Derby B, Czernuszka JT. Novel collagen scaffolds with predefined internal morphology made by solid freeform fabrication. *Biomaterials* 2003;24:1487–97.
- Schaffner AE, Barker JL, Stenger DA, Hickman JJ. Investigation of the factors necessary for growth of hippocampal neurons in a defined system. *J Neurosci Methods* 1995;62:111–9.
- Scholl M, Sprossler C, Denyer M, Krause M, Nakajima K, Maelicke A, Knoll W, Offenhausser A. Ordered networks of rat hippocampal neurons attached to silicon oxide surfaces. *J Neurosci Methods* 2000;104:65–75.
- Segal MM. Epileptiform activity in microcultures containing one excitatory hippocampal neuron. *J Neurophysiol* 1991;65:761–70.
- Sperry RW. Chemoaffinity in the orderly growth of nerve fiber patterns and connections. *Proc Natl Acad Sci USA* 1963;50:703–10.
- St John PM, Kam L, Turner SW, Craighead HG, Issacson M, Turner JN, Shain W. Preferential glial cell attachment to microcontact printed surfaces. *J Neurosci Methods* 1997;75:171–7.
- Turcu F, Tratsk-Nitz K, Thanos S, Schuhmann W, Heiduschka P. Ink-jet printing for micropattern generation of laminin for neuronal adhesion. *J Neurosci Methods* 2003;131:141–8.
- Walker CR, Peacock JH. Development of GABAergic function of dissociated hippocampal cultures from fetal mice. *Brain Res* 1981;254:541–55.
- Watanabe K, Miyazaki T, Matsuda R. Growth factor array fabrication using a color ink jet printer. *Zoolog Sci* 2003;20:429–34.
- Westermarck B. Growth control in miniclones of human glial cells. *Exp Cell Res* 1978;111:295–9.
- Wheeler BC, Corey JM, Brewer GJ, Branch DW. Microcontact printing for precise control of nerve cell growth in culture. *J Biomech Eng* 1999;121:73–8.
- Wilson Jr WC, Boland T. Cell and organ printing 1: protein and cell printers. *Anat Rec* 2003;272A:491–6.
- Wyart C, Ybert C, Bourdieu L, Herr C, Prinz C, Chatenay D. Constrained synaptic connectivity in functional mammalian neuronal networks grown on patterned surfaces. *J Neurosci Methods* 2002;117:123–31.
- Zhang S, Holmes T, Lockshin C, Rich A. Spontaneous assembly of a self-complementary oligopeptide to form a stable macroscopic membrane. *Proc Natl Acad Sci USA* 1993;90:3334–8.
- Zhang S, Holmes TC, DiPersio CM, Hynes RO, Su X, Rich A. Self-complementary oligopeptide matrices support mammalian cell attachment. *Biomaterials* 1995;16:1385–93.
- Zhang SG, Yan L, Altman M, Lassel M, Nugent H, Frankel F, Lauffenburger DA, Whitesides GM, Rich A. Biological surface engineering: a simple system for cell pattern formation. *Biomaterials* 1999;20:1213–20.