A Fast Flexible Ink-Jet Printing Method for Patterning Networks of Neurons in Culture

by

Sawyer Buckminster Fuller

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Submitted to the Department of Mechanical Engineering in Partial Fulfillment of the Requirements of the Degree of

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Abstract

Dissociated nerve cells in culture are an accessible experimental tool for analyzing the behavior of networks of nerve cells that underlie the operation of the brain. Previous work has demonstrated the ability to constrain and influence the behavior of growing neurons by arrangement of substrate-bound chemical patterns, but these techniques have various limitations. In particular, techniques that employ photolithography to form patterns, such as microcontact printing, photolithography, and microfabricated microfluidics, require the use of an un-alterable previously existing mask. In this work a high-resolution ink-jet printer was designed and built to programmably pattern surface materials for neuroscience, overcoming some of the weaknesses of lithographic-based fabrication technology. An ink-jet printer operates by individually ejecting microscopic liquid droplets, 10 to 100 micrometers across, under computer control, as the print head is robotically moved in two dimensions a small distance above a substrate, leaving a pattern of deposited material. A range of biomaterials, including MatriGel, collagen, polylysine, RADA-motif self-assembling peptides, and laminin were printed. Ink-jet printed patterns of collagen/polylysine ink onto inhibitory polyethylene glycol backgrounds constrained the growth of primary rat hippocampal neurons for periods longer than three weeks. Patterns included dots and lines with feature sizes as small as 65 micrometers, connected islands, adjacent parallel lines with gaps as small as 5 micrometers maintained over millimeter distances, text, and out-of-plane wax structure such as walls and tunnels. Neurons on these patterns showed healthy resting potentials, spontaneous activity, and comparable concentrations of inhibitory cells and synaptic puncta to non-patterned control cells. The printer was also able to fabricate surface-bound gradients of laminin, which could enable directional axon specification. The printer thus provided a rapid means for arranging chemical constraints for neuron growth and proved to be a versatile tool able to aid in unique experiments. This work represents the first known application of ink-jet printing to patterning neurons in culture.

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"We Came in Peace for All Mankind."

– Inscription on the plaque left by the first human astronauts on the Moon

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Biographical Note

Sawyer Buckminster Fuller received the Bachelor of Science degree in Mechanical Engineering from the Massachusetts Institute of Technology in June 2000 and received the Master of Science Degree in September 2003 from the same institution.

He is a National Science Foundation Graduate fellow in the area of Neurosciences and an MIT Presidential Fellow. He has published in two conference proceedings and is the first author of the refereed journal article "Ink-Jet Fabricated Nanoparticle Micro-Electromechanical Systems," *IEEE Journal of Micro-Electromechanical Systems*, February 2002. He is the recipient of a NASA Technology Award for the design and construction of a hopping robotic rover at the NASA Jet Propulsion Laboratory, which is described in the February 2003 edition of NASA Tech Briefs. His work on ink-jet printed electronics and MEMS under Prof. Joseph Jacobson was described in the "How Things Work" section of USA Today and was featured in the cover article of the November 2000 issue of *Technology Review,* ("Print Your Next PC: Research at MIT's Media Lab could make it possible to print out hardware right on the desktop"). Sawyer placed 2nd in MIT's famous 2.007 Robotics Design Contest and received the Hoist and Crane Mechanical Engineering Department Award for his undergraduate thesis. Sawyer was an undergraduate teaching assistant in MIT courses 2.007 (Introduction to Mechanical Design) and 2.670 (Autonomous Lego Robotics Contest).

Sawyer enjoys windsurfing, drawing, and painting.

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Chapter 1

MOTIVATION

1.1 Dissociated neurons in culture

The task of understanding the operation of neurons is of legendary difficulty. Mathematical models and experimental technique remain separated by an appreciable divide. And theoretical neuroscientists must choose which of a nearly limitless array of equally mysterious neural behaviors to attempt to explain mechanistically. To make headway, a neuroscientist may choose to invent further refinements to theoretical mathematical models, often inspired by experimental findings, or he or she may attempt to improve their experimental technique. Most techniques in neuroscience to detect the activity of neurons share a common attribute: they detect the aggregate activity of many individual neurons. Developing techniques to reduce the number of neurons whose activity is being monitored is a promising approach for the experimental neuroscientist.

The use of dissociated networks of cultured nerve cells provides an experimentally accessible tool for observing the behavior of relatively low numbers of nerve cells (Banker and Goslin, 1998). In dissociated cultures, the structural integrity of the tissue is lost, but cells soon re-grow neurites and synapses in culture. The low density of nerve cells, in comparison to that found in the brain, and the visible arrangement of neurites, facilitates observation of individual or small-scale patterns

of activity. In comparison to other experimental paradigms that are under active study, including brain tissue slices in culture and electrodes or remote sensing in awake or anesthetized animals, dissociated neuronal networks are more spread out and their connectivity is potentially controllable. For instance, using dissociated cultures, synapses and other characteristics have been analyzed by immunostaining their activity and connectivity has been assessed traditionally by patch clamp recording (Neher and Sakmann, 1976). Other techniques such a calcium-sensitive dye imaging (Grinvald et al, 1981), micro-electrode arrays (Thomas et al, 1972; Gross et al, 1977; Segev et al, 2001; Segev et. a. 2002; Shahaf and Marom, 2001; Demarse et al, 2001) and micro-wells (Maher et al, 1999 and 1999), semiconductor logic elements (Fromhertz et al, 1991; Zeck and Fromherz, 2001), and photoconductive stimulation (Colicos et al, 2001) have added other capabilities for input and output to neurons. Because of their low concentration, and thus their accessibility to experimental control and observation, dissociated neuronal networks are among the best experimental paradigms for deducing the behavior of neurons at the individual level.

1.2 Hippocampal neurons

The use of rat hippocampal neurons balances the need for a simple cell system and the need to tackle some of the most neurologically interesting questions concerning the basis of learning and memory. Hippocampal cells have the additional advantage that they are similar enough to human neurons discoveries pertinent to them are more relevant to medical applications. The hippocampus is an ideal source of neurons because it is a part of the old cortex, having of three layers of neural tissue. The neocortex, which covers the top portion of the brain and has the characteristic wrinkles and serves as the tissue in which much of our conscious thought is carried out, has multiple varying types of cells projecting to six or more different layers and is evolutionarily more advanced and more complex than the old cortex (Kandel et al,

2000). In contrast, the hippocampus has many fewer types of neurons, making cultures much more homogeneous (Banker and Goslin, 1998). In addition, the operation of the hippocampus has been extensively studied, owing to its relatively simpler organization and its role in memory formation, which is a relatively quantifiable neural phenomenon. While it is a relatively small part of the brain, the simplicity, relative familiarity, and the homogeneity of the hippocampus make it an ideal source of dissociated neurons.

1.3 Patterning

Early work in nerve cell culture relied on entirely random dissociated arrangements of connected neurons for a variety of studies. Later work added the capability of making varying sizes of micro-islands (Banker and Goslin, 1998). But recent work has been concerned with more highly-constrained the two-dimensional arrangement of chemical patterns on the growing substrate so that the growth and behavior of neurons is affected. Techniques to do this patterning have included photolithography for surface patterns (Hickman et al, 1994; Wyart et al, 2002), photolithography for surface topography (Dowell et al, 2001; Turner et al., 1997, 1999, 2001), microcontact printing (Branch et al, 2000; Yeung et al, 2001) to microfabricated silicon 3D neuron wells (Maher et al, 1999). These results have demonstrated the ability to pattern individual neurites and to physically constrain cell bodies. Other experiments have used patterned chemicals without photolithography to make simple substrate-bound patterns. For instance, posterior retinotectal neurons have been shown to be repelled by stripes of tissue from the anterior optic tectum (the active proteins later were found to be the ephrins and eph receptors) (Walter et al, 1987). A later experiment used a novel method to generate a surface density gradient of membrane suspension on a porous substrate and found that neurons read the gradient slope rather than its absolute value (Baier and Bonhoeffer, 1992). Stripe assays using silicone rubber fluidic channels have shown that the first neurite among the original

equipotent neurites to reach a laminin surface undergoes a growth spurt and usually becomes the axon (Esch et al, 1999). And surface-bound gradients formed by laminar flow of micro-mixed microfluidic reactors have been shown to cause neurons to preferentially induce axogenesis in a direction that is closer to up gradient than down-gradient (Dertinger et al, 2002).

1.4 Advantages of ink-jet printing

While the approaches described above have been powerful for elucidating certain characteristics of nerve cell behavior, they have limitations that suggest pursuing other possible experimental approaches. In particular, all of the technologies described rely on the use of an unchangeable master pattern: a mask in the case of photolithography, a stamp master in the case of microcontact printing, and other lab apparatuses for other various techniques. Here, we introduce the use of ink-jet printing as a means for patterning arbitrary chemical patterns for use in neuroscience study. Ink jet printing offers many of the capabilities of the techniques described above, including the ability to print narrow lines and surface-bound gradients, with the additional characteristic that it is programmable. In addition, it has an integrating characteristic, in that it can be used to form both gradients and to build other structures connected to them. Ink-jet printing's programmability and versatility should allow the next generation of studies in nervous system development and programmed arrangement of theoretically interesting neural constructs, and they can be done with a rapidly-alterable programmed pattern.

Ink-jet printing is a technology familiar to most by its use in low-cost desktop color printers. Its essence is the computer-controlled ejection of single microscopic droplets of ink into computer-defined arrangements on the page. Though its droplet metaphor limits its ultimate resolution because of fundamental fluid mechanical principles (Heinzl and Hertz, 1985), it is unique among printing technologies in how

amenable it is to use in a biological context. In particular, because material is deposited in liquid form, water-based chemistry, which is prevalent in biology, can be used to bond printed materials onto the substrate or act in more sophisticated catalytic manners. In addition, ink-jet printing can deposit multiple layers of materials, large volumes of soluble factors immobilized in a gel, chemistry designed for timed release should be possible, and substrate-bound gradients, and could aid immensely in developmental studies. Further, ink-jet has the ability to layer material to make out-of-plane structure, such as physical barriers for neurons or liquids. Thus, in addition to the advantages described above, ink-jet printing has other unique advantages that may prove useful for neuroscience study.

The primary limitation for ink-jet printing for certain biological applications is its relatively low resolution compared to other techniques. While droplets ejected by the most sophisticated print heads known will leave dots approximately 20-30 micrometers across, this is larger than the 3 micrometers it has been found is ideal to constrain single neurites (Wyart et al, 2002). However, it may be possible to achieve similar resolution with ink-jet printing by arranging parallel lines of droplets spaced so that the gap between them is that small. This report details attempts to achieve this with ink-jet printing (Figure 13).

1.5 Substrate-bound gradients

Previous work has indicated that laminin and other substrates may act to influence axon specification during the development of neurons. Shortly after neurons settle to the bottom of the well after being dissociated, they sprout multiple processes. Eventually, one undergoes a growth spurt, becoming the axon. This neurite invariably becomes the axon (Bradke and Dotti, 1999; Esch et al, 1999). It has been found that the first neurite to reach an area of laminin and other substrate-bound cell adhesion molecules undergoes a burst of growth, thereby specifying itself as the

axon (Esch, et al, 1999). Further, other studies using microfluidics to generate a substrate-bound gradient of laminin have shown that such gradients can influence the direction of axon specification to be primarily up gradient (Dertinger et al, 2002). The ability to influence the direction of axon outgrowth adds capability to neural circuit assembly control in vitro. For instance, two islands could be influenced to communicate in primarily one direction, important for performing studies of Hebbian learning in culture.

1.6 Approach and summary

This report describes the first-known effort to use ink-jet printing to pattern networks of neurons in culture. The author is aware of no previous work using ink-jet printing for neuroscience. The approach taken was to design and build a technology-intensive custom-made ink-jet printer designed to precisely position droplets of potentially expensive materials, and then to use it to prove its usability for neuron culture. Results show that hot-melt wax ink-jet printing is marginally repulsive to neuron growth and can be used to build non-planar structures. Ink-jet printing is shown to be able to fabricate improved micro islands, an experimental paradigm previously achieved with random dot sizes at random locations using airbrush (Banker and Goslin, 1998). The long-lived micro-islands, made of a collagen/polylysine ink printed onto a uniform inhibitory background coating of poly-ethylene glycol, were of a constant size and placed in arbitrary and repeatable locations. The micro-island neurons were healthy in comparison to non-patterned control cells, as suggested by evidence that patterned cells exhibit comparable resting potentials, membrane resistance and capacitances, spontaneous activity, and distributions of synapses and inhibitory cells. With the same materials, other patterns were printed that showed strong cell adhesion, including islands connected by only neurites and alphanumeric text. Ink-jet printing was then demonstrated to be able to fabricate substrate-bound gradients. Other results included printing an argenine-alanine-aspartate-alanine

(RADA)-motif self-assembling peptide (Holmes et al, 2000; Zhang et al., 1993, 1995, 1999) that was neuron-adhesive and inventing a serine-threonine (TS) repeat hydrophilic peptide designed to mimic the neuron-repulsive properties of poly-ethylene glycol.

Chapter 2

INK-JET PRINTER DESIGN

2.1 History and principles of ink-jet printing

Ink-jet printing has been the primary technology for low-cost desktop printers, with the newest full-color devices costing less than $50. While laser printers and other technologies require high temperatures, complex and precise laser positioning, and other technologies, ink-jet printers seem to offer the least complex means for printing.

The earliest implementation of ink-jet printing was in industrial-grade continuous-jet printers used for covering large areas and for product marking. These printers used an electrically charged continuous stream of ink, which separated into tiny droplets because of a surface tension instability called the Rayleigh instability (Heinzl and Hertz, 1985). An electrical field between two parallel plates electrostatically deflected individual droplets in the charged stream. Dots were deflected along one axis as the substrate was moved along the other axis, and excess droplets were deflected to a catch. Continuous-jet printers offered high throughput, though at the expense of some droplet precision. Such printers are still being used today, but not on the desktop.

A later development was the advent of drop-on-demand (DOD) printing. In DOD printing, droplets were individually ejected by means of a pressure impulse in the ink chamber. Means for generating the pressure impulse ranged from the use of electrically deformable piezo crystals to electrostatic deflection of a membrane to the rapid vaporization of tiny volumes of liquid in thermal communication with a small heater. The pressure wave in the ink chamber was propagated to the nozzle orifice, where a droplet was ejected, but only if the energy content of the pressure wave was of greater value than the surface tension energy of the ejected droplet. The print head was moved robotically in two dimensions over the substrate to leave a computer-controlled arrangement of dots as droplets are ejected on computer command. The individual droplets were ejected on computer command and took a ballistic trajectory at 1 to 10 meters/second to a substrate 1 to 10 mm away. The droplet diameter ranged from 150 to 15 micrometers (Heinzl and Hertz, 1985). Depending on the chemical makeup of the droplet, liquids would evaporate, leaving a round deposit of solids on the substrate (Figure 1). DOD printers have been used in industrial marking, desktop printing, and in other recent non-document applications. They had the advantage that relatively fewer parts were needed, ink cartridges could be replaceable, and that droplet placement repeatability was high.

The first desktop ink-jet printer, the ThinkJet, was released by Hewlett-Packard in the early 1980's and used thermal evaporation technology to generate the pressure impulses. Its droplets were relatively large and its resolution was an unimpressive 150 dots per inch. Later other companies would release piezo-driven printheads (Dataproducts, Epson), and thermal print heads (Hewlett-Packard, Canon, Lexmark). Most print heads were released with water as the ink solvent, rathern than an organic solvent, because of health reasons, but Dataproducts, which released network-class printers that ejected solid hot-melt wax, took a different approach. The advantage of wax was that it solidified on the paper nearly instantaneously, but overall cost of the printer was raised because of the complexity of the wax melting system.

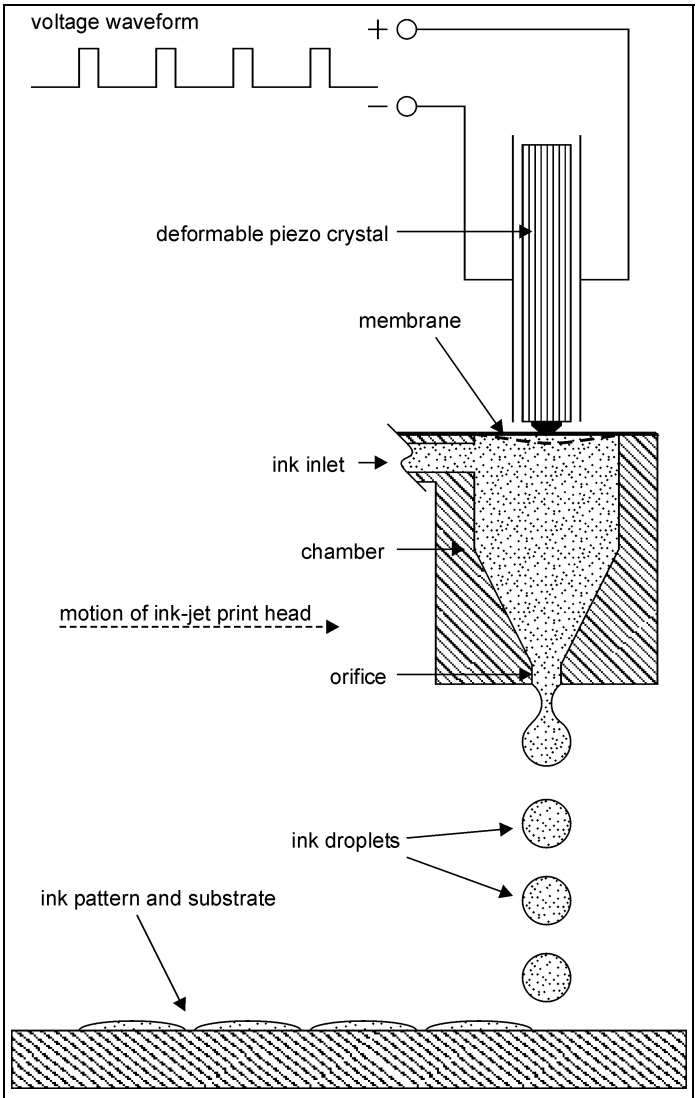


Figure 1. Diagram of piezo ink-jet printer.

Ink-jet printing, however, has more recently emerged as a potential fabrication technology for applications other than document preparation. Notable uses have included three-dimensional rapid fabrication. Research from Elle Sachs' group at MIT (Williams, 1990) and now marketed by Zcorp (Cambridge, MA) uses an ink-jet printed resin binder deposited into successive layers of loose powder, and 3DSystems offers a printer that builds structure from wax using a Dataproducts print

head. Organic light-emitting diodes (OLEDs) may be deposited by ink-jet printing to fabricate full-color flat panel displays (Chang et al, 1998; Hebner et al, 1998). Cambridge Display Technology (Cambridge, UK), among others, is developing an ink-jet printed OLED display technology for market. And ejecting molten solder to make bumps for circuit flip-chip packaging and other applications has been explored in Professor Ain Sonin's lab at MIT (Duthaler, 1995) and at Microfab Technologies (Plano, TX) (Wallace and Hayes, 1997).

Ink-jet printing has been used to build active electronic circuits and micro-electromechanical machines by the author in a previous work, demonstrating its versatility. The author earlier reported the first known ink-jet printed nanoparticle suspensions, and showed the process could produce devices similar to what could be fabricated in a silicon fab (Fuller and Jacobson, 1999; Fuller et al, 2002; Mihm, 2000). Nanoparticles, measuring 1 to 100 nm across, are small clusters of atoms that melt together and fuse into a continuous material that is similar to the bulk material at temperatures as low or lower than 300 C, depending on their size. Printed nanoparticle devices included simple circuits, resonant inductive coils, and micro-electromechanical machines including electrostatic motors and electrothermal actuators. And all devices were printed additively and programmably in a manner of minutes. The devices were made of gold and silver and exhibited conductivity, assessed with the aid of an atomic force microscope to measure cross-sectional area, within a factor of 2 to 4 of the bulk material. In distinct contrast to the extreme processing conditions and mask-based fabrication in modern chip fabrication facilities, the ink-jet printer was able to print relatively inexpensive chip-like devices onto flexible plastic in a programmable fashion. It is speculated that such metal-fabricating devices could potentially be applied to build electrode arrays and other unimagined structures for neuroscience.

Ink-jet printing for bioscience and biotechnology has been explored for DNA microarrays and proteomic sensors, and for building 3D scaffolds for building artificial organs such as a liver (Griffith et al, 1997). However, the author is aware of no results pertaining to the use of ink-jet printing for neuroscience. Unique constraints include the need to print patterns that adhere onto glass coverslips (to have compatibility with the optics and other characteristics of the electrophysiological apparatus), the ability to deposit large volumes of material to act as diffusion point sources of soluble chemoattractant factors such as netrin, and the need for high resolution to pattern single neurite growth.

2.2 Experimental requirements

The printer was designed with the purpose of maximizing the theoretical resolution of ink-jet printing. It was known that desktop printer manufacturers were secretive about their hardware because of competition in the industry so little help could be expected from manufacturers for modifying their hardware to print very specific patterns. While it was known that the dots themselves would measure 20 to 100 micrometers, depending on the printhead technology used, it was possible to arrange neighboring stripes of dots so that the distance between them was less than 10 micrometers, making the technology potentially useful for experiments concerning single axons, dendrites, or other cellular features. In addition, other experiments, such as forming three-dimensional structures were considered interesting, and high droplet placement precision was needed for experiments in that area.

Because the device would be used for biology, another requirement was for it to be both mobile and to use relatively little bench-top space. By being mobile, the device could be moved between labs if necessary for different experiments, or into a cold room, or sterilized and placed inside a sterile laminar flow hood for in-culture

experiments such as injecting material onto living cells or into culture media, or placed in non-air atmospheres.

Lastly, and most importantly, the study proposed would be designed to push the limits of ink-jet printing technology for novel material patterning, so it was required that the priming volume of the print head be relatively small. Expensive proteins or custom-designed peptides could stand as a prohibitive obstacle if large volumes of ink were required.

2.3 Implementation

2.3.1 Print nozzle for water and solvent-based inks

A company that focuses on the technological development of ink-jet printing was selected as the supplier for the print head, primarily because they were the only known company to offer single-nozzle print heads for sale. The single-nozzle design allowed printing to be carried out with very small priming volumes. The liquid dispensing nozzle orifice had a diameter of 30 micrometers (model MJ-AB-030, Microfab Technologies, Plano, TX) and used an annular piezo crystal to generate the pressure impulses necessary to eject droplets (Figure 2). A 30-micrometer orifice was the smallest that operated reliably. Voltage pulses that drove the piezo crystal in the printhead were generated by specially designed driver hardware ("JetDrive III" controller, Microfab Technologies) with a proprietary serial RS-232 command set. Voltage pulses to jet water were 28 V for 20 microseconds, with frequencies ranging up to 1kHz in this study.

The primary problem with the head was that its nozzle was made of glass, the same material that our work was potentially attempting to use as the adhesive substrate. While materials were printed which were known to adsorb to glass, chemicals that were highly reactive with glass, such as silanes, were not pursued because they

would have potentially permanently altered the wettability of the orifice, inhibiting printing.

The ink feed for the liquid print head consisted of a 20 cm length of small-diameter Tygon tubing attached to a 5 mL syringe. Ink was back-filled by generating negative

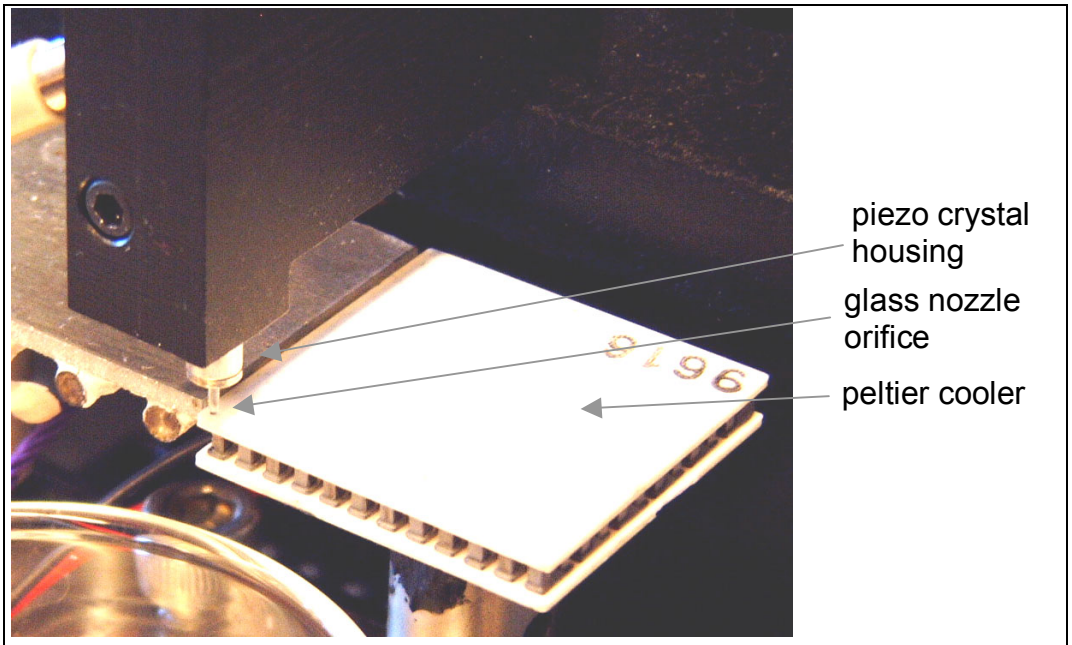


Figure 2. Detail view of the print nozzle showing the glass orifice and the aluminm housing for the annular piezo crystal. A black delrin part acts as the holder for the nozzle. The white peltier cooler, which acts as the support for the substrate, is one inch square.

pressure in the syringe to pull liquid in through the tip of the nozzle, effectively filtering it. The tubing was attached directly to the barbed fitting of the print head and was attached to the syringe's luer fitting through a needle of slightly larger diameter than the inner diameter of the tubing. 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. The plunger of the syringe was adjusted until the printhead was neither experiencing substantial positive or negative pressure. A small low-lint towel, such as a Kim-wipe, 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, so

that the presence and quality of the expulsion could be detected by naked eye as a tiny stream of microscopic droplets leaving the tip of the printhead. Once the stream was satisfactory (visualizing was facilitated by arranging a black backdrop to the stream of droplets), the print head was then commanded to eject droplets at 10 Hz continuously during all idle periods to insure that it does not dry out and clog during periods of inactivity, but a small enough volume to be of negligible ink usage. Because any small piece of dust from the air could clog the microscopic orifice, all surfaces in contact with the ink were repeatedly flushed with filtered water before being assembled.

Prior to printing, all surfaces in contact with the culture were sterilized with a mixture of 70% ethanol: the printing surface upon which the substrate was placed, and the fluid flow channel prior to loading the ink. Prior to cell plating, the printed coverslips were soaked in serum or chemicals designed to assure adherence or repulsion. Before plating, the slides were sterilized by ultraviolet radiation for 15 minutes to reduce the risk of contamination.

2.3.2 Print nozzle for hot-melt wax

For the purposes of solid wax printing, a solid wax print head (“Jetstack” generation II 48-nozzle linear array, kindly donated by Hitachi Koki Imaging Solutions, Inc.), designed for a since-discontinued desktop printer model, was used because of its relative ease of use in comparison to more modern custom-designed print heads with integrated electronics and wax heating elements. The head was modified by bonding a 6 inch piece of insulated 120V heating wire to the aluminum body of the print head using RTV compound. Temperature was controlled in a proportional-integral-derivative closed-loop using a thermcouple and a temperature controller with a power relay operating in pulse-width-modulated (PWM) mode (Series 965, Watlow Electric Manufacturing Company, St. Louis, Missouri). To guard against the hazard

of flammables out-gassing and creating an explosion hazard, the temperature setting was set to below the flash point of the wax. Wax was loaded by using a standard biological-grade all-plastic syringe filled with wax in pellet form, which had been heated in a laboratory oven to its melting temperature. The head was primed by forcefully injecting the wax into one of its inlet ports while plugging the other. (It was important to wear safety goggles during this step because of the potential hazard of streams of high-pressure wax leaking from imperfect seals during priming.) The wax did not need to be filtered because the print head had built-in filters.

2.3.3 X-Y stage

The print heads were fixed over a moving surface controlled by two perpendicular leadscrew-driven servomotors (Model 850-G-HS, Newport Corporation, Irvine, California). These were driven by closed-loop control by a three-axis controller (Model ESP-300, Newport Corporation). Position and initialization commands were sequentially sent to the stage controller via serial RS-232 commands. The printing surface was horizontal, allowing the substrate, such as a round glass coverslip, to be positioned on top. The servomotors were mounted onto linear stages with ball-bearings rolling between stainless steel rods (Newport model 443 for the X-direction and model 423 for the Y-direction). The larger travel (approximately two inches) in the X-direction was needed to allow the use of multiple print heads simultaneously. The extra stiffness of a crossed-roller stage using cylindrical bearings in V-ways was deemed unnecessary considering the negligible load applied to the stage.

While leadscrew-based positioning systems are relatively slower than ballscrew or linear motor drive systems, they have the advantage that they are substantially less costly, and take up less space. And because controlled velocities were needed at relatively high speeds, and precision down to approximately 1 micrometer were desired, it was believed that stepper-motor driven stages would not be acceptable.

2.3.4 Substrate temperature control

In some cases, it was desirable to control the temperature of the substrate, such as a glass coverslip, as the droplet pattern was printed. A temperature controller (Watlow Series 965) was employed in closed-loop form with a temperature-detecting thermocouple to regulate the surface temperature. A small peltier cooler (Tetech, Traverse City, MI), bonded to a heat sink designed for a Pentium II microprocessor in a PC, was used to heat or cool the substrate, depending on the voltage polarity applied to the peltier cooler.

Thermal calculations were performed as follows. The requirement was that the top of the peltier cooler remain at 0 C while in the immediate vicinity of the heated wax

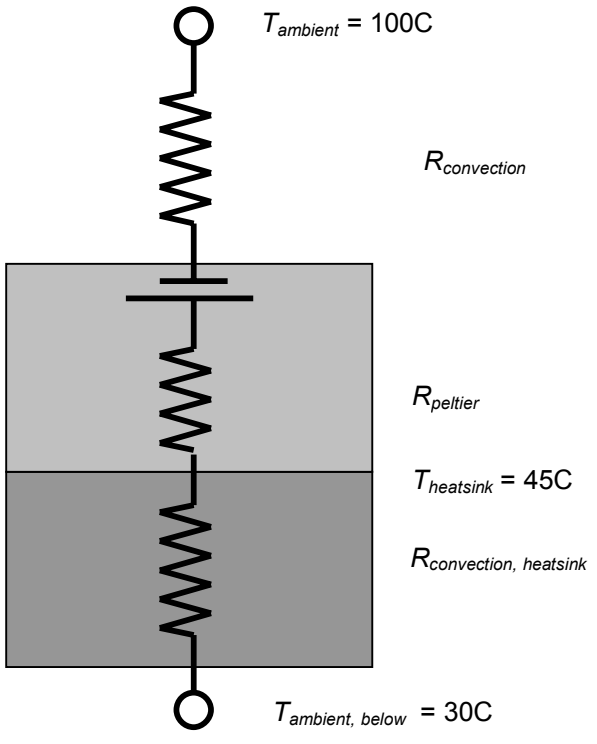


Figure 3. The thermal circuit for peltier substrate cooler.

print head. The ambient temperature of the air above the peltier was assumed to be 100 C as a result of the heating from the print head, and the ambient temperature of the air passing through the heatsink, which was separated from the hot printhead, was assumed to be 30 C. A gas convection heat transfer coefficient, h, for the top of the peltier cooler was assembed to be 20 $Wm^{-2}K^{-1}$, out of a possible range of 1 to 30 cited for gas convection, because the motion of the print head over the substrate was expected to increase convection slightly and because a higher convection coefficient gave an upper bound to the required amount of thermal dissipation needed. Using the equation $Q=hA\Delta T$ where Q is the thermal power in watts and A is the area in square meters, the thermal power needed by 1-inch square peltier was 1.2 W, which was rounded up to 2 W for further safety margin. Each peltier cooler comes specified with a parameter called Q_{max} that specifies the maximum thermal energy it can transfer, though at relatively low efficiency, and normal operation should be specified so that the cooler runs at a fraction of that value. A 5V power supply was selected that could drive the peltier at up to Q_{max}. The heatsink was assumed to be a small temperature above ambient, 45 C, so that the ΔT for the peltier was not outside specification (45 C as opposed to 70 C). For added safety, a heatsink was selected that could sufficiently cool the substrate without using a fan, though a fan was included, assuming a low thermal efficiency of the peltier cooler. For a $Q_{peltier}$ of 15 W, the thermal resistance $R_{heatsink} = (T_{ambient} - T_{heatsink})/ Q_{peltier}$ = 1 W/C. A Pentium II heatsink (Wakefield, part 863X1-100AB) was of sufficient size. While these parameters were not heavily optimized, that was not necessary because of the small power being used and because of the author designed conservatively because of inexperience.

An additional valuable aspect of the peltier cooler was that the polarity of the voltage and the control loop could be reversed to enable substrate heating, which was useful for increasing the evaporation rate.

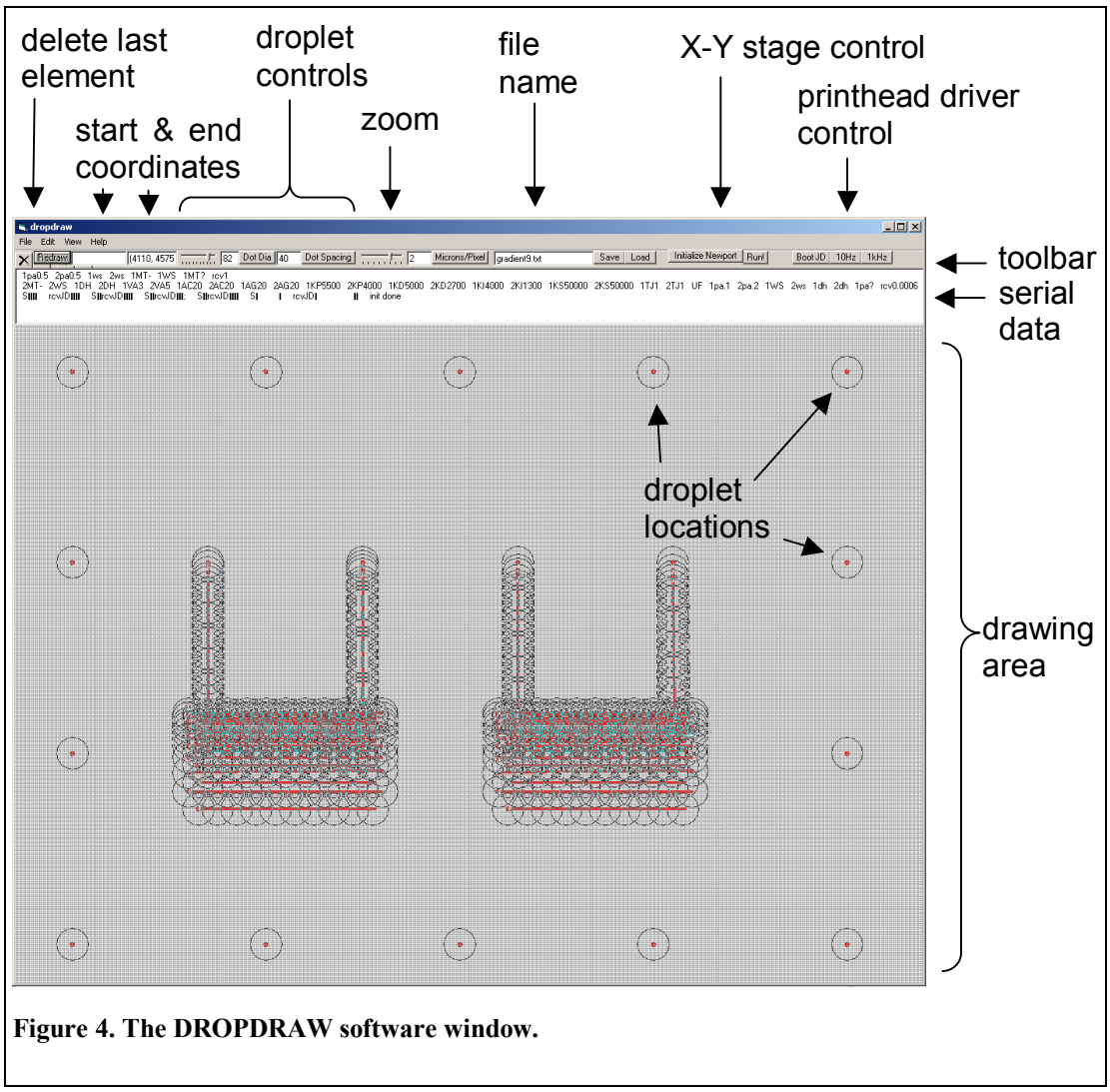


Figure 4. The DROPDRAW software window.

2.3.5 CAD interface

To facilitate the design of patterns, a custom CAD interface, named DROPDRAW, was developed to visualize the expected shape of the printed pattern. To allow for varying dot sizes that result from differing substrate wetting angles, the size of circles, drawn to represent dots, is variable from program to program. In this way, once the size of a dot was measured on a given substrate, the size of the circle could be calibrated for the purposes of visualization during drawing patterns.

The software was developed explicitly for this purpose in Microsoft Visual Basic, with which the author was familiar for the purposes of communicating with other devices over a serial RS-232 port. The interface was designed to present the user with a rendering of the drawing to be printed in the main window, with a series of command buttons across the top of the screen and a text box with status information about the printing process below the command button toolbar (Figure 4). Using this software, a drawing could be developed, and then printed when ready.

In addition, a secondary piece of software was developed to control the waveform of the voltage signal to the print head before it was integrated into the DROPDRAW interface (Figure 5). This software was used to find the driving voltage and pulse duration that worked under most circumstances for the different inks and print heads.

A given drawing in the DROPDRAW software consisted of a set of initial parameters for printing (such as the expected dot size, initial zoom state of the drawing, etc.), and a series of elements. The elements were either be large dots left after an arbitrary number of droplets are deposited at a given position, or arbitrary-length lines over which droplets were deposited, evenly spaced. For dot-type elements, the nozzle was positioned and then commands were sent to the print head driver controller to expel a certain number of droplets while the nozzle remained stationary. Depending on the number of droplets, a dot of a chosen size was left behind. This type of element was useful for making micro-islands of controlled size. The second type of element was useful for making lines of droplets. The nozzle was commanded to move in an arbitrary angle and distance, and the print head driver box was instructed to eject the correct number of droplets at the correct temporal frequency so that they were deposited at a specified spatial frequency on the substrate. The system relied on closed-loop control of the velocity of the motors to insure the trajectory was precisely followed. To insure that motor velocity was consistent throughout the trajectory, the motors were commanded to move so that

they had a lead-in and follow-through distance of sufficient length to insure that they had reached full speed before reaching the position of the first droplet. After the motors reached full speed during the lead-in stage, the motor controller hardware sent a voltage pulse through one of its digital input/outputs directly to the JetDrive box to begin ejecting the string of droplets at the commanded frequency. Experiments to verify the positional accuracy of the voltage pulse by comparing droplet position left during a purely X-motion to that of a purely Y-motion showed that this method was able to place droplets at precise locations with accuracy greater than that of droplet placement (at best, approximately +/- 5 micrometers).

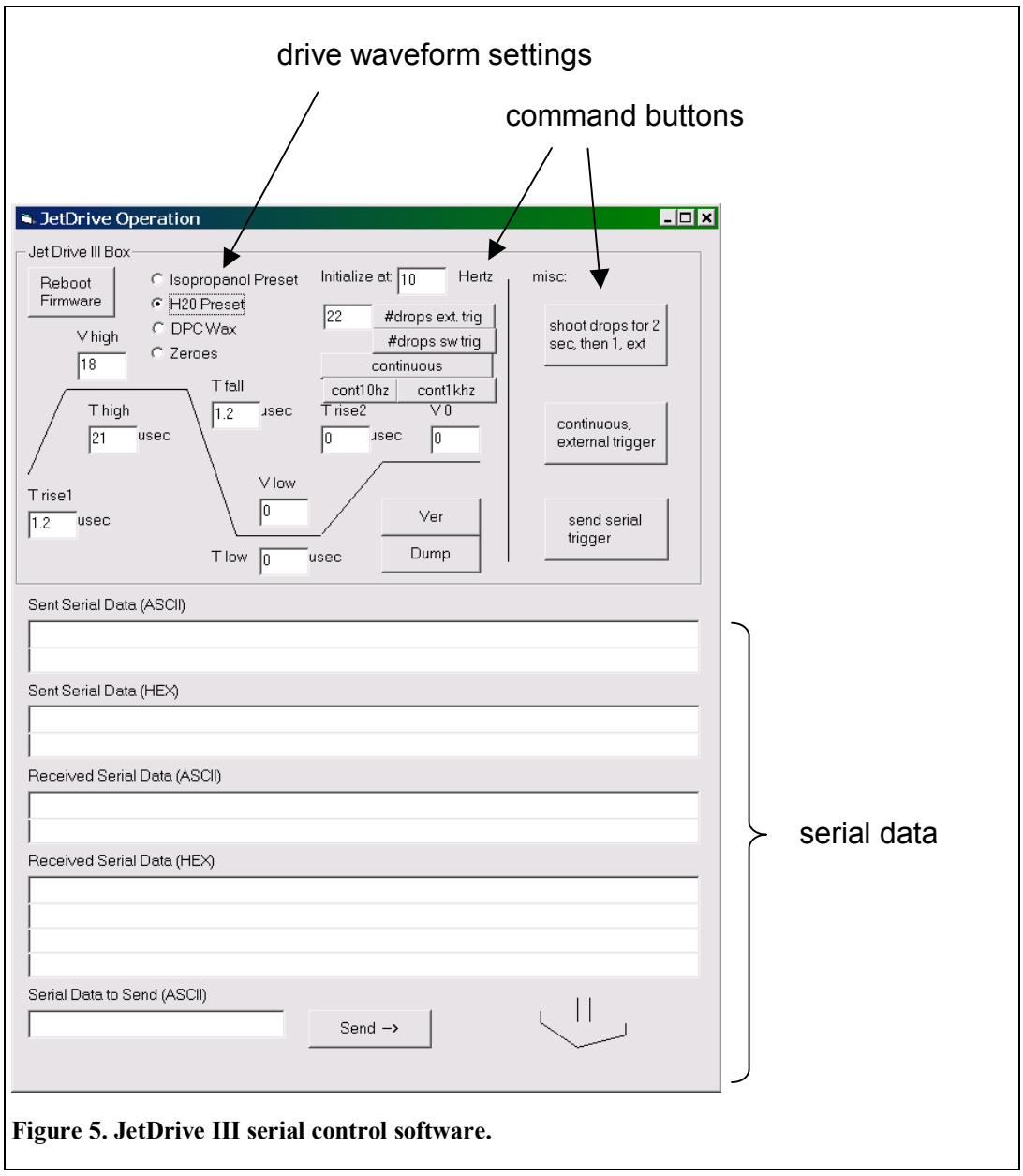


Figure 5. JetDrive III serial control software.

A proprietary text-based file format was developed to save and load drawing patterns. The file format utilized Visual Basic's built-in mechanism to save and load variable values to text format. Each line of the file contained start and stop coordinates, dot spatial frequency, and/or number of droplets to deposit at a given location. Additional global information was stored on the first line of the file, such as

the expected dot size, zoom level of the graphics window, and other information such as whether to offset the pattern with respect to the zero position and whether to repeat the pattern and how many times. The format was extensible in that space in each file was allocated for future parameters for later versions of DROPDRAW, which will still be able to read older drawing files without the need for extensive contingency code. In this way more parameters could be added to each file without breaking DROPDRAW's ability to read older files. A G-code format was considered, to make it compatible commercial CAD-based path planning tools, but the author was unfamiliar with whether programs such as AutoCAD could be used to generate G-code with the specific requirements for this system. In particular, it was not expected that a commercial G-code generator could be found to satisfy the requirement that lead-in and follow-through movements be added to commanded trajectories. In addition, the feature that the individual droplets can be visualized as they are added is a helpful feature when designing patterns. However, it is expected that a parser could be written to decode commercial drawings, such as AutoCAD .DXF files, to extract line elements and points, and load them into the DROPDRAW software for printing. In this way the numerous tools and user interface improvements written for such programs could be leveraged for use in generating sophisticated patterns. For the purposes of this study, however, the combination of visual drawing and text-based modification when necessary was sufficient. On average, a new pattern to be printed would take approximately an hour to be developed and refined before being ready to print.

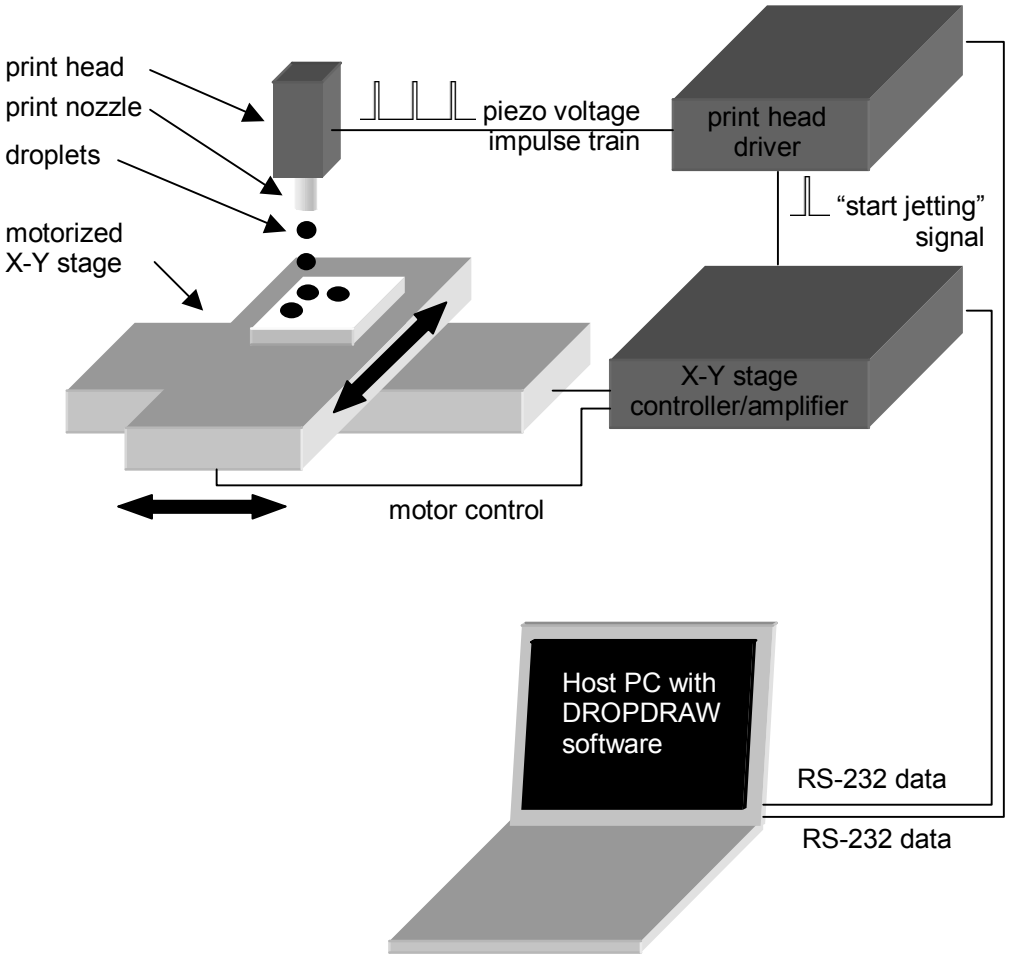


Figure 6. Schematic diagram of the ink-jet printer.

A schematic diagram of the control and communication system of the printer as realized is shown in Figure 6.

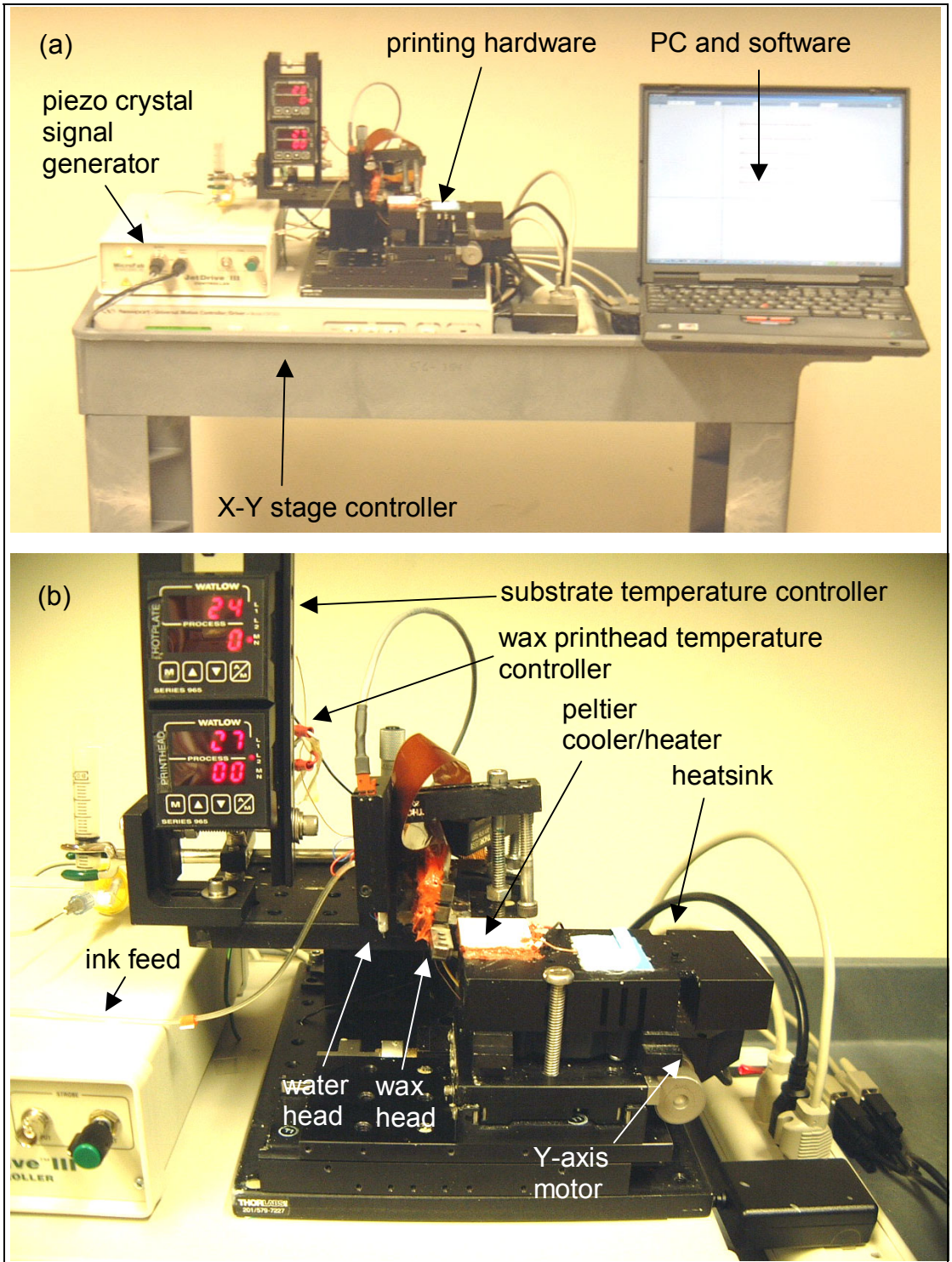


Figure 7. The ink-jet printer. (a) The entire printer is shown on a laboratory cart with control computer, X-Y stage controller hardware, and piezo driver hardware. (b) A close-up view, showing more detail.

2.3.6 Result

The realization of the printer is shown in Figure 7.

Chapter 3

METHODS

3.1 Inhibitory background chemistry

Constraints and capabilities of the ink jet printer necessitate a unique approach to chemical patterning. The ink-jet printing nozzle used in our work was made of glass, so printed materials could not be designed to adhere directly to glass. Other metal printheads, such as a single-nozzle device being developed by PicoJet, Inc., might allow the dispensing of glass-adhesive materials, but for the purposes of this study the glass head was the simplest and most available.

The repulsive background was adapted from a protocol in Cass and Ligler (1998) for immobilizing biomolecules containing an amino groups to glass substrates. Based on earlier work, a substrate of poly-ethylene glycol was chosen because it offered a demonstrated means for inhibiting growth (Branch et al, 2000) and because versions of the molecule were available with an amino terminus (Nektar Pharmeceuticals, Huntsville AL, product No. 2M2V0H01). The amino-terminated PEG was crosslinked to an amino-terminated silane through the homobifunctional crosslinker glutaraldehyde. The silane was employed for its ability to covalently bond to glass. Another route to PEG immobilization might forego the use of toxic glutaraldehyde

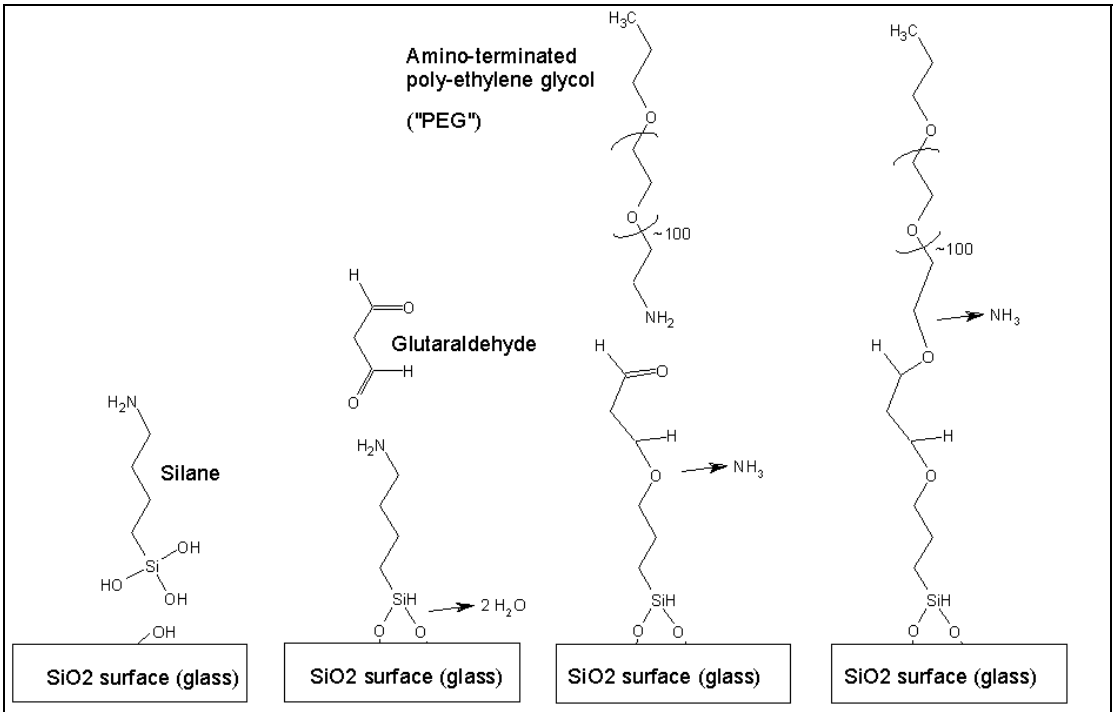


Figure 8. Covalent binding of poly-ethylene glycol (PEG) to glass.

by using a PEG variant with an end group that was reactive with the amino group of the silane. This molecule, called PEG-SPA (Nektar), however, carried a significantly higher price, so it was not used.

The chemistry procedure was as follows. Round 12 mm germanglass coverslips (Electron Microscopy) were cleaned overnight in a 10% KOH solution to remove contaminants, and then soaked in a 2.5% vol. aqueous solution of amino-propyl-triethoxysilane to expose reactive amino groups for 30 minutes and then rinsed in water and absolute ethanol before being dried in a vacuum oven overnight at 80 C. Coverslips were then soaked in the homobifunctional crosslinker glutaraldehyde in a 0.1 M aqueous solution of sodium carbonate (Na_2CO_3) for 2 hours, exposing an aldehyde group that is reactive with amino groups. Coverslips were then soaked in a 0.5% wt. aqueous solution of amino-terminated poly-ethylene glycol, 5000 MW for 1-2 days prior to printing. Please see Figure 8 for a chemistry schematic.

Though it is possible that the printing step could be performed at any step prior to the completion of the inhibitory background chemistry, it was found that the printed positive adhesion materials could be printed directly on top of the inhibitory background and remain adhered. This presented the advantage that no non-adsorbed proteins in the printed ink could dissolve away and contaminate any of the chemistry steps, thereby assuring the strength of the inhibitory background. It is recognized that it was more challenging to create a neuron- and glia-inhibiting background than an adhesive background.

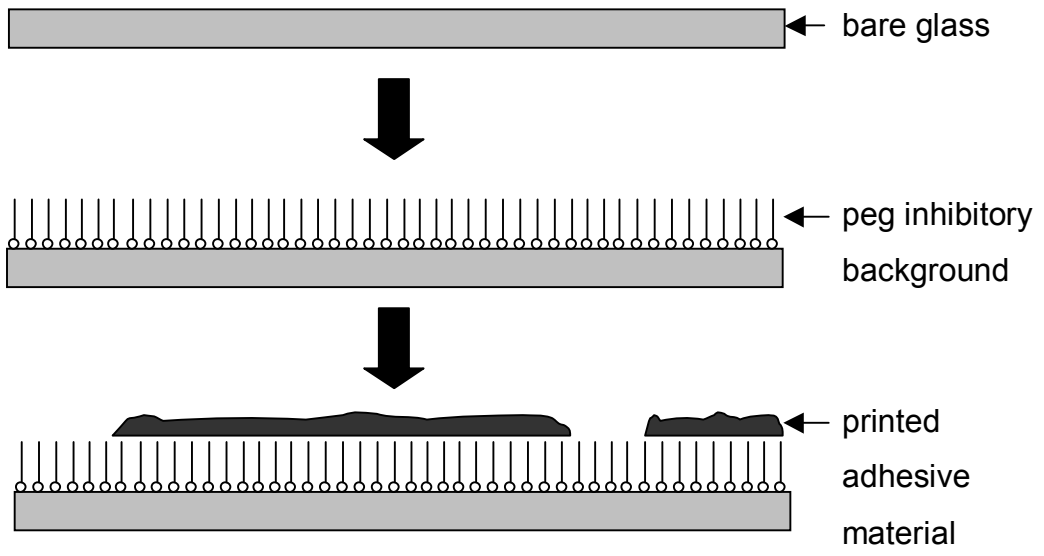


Figure 9. Diagram of the surface patterning process. The bare glass was first chemically modified to have a uniform coat of PEG inhibitory background. Then, adhesive ink was printed by ink-jet and adsorbed to the PEG bacground, leaving local areas of adhesion for neurons.

The degree of repulsion of the inhibitory PEG background was compared to that of the chemistry intermediates, and with a self-assembling peptide analogue to PEG that was thought to potentially be less vulnerable to oxidation (see section 4.1 for results). That peptide was a 10-mer serine-threonine alternating repeat with an amino

terminus ("TS" peptide). It was soaked in 5% by weight water in place of the PEG treatment step above, and its amino-terminus was expected to chemically react with the glutaraldehyde cross-linker like the PEG.

Other attempted backgrounds included a chemically bonded hydrophobic fluorosilane (Fluka 77279), bonded in a bath of N-decane (Aldrich, D-90-10), and solution-deposited agarose.

3.2 Printing and inks

For the cell adhesion tests of micro-islands and other patterns printed onto the PEG-treated backgrounds, the ink material was printed onto a substrate sterilized using an antiseptic alcohol pad. The substrate was heated to 34 C to improve drying rate but cool enough to insure that temperature would not affect the printed materials. Slides were bathed in germicidal UV light for 15-30 minutes from the source in a laminar flow hood, on one or both sides, to sterilize them after printing.

Inks that were printed included poly-ethylene glycol (molecular weight 5000), a self-assembling peptide with the RADA motif (Zhang et al, 1993, 1995, and 1999; Holmes et al, 2000) (Synpep, Dublin, CA) (0.1% by weight), a collagen/polylysine mixture known to be effective as a general-purpose adhesive background (2 mL of 17 mM acetic acid, 3.6 mg of rat tail collagen [Becton-Dickinson], and 40 micrograms of poly-D-lysine [Sigma], in 6 mL of water, multiplied as needed for the desired ink quantity), the protein laminin (Sigma L-2020 0.1 and 0.001% by weight in 0.05 M HEPES and 0.15 M sodium chloride), and the cell adhesion material MatriGel (Becton-Dickinson). The latter rapidly gelled at room temperature and so it was required that the printer and control computer be brought into a cold room for printing. While the ability to print MatriGel was a strong example of the flexibility of the printer, printing in a cold room was a more arduous process than room temperature printing and was avoided. The collagen/polylysine mixture was used for

the experiments with neuron health and pattern longevity on the printed patterns, and the laminin was used for the gradient experiments.

Gradients were fabricated with the assumption that the entire content of laminin in a given ink droplet was less than the maximum that could be adsorbed to the substrate in a given area. In this way multiple droplets left in a given position would leave proportionally more laminin, and controlling the number of droplets deposited could control the density of laminin.

Solid wax printing was done with the print head at 200 C and the substrate actively cooled to 10 C. The wax was candelilla wax from Sigma. It is believed better repulsion could be achieved with a purer wax, such as a microcrystalline wax from Reed.

3.3 Cell culture

The experimental slides were prepared as above with a covalently bound PEG inhibitory background underneath a printed neuron-adhesive pattern of polylysine/collagen (Figure 9). The control slides were un-patterned and treated with a uniform adhesive coating. The coating, the same collagen/polylysine mixture used as the ink in the adhesion experiments, was adsorbed onto glass coverslips that had been cleaned with the following protocol: soak for 30 minutes in 10 M hydrochloric acid, sonicate in 95% ethanol three times for 30 minutes, and then sonicate in double-distilled Millipore water.

The slides with printed patterns were soaked in the serum medium overnight prior to cell plating. This step appeared to yield healthier-looking cells at the expense of some of the cell growth inhibition ability of the PEG background.

(This work was performed in collaboration with Jung Choi.) Cells from P1 rat hippocampi were obtained using a protocol based on one reported in Banker and

Goslin (1998). All materials were ordered from Sigma unless otherwise specified. One-day old rat pups were sacrificed and their hippocampi were removed and sliced into small pieces in buffered Hank's Balanced Salt Solution (HBSS) at standard concentration at room temperature. Cells were enzymatically dissociated using a papain enzyme solution. Papain is a protein-cleaving enzyme similar to trypsin but has been found to be less harmful to neurons. Papain (Worthington) was mixed at 100 units of strength in 5mL of dissection solution, 75 microliters of 100 mM $CaCl_2$, 50 microliters of 50 mM EDTA (a chelator to remove excess metal ions from the solution), 1 mg of cysteine to activate the papain enzyme, and 10 micrograms of DNAse enzyme to reduce gelling that results from ruptured cells. Cells were digested in papain in an incubator at 37 C for 40 minutes, and then rinsed three times in HBSS. The remaining HBSS was removed, and the serum plating medium was added. In the case of the control cells, the plating medium was glia-conditioned, while the experimental cells were plated in serum that was not glia-conditioned. To make glia-conditioned medium, dissociated cells were plated at a sufficiently low density (less than 5,000 cells/mL) in culture flasks that neurons died, leaving only glia after a few days. Cell plating serum was conditioned in the presence of the glia for one day prior to its use. The serum consisted of a mixture of the following, multiplied as necessary to insure a quantity of at least 500 microliters per well: 430 mL of Eagle Basal Medium (Invitrogen), 1.8 g glucose, 5 mL of Sodium Pyruvate 100 mM (Invitrogen), 50 mL of Fetal Bovine Serum (Hyclone), 500 microliters of Mito Serum Extender (Beckton-Dickinson), B27 (Invitrogen), and 5mL of 1 M HEPES. Cell plating density ranged from 10,000 to 30,000 cells/mL, measured with a hemacytometer, or approximately half that number per coverslip, with each well receiving 500 mL of medium. Cells were incubated in a 5% CO_2 atmosphere at 37 C.

After four hours in culture, the medium used to plate the experimental cells was removed and replaced with Neurobasal neuronal maintenance medium (Life

Technologies), while the control cells were left in serum. Neurobasal was found to extend the life of neurons in very low-density cultures.

Feeding was performed once each week by removing the entire medium and replacing it with the same material. Removing only half of the medium led to badly smelling cultures, and no change in neuron survivability was observed as a result of the presumed shock of the sudden change in medium when it was entirely replaced.

3.4 Electrophysiology

(This work was performed in collaboration with Neville Sanjana.) The patch-clamp technique was used to measure electrophysiological properties of the cultured cells (Neher and Sakmann, 1976). Cells were probed using ruptured-patch whole-cell recording between day 10 and 12 in culture. Patch recordings were made with a HEKA EPC-8 amplifier and custom software written in the MATLAB programming environment. During the recordings, the culture medium was replaced by a continuously perfused extracellular bath solution containing (in mM) NaCl (145), HEPES (10), glucose (8), KCl (3), $CaCl_2$ (3), and $MgCl_2$ (2) with pH adjusted to 7.3. Pipetttes made of borosilicate glass were pulled on a Flaming/Brown puller (Sutter Instruments) so that that pipette resistance was kept between 3 and 7 MΩ. Pipettes contained (in mM) K gluconate (130), KCL (10), $MgCl_2$ (5), EGTA (0.6), HEPES (5), $CaCl_2$ (0.06), Mg-ATP (2), GTP (0.2), leupeptine (0.2), phosphocreatine (20), creatine phosphokinase (50 U/mL) (Arancio et al., 1995). For all cells, the following parameters were continuously calculated and recorded: access resistance, membrane resistance, and membrane capacitance. Resting membrane potentials were recording immediately after entering the cell. All seals were above 1 GΩ and data was kept only for cells with average access resistance below 20 MΩ for all recordings.

3.5 Immunostaining

Cells were fixed and stained with fluorescent-conjugated antibodies for imaging. Primary antibodies were mouse monoclonal anti-GABA (1:200, Sigma A0310) to detect inhibitory cells, rabbit polyclonal anti-synapsin I (1:750, Chemicon AB1543) to detect synaptic puncta, and rabbit anti-laminin (1: 40, Sigma L-9393) to detect laminin surface concentration. Secondary antibodies from Molecular Probes were goat IgG anti-rabbit (Alexa Fluor 350) and anti-mouse (Alexa Fluor 488); both used at 1:200 dilution. Cells were prepared for immunochemistry by fixing for 20 minutes in a 4% paraformaldehyde in phosphate-buffered saline (PBS), with 250 g/L sucrose, permeabilized in 0.25% TritonX for 10 minutes and then rinsed in PBS, and blocked for 1 hour in 10 % goat serum. Incubation in primary antibodies was at 4 C overnight at their respective concentrations in 5% goat serum in 1X PBS, and rinsed three times afterward in PBS for five minutes. Secondary fluorescent-conjugated antibodies were incubated in 2% goat serum in 1X PBS for 1 hour and rinsed 3 times in PBS. 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 GABA-ergic 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 were touching or overlapping did we adjust cell counts accordingly. For synapsin I puncta detection, we first applied a radial tophat filter and then a single erosion-dilation filter cycle to isolate puncta.

Chapter 4

RESULTS

4.1 Island fabrication

Solids in the droplets were left on the substrate after the water solvent evaporated, leaving patterns of dots on the substrate that were visible using phase-contrast microscopy. For the purposes of making micro-islands, an assessment of dot size versus number of droplets placed in a given X-Y coordinate was made so that micro-

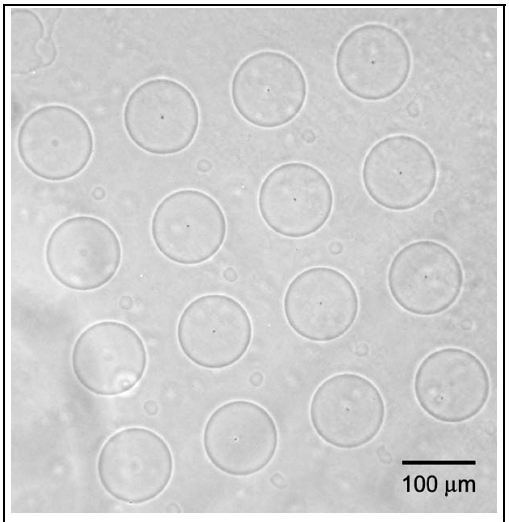


Figure 10. Islands fabricated by ink-jet printing 4 droplets at each location.

island fabrication could be regulated. Islands ranged in size from 65 +/- 5 micrometers in diameter (n=8) for a single droplet of collagen/pdl mixture on PEG to 460 +/- 5 micrometers for 512 droplets, the highest number we attempted (Figure 12). For comparison, the dot diameter left on other substrates, such as glass cleaned with hydrochloric acid, was measured. Because of the variation in 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.

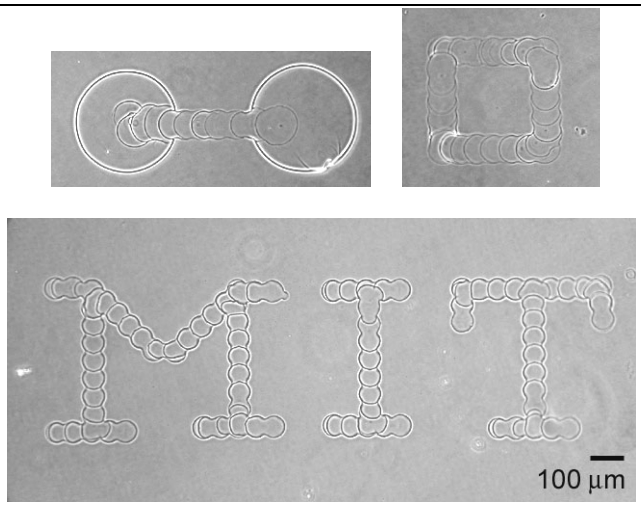


Figure 11. Connected islands, a box, and text printed by ink-jet.

4.2 Connected islands and other patterns

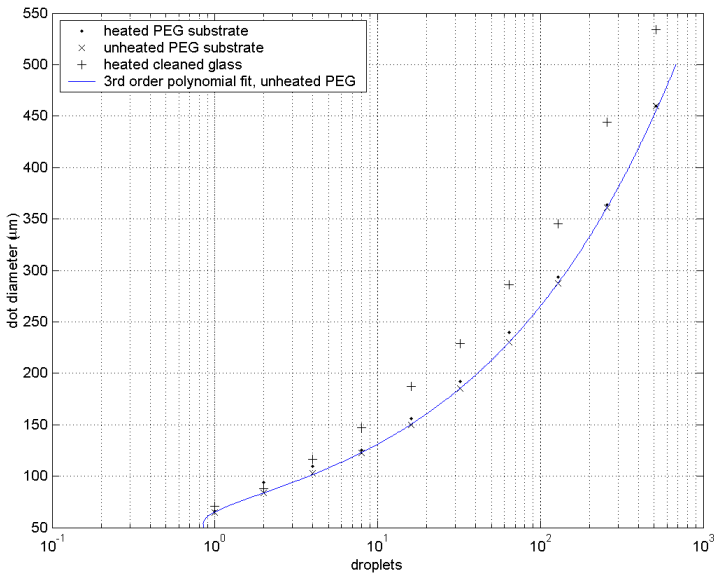


Figure 12. Dot size left on the substrate as a function of the number of droplets deposited. Variables include substrate composition and substrate temperature (n=8 for each data point).

Other patterns, such as micro-islands connected by narrow bridges, box shapes, and alphanumeric text were rendered using trains of single droplets deposited slowly enough to insure complete evaporation of solvent between successive droplets (Figure 11). All presented results were printed using a droplet frequency of 1 droplet per second.

4.3 Solid wax printing

Structures were fabricated in 2D and 3D using the ink-jet printer. Features included evenly spaced grids of droplets, corral-shaped patterns intended to constrain connections between groups of neurons, and narrow gaps as small as 5 micrometers, and gaps retained around corners (Figure 13).

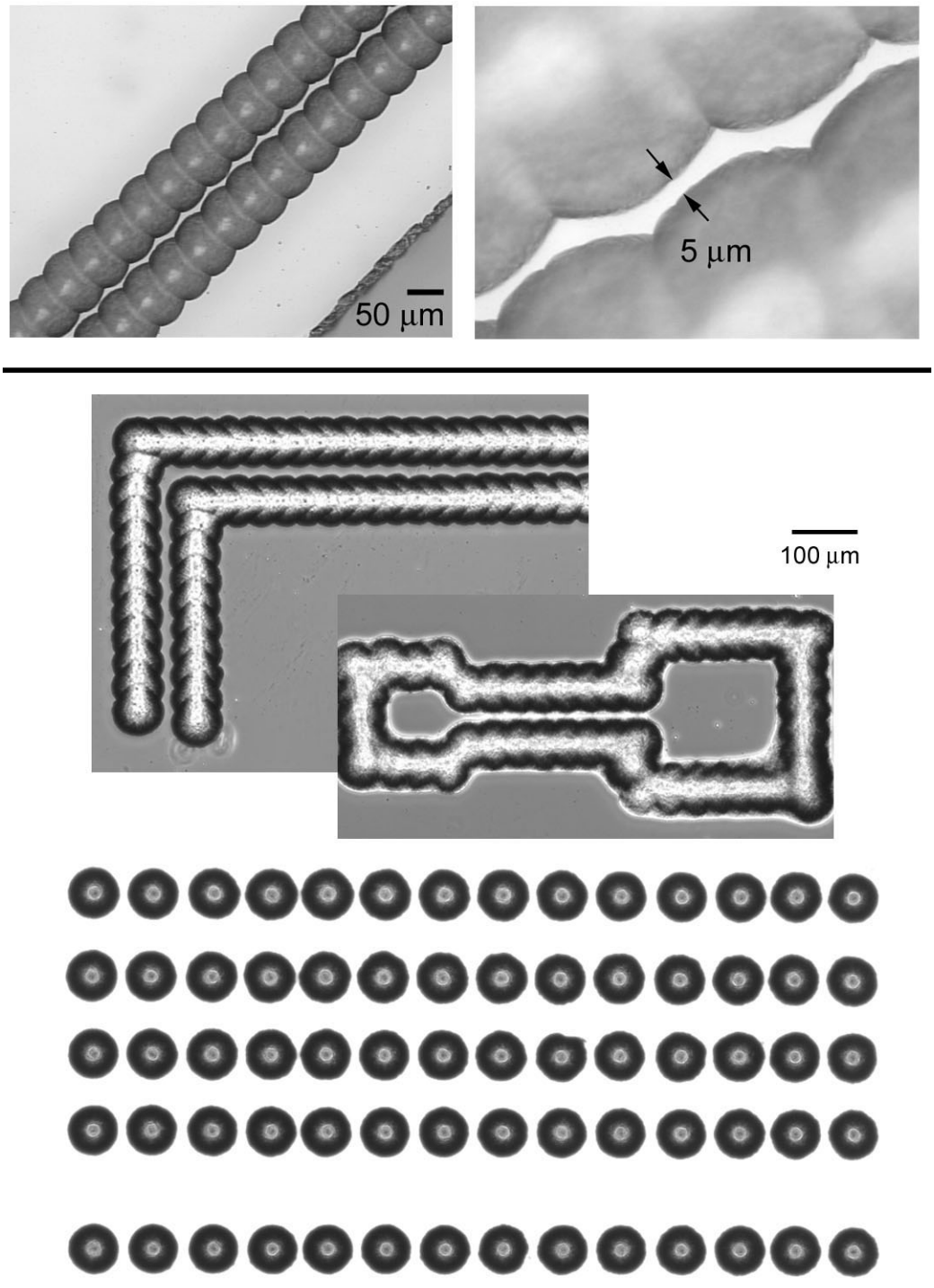
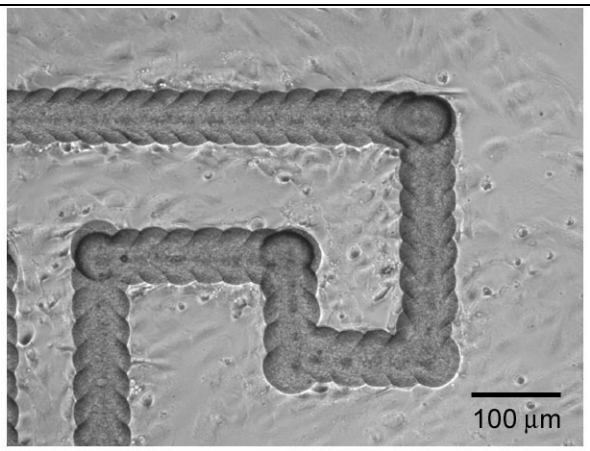


Figure 13. Ink-jet printed wax patterns, including a line a line of closely-spaced droplets (top), a small gap maintained around a bend, a two-island corral structure, and a grid of dots (bottom).

Adhesion tests on the wax included growing epithelial cells on patterns (Figure 14) and growing PC12 neurons (in collaboration with Rutledge Ellis-Behnke) after soaking the pattern in poly-L-lysine. While it was found there was marginally less adhesion to the polylysine than to the wax, further experimentation was not pursued because the wax was not strongly non-adhesive. The complex wax pattern shown at the bottom of Figure 14 was to be used in a later experiment to attempt to induce nerve fasciculation (bundling) in vitro. This experiment was not completed.



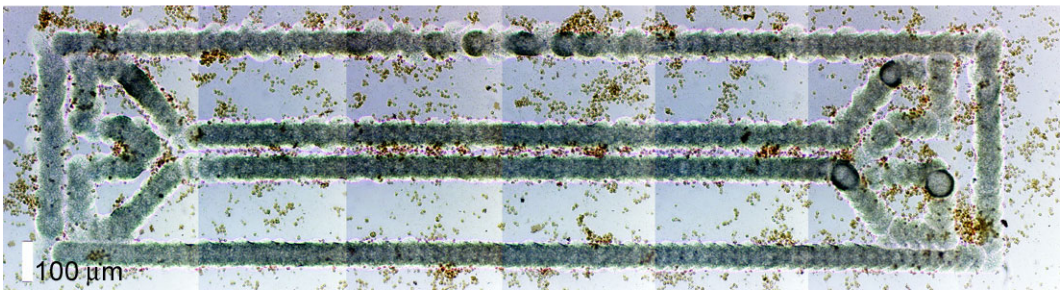


Figure 14. Epithelial cells (top) and PC12 neurons (bottom) grown on wax patterns. Bottom image source: Rutledge Ellis-Behnke.

Three-dimensional structures fabricated by layering wax droplets were fabricated and imaged with the environmental scanning electron microscope (ESEM). The ESEM allows visualization of targets that are not conductive, in contrast to standard electron microscopy, and in addition does not require extreme vacuums.

Overhanging structure was achieved to enable short tunnels, intended to physically constrain the growth of neurites (Figure 15). Droplet placement repeatability problems, likely the result of oxidation at the nozzle as a result of high temperatures, limited the quality of the features shown.

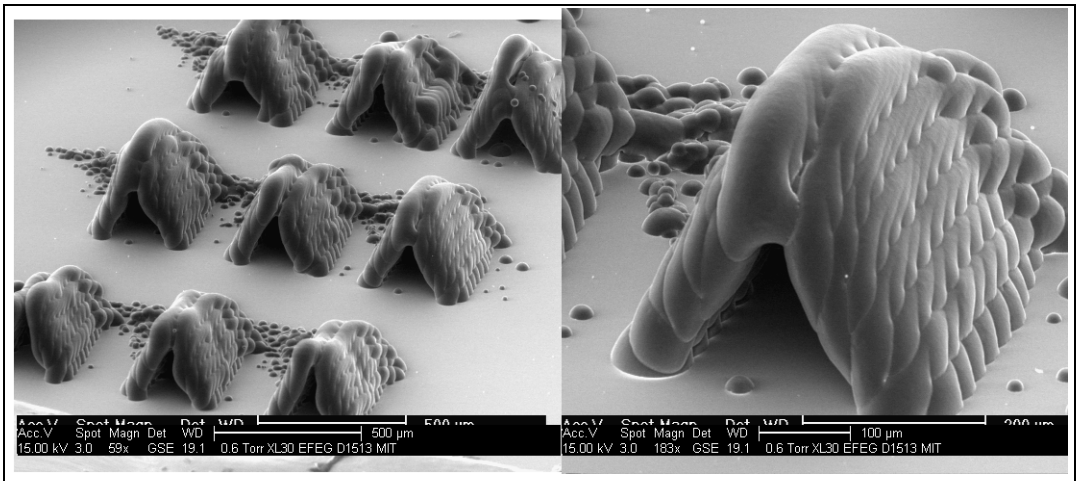


Figure 15. Wax tunnels imaged by enviromental scanning electron microscope. Extra badly-position droplets on the far side of each tunnel are indicative of clogging in the nozzle.

4.4 Cell adhesion

The strongest results for cell adhesion/repulsion were found with the combination of PEG as the inhibitory background and collagen/polylysine ink as the adhesive foreground. While the RADA peptide was found to attract neurons to it, it was found that the gelled material became delaminated from its PEG substrate. Over time, dots folded in half or into small clumps within a week or two of plating neurons (Figure 16). It is not known why the collagen/polylysine matrix adheres in a way that inhibits this phenomenon. While MatriGel was found to induce neuron adhesion, it had the problem that it had to be printed in a cold room to insure that it did not gel inside the print head, making it more difficult to use.

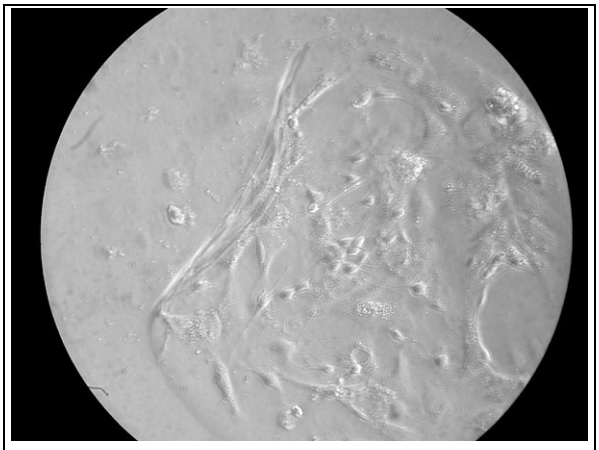


Figure 16. A printed dot of RADA delaminating from the substrate through action of neurons after two days in culture.

To be sure that the inhibitory action was due to PEG and not due to one of the underlying chemical crosslinkers, a study was done to compare the inhibition of the chemicals in intermediate steps (data not presented). Neurons and glia adhered well to bare cleaned glass, slightly worse to APTS-treated glass, well to glutaraldehyde, and very poorly to PEG (as desired). It has been noticed that glia adhere much more readily to varying substrates than do neurons. However, in this study it was intended that neurons be as healthy as possible, so patterning was done in a way that was compatible with glia substrates for the neurons, so the glia needed be compliant to the patterns. Results indicated that the TS peptide described above fared only

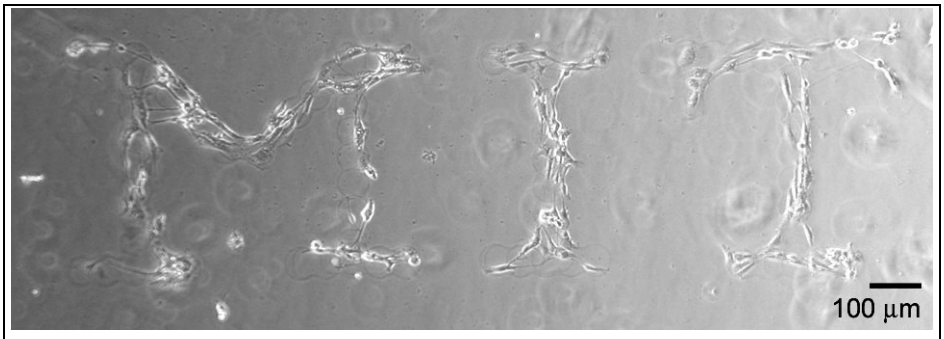


Figure 17. Text pattern of RADA peptide printed on TS peptide inhibitory background shows strong pattern compliance after 2 days in culture.

marginally better than did bare glass as an adhesion inhibitor. The best example of TS peptide successfully acting as a growth inhibitor is shown in Figure 17, after two days in vitro.

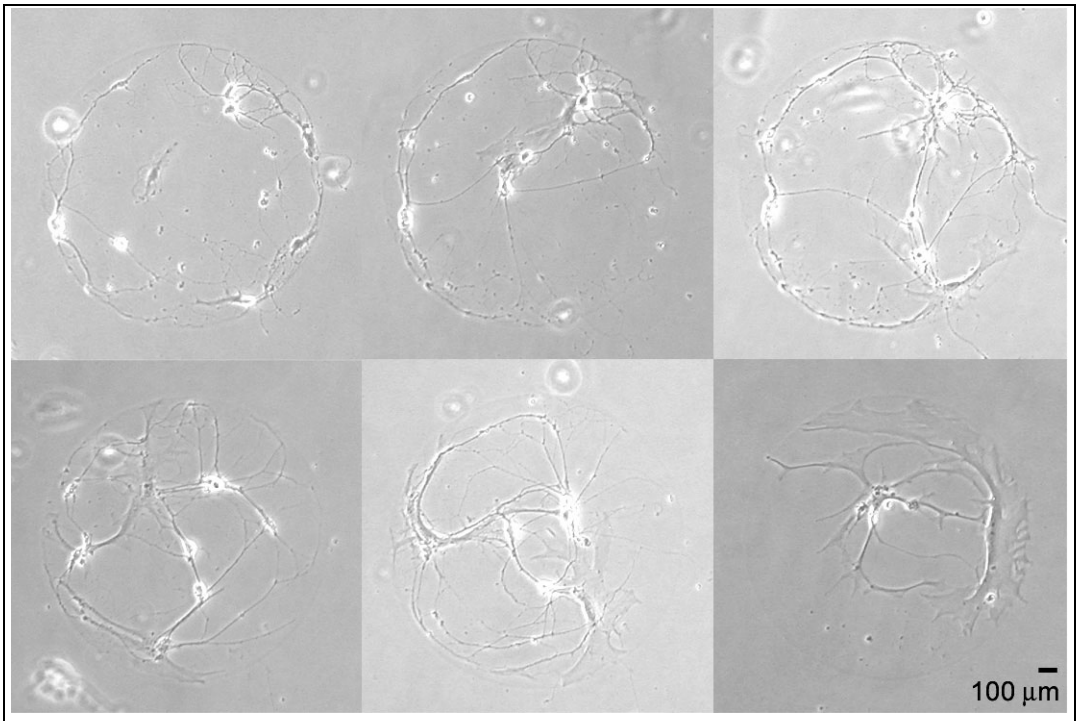


Figure 18. A timelapse image of an island of neurons, taken at days 5, 8, 10, 13, 15, 20, and 25 days in vitro.

The best and easiest results were with patterns printed out of collagen/polylysine onto PEG. Patterns included islands, connected islands, arbitrary shapes, and text (Figure 19).

Neurons and glia growing in culture dynamically moved around within islands. A timelapse image of neurons is shown in Figure 18.

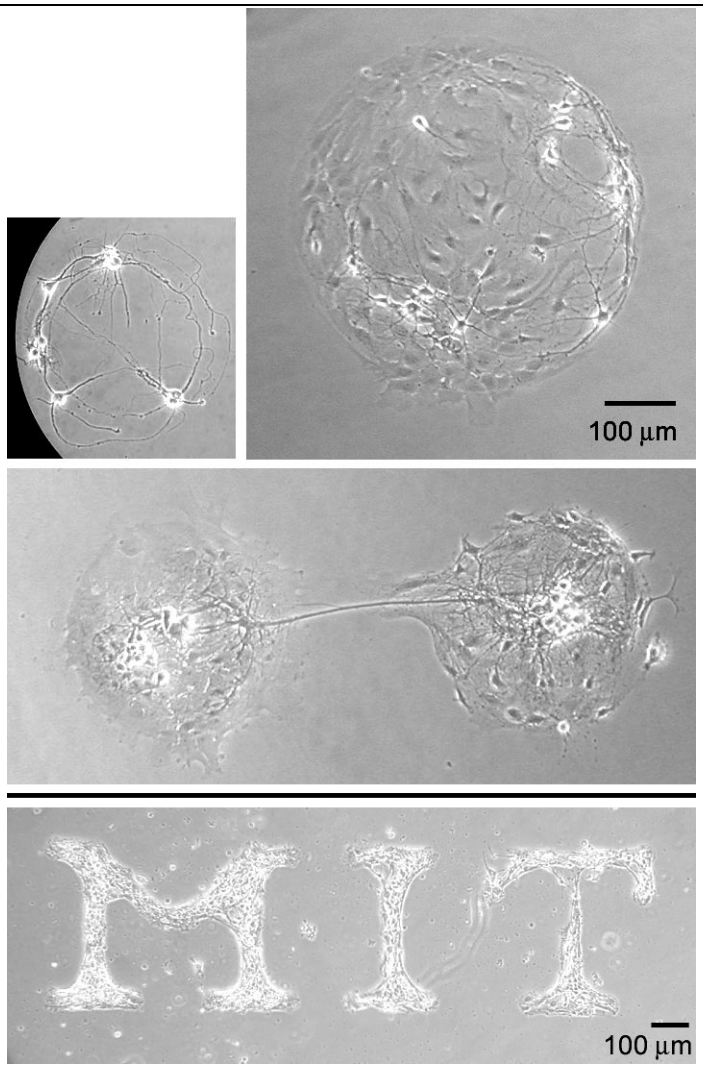


Figure 19. Neurons and glia patterns adhering to polylysine/collagen ink on PEG inhibitory background. Islands with few glia (top left) many glia (top right) after 6 days in culture, connected islands after 10 days, and text, (bottom) have been demonstrated.

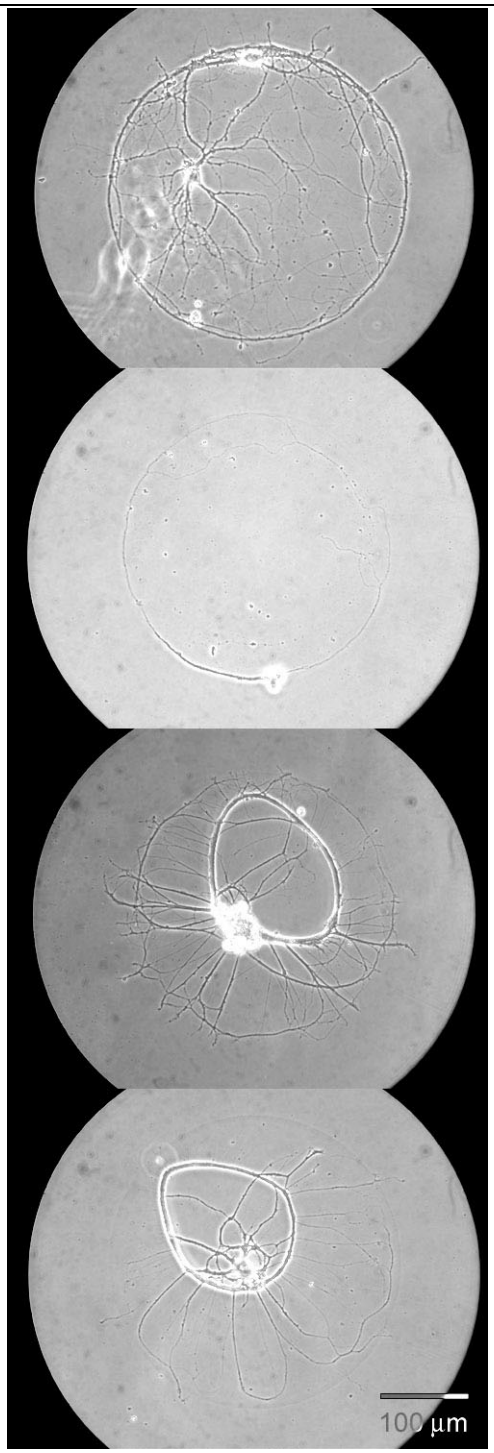


Figure 20. Neurons growing on glia-free islands after 25 days in vitro. These neurons exhibit autaptic connections and ring morphology. Some appear to keep neurites encircling the edge of the droplet, while in other islands it appears neurites contract after encircling the island, making a ring formation smaller than the original dot. Neuron somata growing on glia invariably pull toward the middle.

Neurons adhered in varying degrees to the islands. To assess the viability of the islands over time, images were taken at different days in culture. Islands physically distinct from other islands were considered "intact", while islands that were connected to other islands or far outside of their boundaries were not. Compliance fell from approximately 75% after 5 days in culture to 45% at day 25 (n=36 islands) (Figure 21).

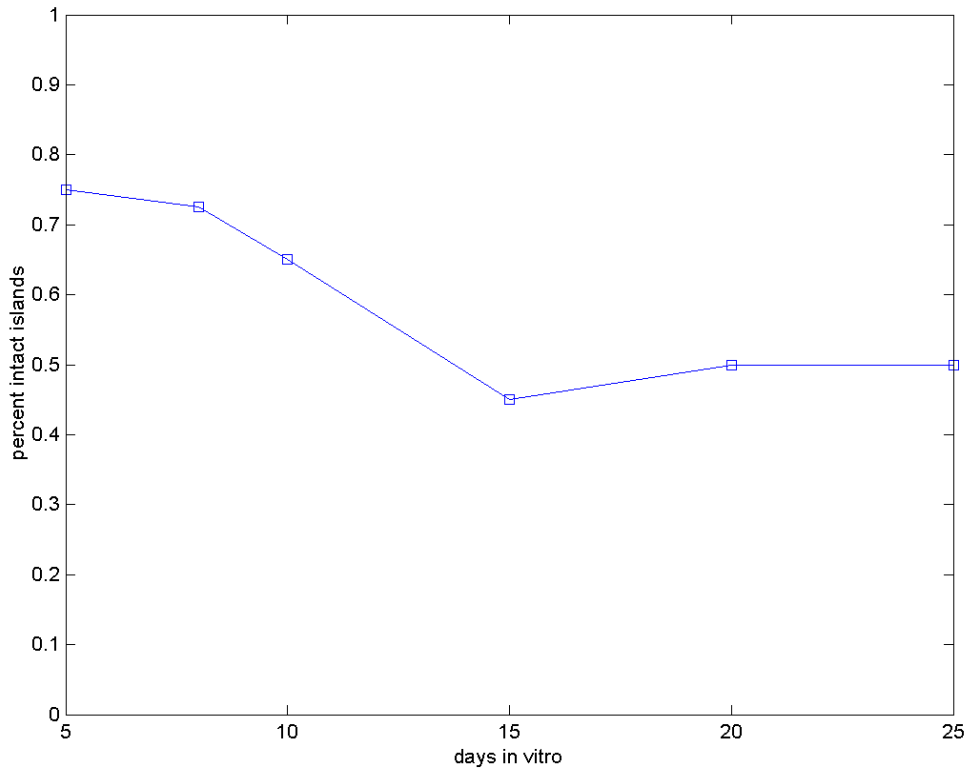


Figure 21. Effectiveness of PEG inhibitory layer (n=25 islands).

4.5 Cell health

Neurons on islands were probed by patch clamp to find their resting potential and other relevant electrophysiological data. Cells exhibited healthy resting potential in

comparison to un-patterned control cells, as well as normal access resistance and membrane capacitance (Table 1).

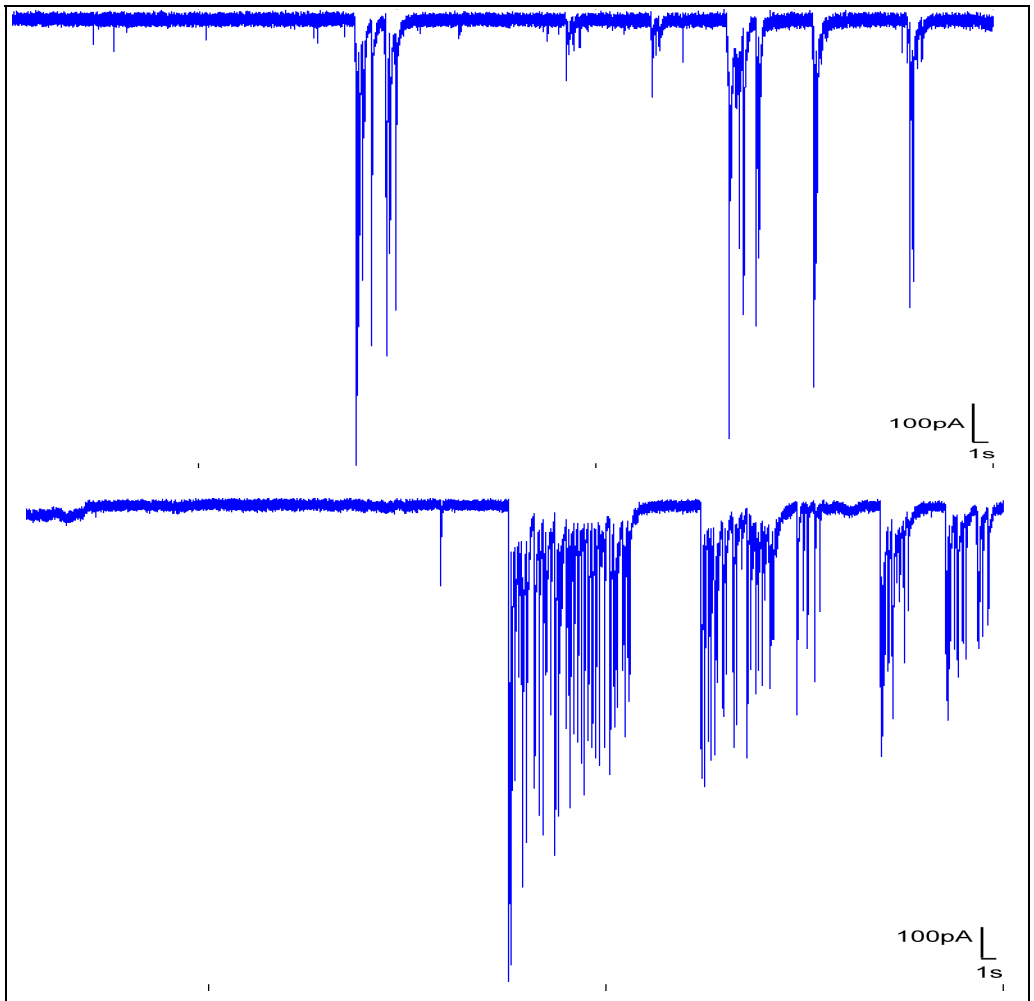


Figure 22. Spontaneous activity detected by patch clamp in voltage clamped mode in control unpatterned cells (top) and patterned cells (bottom).

Spontaneous activity was detected in the form of mini-EPSP's (excitatory post-synaptic potentials) that arose as a result of the spontaneous release of single vesicles of neurotransmitter. Other phenomena observed included detection of AMPA-minis (fast, larger minis) and NMDA-minis (slower, lower minis) (data not shown), detection of evoked EPSP's (that arise as a result of an action potential reaching a

presynaptic terminal and inducing the release of neurotransmitter, depolarizing the patch clamped postsynaptic cell), and action potentials in the cell body (Figure 22).

	resting potential (mV)	membrane resistance (MΩ)	membrane capacitance (pF)
control cells (n=12)	-55.3 (± 8.1)	402 (± 163)	57 (± 20)
patterned cells (n=12)	-54.7 (± 6.6)	277 (± 125)	80 (± 29)

Table 1. Electrophysiological properties of control and patterned cells are comparable after 10 to 12 days, indicating patterned cells have comparable health.

Cells were stained with the calcium-sensitive dye Fluo-4 and showed waves of calcium activity in glia and occasional spikes detected in the neurons. An image of a ring of neurons stained with fluo-4 is in Figure 23 (top). Slow transients detected by brightening of the fluorescent dye in response to increased intracellular levels of calcium appeared to propagate around the patterned ring structure. Brightness was measured as the average relative brightness of three soma (Figure 23, bottom left) and plotted over time (Figure 23, bottom right).

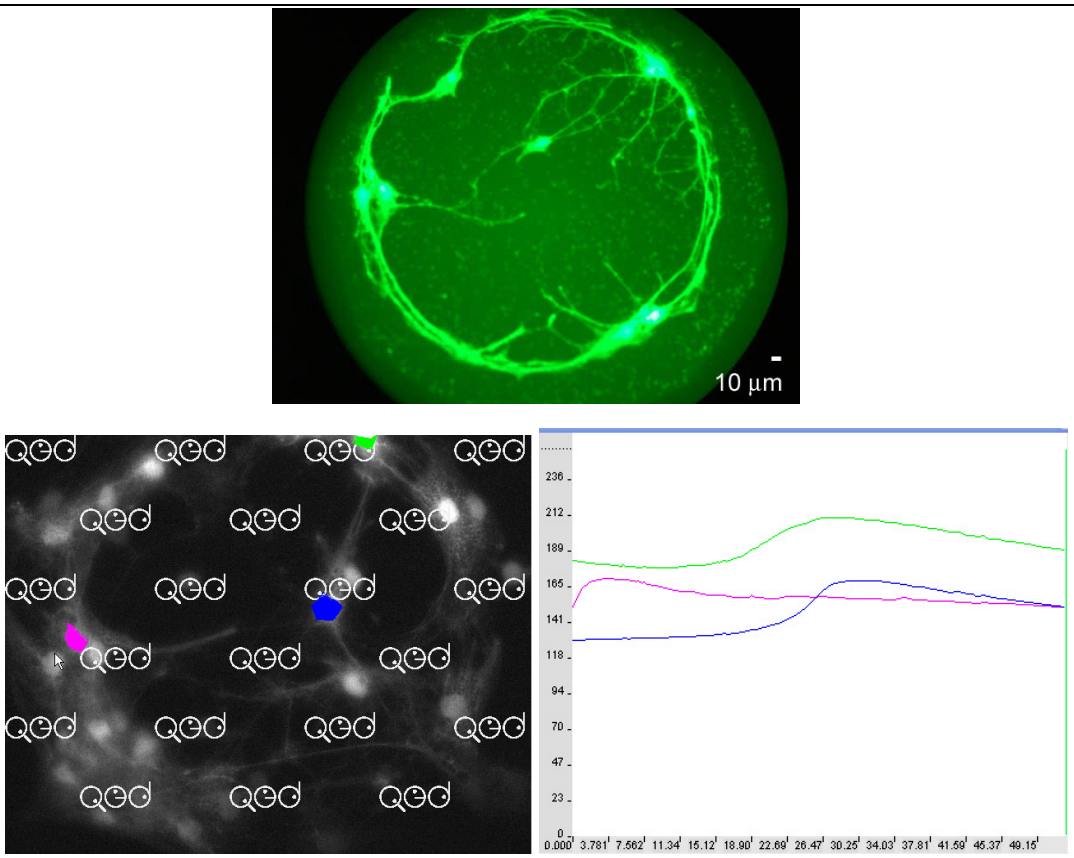


Figure 23. Slow calcium transients detected sequentially around a patterned island of neurons. (top) A ring of neurons stained with the calcium-dependent dye fluo-4. (Lower left) The average relative brightness of the three colored areas, corresponding to three different neurons is plotted in the graph (right). The diameter of the ring on the left is approxmately 350 micrometers, and the X-axis units on the right are seconds.

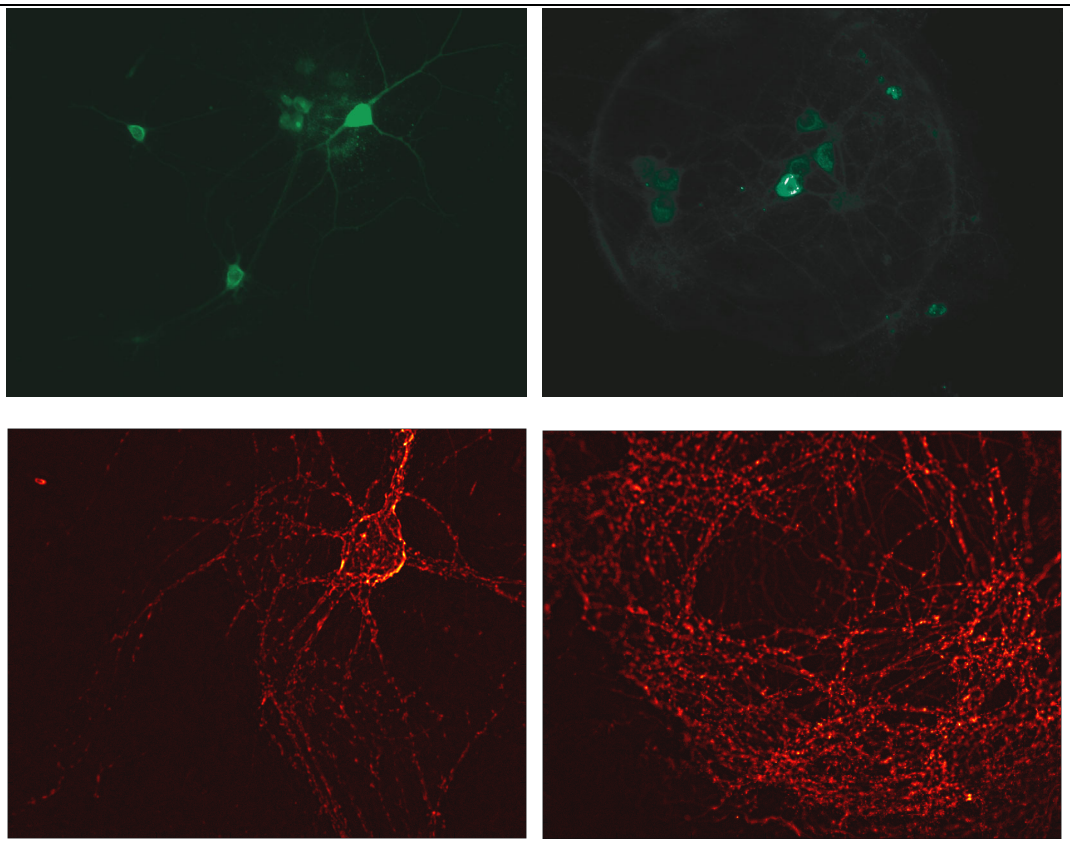


Figure 24. Patterned cells (right) show normal distributions of inhibitory cells and synaptic puncta in comparison to unpatterned control cells (left). Top: false-color images of fluorescent marker for GABA. Bright cells are inhibitory. Bottom: false color images of fluorescent marker for synapsin I. Each excitatory synapse is labelled as a puncta. Scale: the uper image was taken at 10X magnification, and each cell body is approximately 20 µm. The lower image was taken at 20X.

4.6 Immunostaining

Immunostaining assays indicated that patterned cells showed a lower percentage of inhibitory cells, which stained brighter for GABA (22%, n=176) in comparison to excitatory cells, than do control un-patterned cultures (51%, n=171). Staining for synapsin I showed a roughly comparable synaptic density (0.16 stained puncta per μm^2, n=5 neurites) in comparison to control cultures (0.15 stained puncta per μm^2, n=5 neurites).

4.7 Gradient fabrication

Laminin was found to adsorb onto substrates of both PEG and poly-L-lysine. On PEG, it was found that neurons behaved toward laminin in a similar way to that of collagen: they appeared to migrate to the laminin surface and away from PEG, making printed laminin patterns usable as islands (Figure 25).

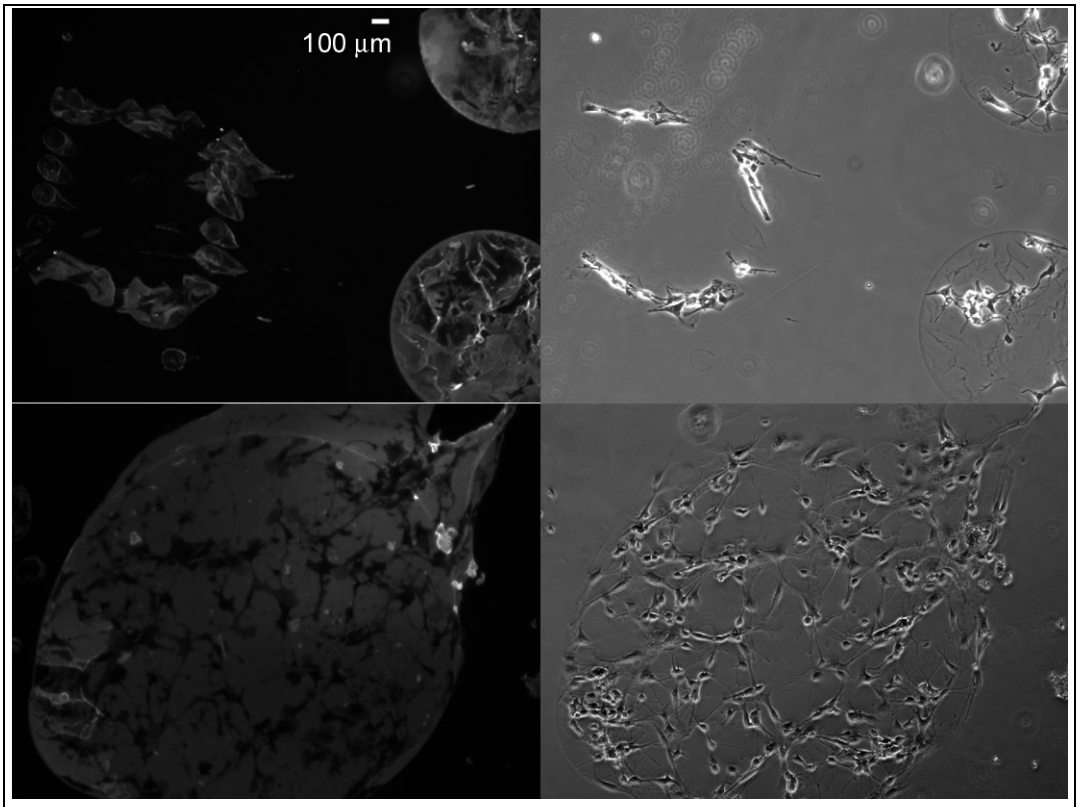


Figure 25. Islands and patterns of laminin printed on PEG are adhesive to neurons. Fluorescently tagged by anti-lamin (left) and phase-contrast brightfield (right).

Gradients of laminin were visualized using fluorescent immunostaining (Figure 26). Printing time was approximately 4 minutes for the large-area gradients and 2 minutes for the smaller linear gradients. Excess non-adsorbed laminin appeared to become a non-evaporating liquid, perhaps an oil, that presumably rinsed away when the antibody solution was added (Figure 26, middle figures).

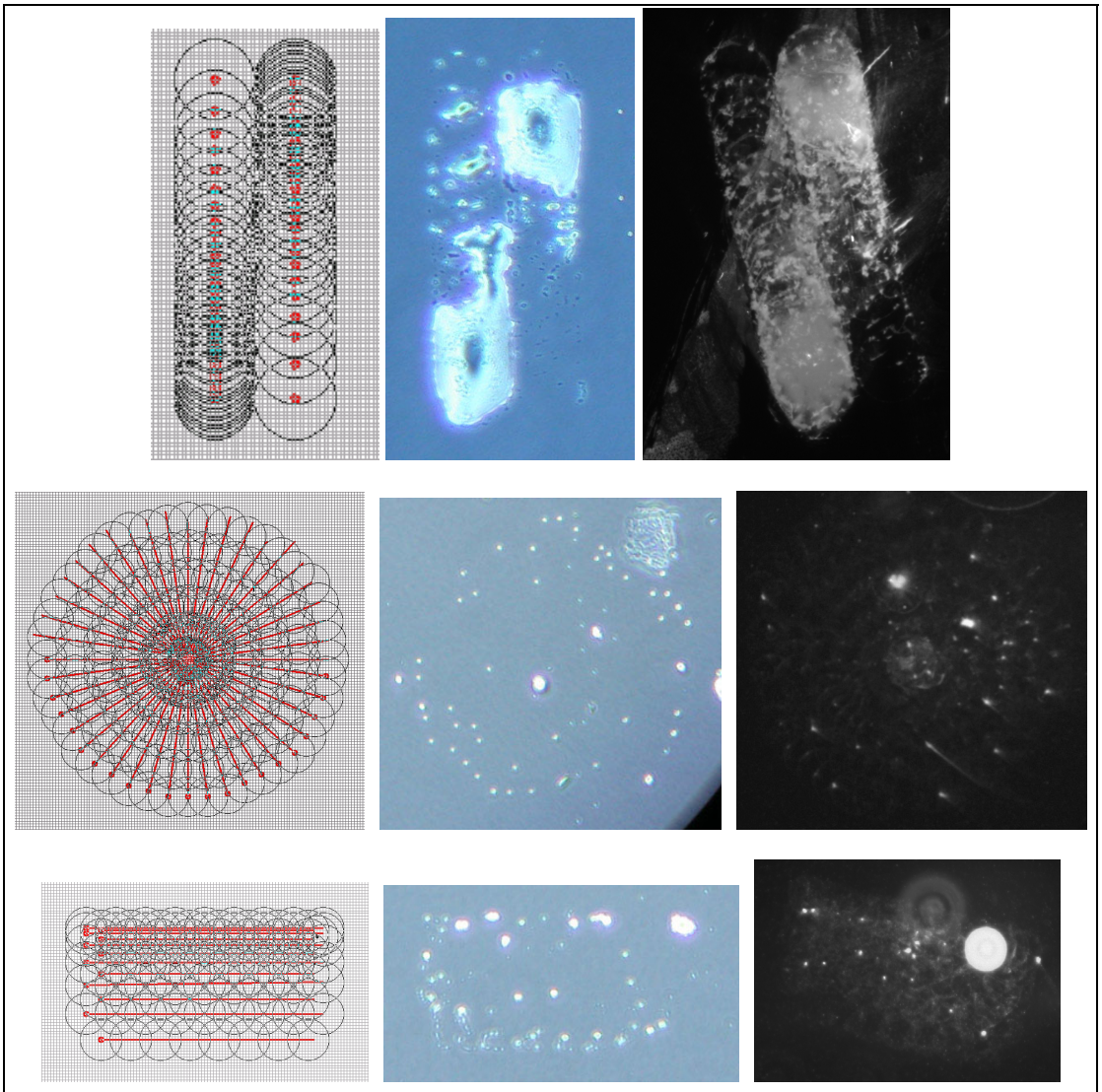


Figure 26. Ink-jet can produce laminin gradients. Images show CAD representation of gradient on left, brightfield phase-contrast image immediately after printing in middle, and fluorescent anti-laminin antibody image on right. Ink density is 25 micrograms/mL on top image, 1 microgram/ml in bottom two. Scale: droplets are 100 micrometers in diameter for top image, 70 micrometers for lower two images.

Chapter 5

DISCUSSION

This report describes the design and construction of an ink-jet printer that programmably patterned surface factors that influenced the growth and assembly of neuronal networks and other cells in culture. Cells adhered to the printed patterns, and neurons in particular showed long-term adhesion to the patterns and characteristics of health, including exhibiting normal resting potentials, spontaneous activity, and normal distributions of synapses and inhibitory cells.

While previously published work on patterning using technologies such as photolithography and microcontact printing has relied on the use of an un-alterable master pattern, the ink-jet printing method reported here had the advantage that it was programmable on the fly. Further, it had the capability to additively deposit relatively larger volumes of material, which could be useful for patterning sources of diffusible factors, and it has the ability to make surface bound gradients by varying the number of layers of a given ink. It is believed there may be other applications for ink-jet, such as injecting diffusible factors into solution or even potentially ejecting whole cells. In this way it is expected that ink-jet printing can play a complementary role to other methods in neuroscience study.

5.1 Feature size

For certain aspects of patterning, an important characteristic is the ability to pattern at very high resolution. In this work, the nozzle ejected droplets approximately 40 micrometers across (35 picoliters), leaving dots on the substrate from 65-80 micrometers across, depending on surface wettability, temperature, and the content of the ink. The smallest droplets reported in industry are approximately 16 micrometers across (2 picoliters), which would leave dots approximately 35 micrometers across. While this is still larger than the size of the ideal line width of approximately 3 micrometers for a neurite (Wyart et al, 2002), I have demonstrated the ability to pattern lines of dots with gaps as small as 5 micrometers between them for distances of millimeters (Figure 13). If the printed material were inhibitory on top of an adhesive background, and the technique improved, this system could be used to generate small channels for neurites. However, neurons appear to be very mobile, so any such small-scale features would require very strong adhesion and repulsion characteristics.

5.2 Programmability

Previous work has demonstrated the ability to constrain neurites in culture, but all work thus reported has relied upon a previously existing master pattern. While this is definitely not a fundamental limitation, the technique of ink-jet printing has the advantage that patterns can be rebuilt on the fly. For instance, results with fabricating gradients came within a week of discovery that gradients of laminin fabricated by microfluidics could be used to influence the direction of neurite outgrowth (Dertinger et al, 2002). A further advantage was that while microfluidic technology could fabricate the laminin gradient, it did not have a convenient means for fabricating any structures adjacent to it. With ink-jet printing, the author was able to generate a pattern that could potentially direct neurites that were growing up-gradient toward islands of neurons elsewhere. This capability could thus influence higher-

level neural connectivity, such as directional connectivity and eventually relatively complex arrangements of connections.

5.3 Gradients and large volumes

A second fundamental advantage of ink-jet printing is its ability readily layer multiple materials and to deposit relatively large volumes of material. While other techniques can do this, none has the ability to combine large volumes with multiple layers and programmable arrangement. Large volumes of materials and layers may enable the fabrication of slow-release and/or timed/release of secreted factors. And the author is not aware of a method employing photolithography and/or microcontact printing to generate surface-bound gradients, aside from the use of dithering. It is believed that these unique capabilities of ink-jet printing represent a potentially powerful tool for combinatoric analyses of axon, dendrite, and migration guidance cues.

5.4 Clogging and partial clogging

One of the primary challenges encountered during the course of this work was to achieve consistent results with the print head. For instance, occasionally the nozzle would clog or dry out, or it would eject droplets at an angle far from vertical, or the pressure in the ink feed would fall sufficiently that the meniscus at the orifice would pull inside the ink chamber of the head. While many of the problems are solvable after a bit of frustration by simply re-priming the head and re-starting the print command on a fresh coverslip, and further refinement to the existing protocol, the problem of oblique jetting deserves further discussion. The author contacted the manufacturer of the head with this problem, and their suggestion was that the nozzle be made hydrophilic by soaking in the commercial windshield water repellent rain-X (though it was important to be ejecting air from the orifice while doing so to make sure that the interior of the nozzle did not become hydrophobic). Further, with a

microscope camera and a strobe LED mounted in such a way that the droplets could be visualized, it would be possible to improve the quality of droplet ejection by visual verification. Optical imaging equipment was something I did not have for this study. The patterns employed in this work purposefully did not rely on highly repeatable droplet placement, but with these tools, it is expected that such studies could be more readily undertaken.

5.5 Printer design

The printer itself consisted of hardware costing approximately $7000, not including the host PC, which is considered a commodity item in a research lab. It is believed that the relatively custom hardware of the JetDrive III printhead driver from Microfab could be replaced in favor of a relatively simple custom circuit consisting of an op-amp, a power supply, a few resistors, and judicious control of the PC's parallel port as digital droplet command generator, bringing the price down to approximately $4000.

The primary critique of the hardware is that its top speed was slow enough that printing time was longer than it could be. Further, after long periods of continuous printing, the motors occasionally would be unable to keep up with the command velocity when moving against the spring-return of the linear stage, perhaps because of heat, and their error would reach threshold and the print job would abort. However, despite these limitations, motor solutions that could overcome these limitations, such as linear magnetic motors, would be substantially more expensive and heavier. A significant obstacle during programming was repeated problems with timing issues between the three processing devices. Occasionally a command would reach a device in the wrong order, or the DROPDRAW program would timeout while waiting for a response "buffer no longer full" from the serial port. It is suggested that future printer implementations would be simpler if one or more of the

three was not required to communicate via serial port. For instance, it is believed that not all of the sophisticated features of the JetDrive III control box were needed, and a surrogate system using the computer's parallel port could be adapted. While the Newport ESP300 controller provided all of the necessary capabilities needed to implement the features of the printer, it is the author's opinion that the device was intended for simpler control problems. However it was able to perform well nonetheless, despite requiring some convoluted command structures and modes to operate as desired (such as the need to "group" axes that were intended to move in concert). The author vastly underestimated the time it would take to implement the DROPDRAW software and printer, and would have looked more closely at commercial systems were he to attempt it again. However, the author is not aware of any software system that is tailor-made for ink-jet printing of the sort reported here.

Another option would be to further study the possibility of using a modified commercial ink jet printer. Such devices have the advantage that there is a wide range of refined software designed to generate images for their output, and printing time would be on the order of seconds, instead of minutes for the reported hardware. However, it is believed that precise control of individual droplet placement could be hard to achieve, and depositing structures such as islands could be problematic. It is the author's experience that desktop printer manufacturers are very reluctant to divulge any information about the systems employed by their hardware, owing to the substantial competition in that market. Hence attempts at contacting the manufacturer for means to precisely control droplet placement have not yet been undertaken. Preliminary attempts at simply drawing small-scale features and observing the resulting printed output has shown that small features at the scale of single droplets and/or single-wide lines of droplets on commercial printers are hard to achieve. However, it is conceivable that shapes such as thickly-layered islands and gradients could still be fabricated using a technique in which the paper-feed mechanism of the printer is disabled and the drawing consists of repeated patterns or

patterns of nearly repeated patterns that become repeatedly deposited in the same location. However, for this work, the ability to print relatively ambitious ink types was an advantage. Commercial printers have problems in this regard because of their use of multiple simultaneous nozzles, which compounds the likelihood of clogging, and the expectation of having surfactants in their inks, which may be harmful to protein-based inks. Further, and perhaps most significantly, the large priming volumes that are required to start printing with commercial ink cartridges makes using expensive proteins such as laminin or custom-designed peptides problematic. The priming volume needed in the printer reported here was often less than 1 mL. In sum, while commercial printers could offer potential benefits, the technology-centric nature of this work required a very custom design, and enabled results that would not have been attainable with desktop printer hardware.

5.6 Wax printing

Wax printing was explored as a means for building three-dimensional structure to constrain neurite growth, but the conclusion reached in this work was that that the low resolution of wax printing did not justify its further use for studying cultured neurons. Further, problems with consistently getting quality jetting were endemic: only very rarely did droplets place repeatably, though when they did the results were stimulating, as in Figure 13. After discussion with Prof. Ain Sonin of MIT, who pioneered 3D fabrication with wax, it was decided that the print head was being heated too high and was likely oxidizing wax at the nozzle orifice, causing clogging. It was found that cells were repelled only marginally from wax patterns, as compared to their adhesion to bare glass or polylysine-treated glass. It is speculated that the marginal repulsive strength of the wax may be attributable to the fact that its source is a plant, and so there may be adhesive contamination. A further experiment could be to test out the repulsive properties of more pure microcrystalline wax. The idea of printing gels was briefly explored, because of the ability of neurites to pass through

gel walls, but no gel was passed through a nozzle because it appeared to be far too viscous and non-Newtonian at the relevant temperature range. It may be worth exploring the idea of printing a very low-viscosity gel in the future, however.

5.7 Chemistry

Preparation of the PEG inhibitory background layer was a time-intensive process taking over three days and it utilized the toxic substance glutaraldehyde. Thus there is room for improvement in this protocol, primarily in reducing the number of steps. The step of soaking the patterned coverslips in serum overnight before plating weakened the inhibition of the PEG. However, this study sought to primarily optimize cell health, and cells were healthier on serum soaked PEG, though much more compliant on non-soaked PEG. This is an area for further study.

5.8 Future work

Most promising for theoretical study appears to be ink-jet printing's ability to make rings of neurons where there are no glia, which could be useful for experiments to compare theoretical ring networks to actual ones (Hahnloser et al, 2000; Xie et al, 2002) and its ability to make rapidly adjustable patterns of connected islands for studying long-term potentiation (LTP) and/or the Hebbian learning paradigm. Its ability to fabricate gradients could be useful both for developmental studies in which physiological mechanisms for axon outgrowth could be studied, and for theoretical studies in which axon direction could be useful, such as for in multi-layered networks. It is the author's opinion that using a commercially-available desktop printer may be more practical for the simpler-to-print structures, such as connected islands.

For axon growth and other physiological studies, the printer may prove to be a powerful tool for combinatoric analysis of the array of behaviors of neurons during

neurite outgrowth and migration. And there is substantial room for exploring the effect and significance of substrate-bound gradients (Dertinger et al, 2002), which have shown the ability to orient axon growth. Printed

In the area of biomaterials, ink-jet printing was adept at printing self-assembling peptide materials that gelled into structures resembling extracellular matrix proteins that were neuron-adhesive. Further study could explore the idea of depositing islands or other structures of soluble factors in gel for making diffusion gradients in the culture medium. Fabricating point sources of soluble factors requires assessing the rate of diffusion out of the gel and through the solution to determine the gradient steepness, insuring that such factors retain their activity after exposure to open air during the print step, and insuring that their levels are detectable by neurites and cells. It is likely that an improvement in substrate chemistry could be found to either reduce the time needed to make the inhibitory background, improve the health of the cells, or improve pattern fidelity.

REFERENCES

Arancio O, Kandel ER, Hawkins RD (1995) Activity-dependent long-term enhancement of transmitter release by presynaptic 3',5'-cyclic GMP in cultured hippocampal neurons. Nature 376:74-80.

Baier H, Bonhoeffer F (1992) Axon Guidance by Gradients of a Target-Derived Component. Science 255:472-475.

Banker G, Goslin K (1998) Culturing Nerve Cells, 2nd. Edition. Cambridge, MA: MIT Press.

Bradke F, Dotti CG (1999) The role of local actin instability in axon formation. Science 283:1931-1934.

Branch DW, Wheeler BC, Brewer GJ, Leckband DE (2000) Long-Term Maintenance of Patterns of Hippocampal Pyramidal Cells on Substrates of Polyethylene Glycol and Microstamped Polylysine. IEEE Transactions on Biomedical Engineering 47:290-300.

Cass T, Ligler FS (1998) Immobilized Biomolecules in Analysis: A Practical Approach. Oxford: Oxford University Press=.

Chang S-C, Bharathan J, Yang Y (1998) Dual-color polymer light-emitting pixels processed by hybrid ink-jet printing. Applied Physics Letters 73:2561-2563.

Colicos MA, Collins BE, Sailor MJ, Goda Y (2001) Remodeling of synaptic actin induced by photoconductive stimulation. Cell 107:605-616.

Demarse TB, Wagenaar DA, Blau AW, Potter SM (2001) The Neurally controlled Animat: Biological Brains Acting with Simulated Bodies. Autonomous Robots 11:305-310.

Dertinger SKW, Jiang XY, Li ZY, Murthy VN, Whitesides GM (2002) Gradients of substrate-bound laminin orient axonal specification of neurons. Proceedings of the National Academy of Sciences of the United States of America 99:12542-12547.

Dowell N, Perez-Turner AM, Hussain RJ, Smith K, Russo A, Craighead HG, Turner JN, Shain W (2001) The role of topographical cues on the development of rat

hippocampal neuron polarity and synapse formation in vitro. Molecular Biology of the Cell 12:377a-377a.

Duthaler G (1995) Design of a drop-on-demand delivery system for molten solder microdrops. In: Mechanical Engineering. Cambridge, MA: Massachusetts Institute of Technology.

Esch T, Lemmon V, Banker G (1999) Local presentation of substrate molecules directs axon specification by cultured hippocampal neurons. Journal of Neuroscience 19:6417-6426.

Fromherz P, Offenhausser A, Vetter T, Weis J (1991) A Neuron-Silicon Junction: A Retzius Cell of the Leech on an Insulated-Gate Field-Effect Transistor. Science 252:1290-1293.

Fuller SB, Jacobson JM (2000) Ink-jet fabricated nanoparticle micro-electromechanical systems. Proceedings of the IEEE conference on micro-electromechanical systems:138-141.

Fuller SB, Wilhelm EJ, Jacobson JA (2002) Ink-jet printed nanoparticle microelectromechanical systems. Journal of Microelectromechanical Systems 11:54-60.

Griffith LG, Wu B, Cima MJ, Powers MJ, Chagnaud B, Vacanti JP (1997) In vitro organigenesis of liver tissue. Annals of the New York Academy of Sciences 831:382-297.

Grinvald A, Ross WN, Farber I (1981) Simultaneous Optical Measurements of Electrical-Activity from Multiple Sites on Processes of Cultured Neurons. Proceedings of the National Academy of Sciences of the United States of America-Biological Sciences 78:3245-3249.

Gross GW, Rieske E, Kreutzberg GW, Meyer A (1977) A new fixed-array multi-microelectrode system designed for long-term monitoring of extracellular single unit neuronal activity in vitro. Neuroscience Letters 6:101-105.

Hahnloser RHR, Sarpeshkar R, Mahowald MA, Douglas RJ, Seung S (2000) Digital selection and analogue amplification coexist in a cortex-inspired silicon circuit (vol 405, pg 947, 2000). Nature 408:1012-U1024.

Hebner TR, Wu CC, Marcy D (1998) Ink-jet printing of doped polymers for organic light emitting devices. Applied Physics Letters 73:2561-2563.

Heinzl J, Herz CH (1985) Ink-jet printing. Advances in Electronics and Electron Physics 65:91-171.

Hickman JJ, Bhatia SK, Quong JN, Shoen P, Stenger DA, Pike CJ, Cotman CW (1994) Rational Pattern Design for in-Vitro Cellular Networks Using Surface Photochemistry. Journal of Vacuum Science & Technology a-Vacuum Surfaces and Films 12:607-616.

Holmes TC, de Lacalle S, Su X, Liu GS, Rich A, Zhang SG (2000) Extensive neurite outgrowth and active synapse formation on self-assembling peptide scaffolds. Proceedings of the National Academy of Sciences of the United States of America 97:6728-6733.

Kandel ER, Schwartz JH, Jessel TM (2000) Principles of Neural Science. New York, NY: McGraw-Hill.

Maher MP, Pine J, Wright J, Tai YC (1999) The neurochip: a new multielectrode device for stimulating and recording from cultured neurons. Journal of Neuroscience Methods 87:45-56.

Maher MP, Dvorak-Carbone H, Pine J, Wright JA, Tai YC (1999) Microstructures for studies of cultured neural networks. Medical & Biological Engineering & Computing 37:110-118.

Mihm S (2000) Print Your Next PC. In: Technology Review.

Neher E, Sakmann B (1976) Single-Channel Currents Recorded from Membrane of Denervated Frog Muscle-Fibers. Nature 260:799-802.

Segev R, Shapira Y, Benveniste M, Ben-Jacob E (2001) Observations and modeling of synchronized bursting in two-dimensional neural networks. Physical Review E 6401:art. no.-011920.

Segev R, Benveniste M, Hulata E, Cohen N, Palevski A, Kapon E, Shapira Y, Ben-Jacob E (2002) Long term Behavior of lithographically prepared in vitro neuronal networks. Physical Review Letters 88:art. no.-118102.

Shahaf G, Marom S (2001) Learning in networks of cortical neurons. Journal of Neuroscience 21:8782-8788.

StJohn PM, Kam L, Turner SW, Craighead HG, Issacson M, Turner JN, Shain W (1997) Preferential glial cell attachment to microcontact printed surfaces. Journal of Neuroscience Methods 75:171-177.

Tessier-Lavigne M, Goodman CS (1996) The Molecular Biology of Axon Guidance. Science 274:1123-1133.

Thomas CA, Springer PA, Loeb GE, Berwald-Netter Y, Okun LM (1972) A miniature microelectrode array to monitor the bioelectric activity of cultured cells. Experimental Cell Research 74:61-66.

Turner S, Kam L, Isaacson M, Craighead HG, Shain W, Turner J (1997) Cell attachment on silicon nanostructures. Journal of Vacuum Science & Technology B 15:2848-2854.

Turner JN, Shain W, Szarowski DH, Andersen M, Martins S, Isaacson M, Craighead H (1999) Cerebral astrocyte response to micromachined silicon implants. Experimental Neurology 156:33-49.

Turner AMP, Dowell N, Turner SWP, Kam L, Isaacson M, Turner JN, Craighead HG, Shain W (2000) Attachment of astroglial cells to microfabricated pillar arrays of different geometries. Journal of Biomedical Materials Research 51:430-441.

Wallace DB, Hayes DJ (1997) Solder-jet technology update. In: SPIE International Symposium on Microelectronics, pp 681-684.

Walter J, Henke-Fahle S, Bonhoeffer F (1987) Development 101:685.

Williams PA (1990) Three dimensional printing: A new process to fabricate prototypes directly from CAD models. In: Mechanical Engineering. Cambridge: Massachusetts Institute of Technology.

Wyart C, Ybert C, Bourdieu L, Herr C, Prinz C, Chatenay D (2002) Constrained synaptic connectivity in functional mammalian neuronal networks grown on patterned surfaces. Journal of Neuroscience Methods 117:123-131.

Xie XH, Hahnloser RHR, Seung HS (2002) Double-ring network model of the head-direction system. Physical Review E 66:art. no.-041902.

Yeung CK, Lauer L, Offenhausser A, Knoll W (2001) Modulation of the growth and guidance of rat brain stem neurons using patterned extracellular matrix proteins. Neuroscience Letters 301:147-150.

Zeck G, Fromherz P (2001) Noninvasive neuroelectronic interfacing with synaptically connected snail neurons immobilized on a semiconductor chip. Proceedings of the National Academy of Sciences of the United States of America 98:10457-10462.

Zhang SG, Holmes T, Lockshin C, Rich A (1993) Spontaneous Assembly of a Self-Complementary Oligopeptide to Form a Stable Macroscopic Membrane. Proceedings of the National Academy of Sciences of the United States of America 90:3334-3338.

Zhang SG, Holmes TC, Dipersio CM, Hynes RO, Su X, Rich A (1995) Self-Complementary Oligopeptide Matrices Support Mammalian-Cell Attachment. Biomaterials 16:1385-1393.

Zhang SG, Yan L, Altman M, Lassle M, Nugent H, Frankel F, Lauffenburger DA, Whitesides GM, Rich A (1999) Biological surface engineering: a simple system for cell pattern formation. Biomaterials 20:1213-1220.