# Laser Micropatterning of Polylactide Microspheres into Neuronal-Glial Coculture for the Study of Axonal Regeneration

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**Summary:** 250,000 Americans suffer from spinal cord injury caused by vehicular, work, and sports related accidents[1]. After injury to the spinal cord, axons do not have the capability of regenerating across the lesioned site. To understand the mechanisms of axon regeneration and to identify a better method of regeneration, a new technique for mimicking and studying the *in vivo* cellular environment is needed. To meet this goal, we have developed a laser cell micropatterning system that uses a weakly focused laser beam to pattern both biological cells and nonbiological particles for the study of various cell-cell-polymer interactions. Using this system, we have successfully copatterned neurons, glial cells, and polymer microspheres into a viable matrix. These copatterns allow us to study the effects nerve growth factor has, when released from a degradable polymer microsphere, on a single neuron within a specific arrangement of multiple cell types.

**Keywords:** axon regeneration; biodegradable; drug delivery system; laser cell micropatterning; microspheres

## 1. Introduction

Spinal cord injury (SCI) currently affects 250,000 Americans, and 10,000 new individuals are added to this list each year[1]. This disabling injury causes enormous physical, psychological, and economical stresses on the victims themselves, as well as their family and friends.

To effectively treat SCI, it is necessary to understand the *in vivo* environment that is found in the central nervous system (CNS) prior, during, and post injury. Within the CNS there are two major cell types: the neuron, which conducts signals to and from the brain, and the glial cells which are the support cells that form the myelin sheath (oligodendrocytes) and blood brain barrier (astrocytes). These cells are arranged in specific patterns to insure stimuli are recognized, transferred, and processed correctly.

When SCI occurs, microglia migrate to the site to remove any debris in the lesion. During the same time period, oligodendrocyte precursor cells and reactive astrocytes enter

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the lesioned site, forming a dense glial scar. This dense glial scar creates physical as well as chemical barriers, caused by various proteoglycans and glycoproteins released by different glial cells[2]. These barriers inhibit axonal regeneration after injury. The physical barrier blocks axons from crossing the lesion site while the chemical barrier causes the growth cone to collapse[3, 4].

Though neurons do not regenerate within the CNS, Cajal proved that, in a permissive environment, neurons from the CNS do have the inherent capability to regenerate axons[5]. This environment includes the surface in which cells attach to as well as the chemicals that are present in the extracellular fluid. This can best be seen during development, where four different environmental cues that guide axons to their final target are expressed. These cues are attractive and repulsive contact guidance cues and attractive and repulsive diffusive cues[6].

To implement these desired cues, researchers have investigated the use of nerve conduits. Nerve conduits are simple cylindrical tubes that help protect regenerating axons from the harsh environment as well as guide neurons to induce axonal regeneration through the selective use of degradable polymer chemistry[7], electrical conductivity[8], Schwann cell encapsulation [9], and neurotrophic factor release[10].

The first and most widely studied neurotrophic factor, nerve growth factor (NGF), has been shown to aid in the survival of neurons, promote the growth of processes, as well as to stimulate the growth of axons and the formation of synapses[11]. Even more specifically, with a NGF concentration gradient, the growth of the axons will be directed along the path of low concentration to that of high concentration[12].

A common way to form this concentration gradient is through the release of NGF from a degradable polymer microsphere. One of the most widely used degradable polymers is polylactide (PL). PL is an α-hydroxy polyester found in three isomeric configurations: PLLA, PDLA, and PDLLA. PL undergoes degradation by hydrolysis of the ester bond. Through the use of a double emulsion technique, NGF can be easily encapsulated into PL microspheres of various sizes and loading concentrations[13-15].

When studying the effects of NGF released from degradable polymers, it is common to place the polymer microspheres into the sample medium and allow NGF to be released. This medium, minus the polymer microspheres, is then applied to a standard culture of neurons and the effects of the NGF are recorded[13, 15]. The described study is only sufficient to understand the macroscopic effects of NGF on developing neurons and

to determine the bioactivity of the released NGF. With this conventional approach, it is not possible to study the effects of NGF released from a single microsphere on individual neurons, therefore obscuring assessment of directional axon guidance. In addition, current techniques to study cell-cell interactions have relied heavily on micropatterning the culture surface with different substrates, which either inhibit or permit cell attachment to occur[16, 17]. It is not feasible to assess the temporospatial interactions of a single cell with its surroundings, including neighboring cells and adjacent extracellular matrix (ECM). Systematic evaluation is limited using conventional methodology because the site- and time-specific placement of an individual cell cannot be repeated for investigation.

To achieve temporally and spatially resolved cell-cell-polymer interactions, a new laser cell micropatterning system was developed, using the principles of laser-guided direct-writing described by Odde and coworkers.[18]. In this system, a weakly focused laser beam traps and guides both biological and nonbiological particles using the optical force associated with the beam. The radial force component traps a particle in the center of the beam, while the axial force component propels the particle onto the surface of the substrate. By moving the substrate relative to the stationary beam, a specific pattern can be obtained with a spatial resolution of $< 1$ $\mu$m. Various patterns created using this technique can then be used to study the effects caused by different cell and polymer arrangements.

Results from this research demonstrated that we were able to 1) generate the necessary sized PLLA microspheres for laser micropatterning 2) pattern neurons into various kinds of matrices, 3) show neuron viability after patterning, 4) copattern neurons, glial cells, and polymer microspheres to form a specific structure.

## 2. Methods and Materials

### 2.1 Polymer Microspheres[19]

Polymer microspheres were formed using a modified double emulsion technique, described in brief as follows. First, 500 mg of PLLA (Intrinsic viscosity = 1.09 dL/g; Absorbable Polymers International; Birmingham, AL) was dissolved in 2 mL of chloroform (Sigma; St. Louis, MO). The PLLA solution was then injected into a 5% poly vinyl alcohol (PVA; $M_w$ = 13,000-23,000 g/mol, 87-89% hydrolyzed; Sigma; St. Louis, MO) solution, stirred at 2000 RPM using an IKA* Eurostar Power Control-Visc Stirrer (IKA; Wilmington, NC) with a syringe and 22-gauge needle. The microspheres were

rinsed once with a 2% isopropanol (VWR; West Chester, PA) solution for 10 minutes and stirred at a rate of 700 RPM. The microspheres were then removed and placed into a 1-liter media bottle, and 200 mL of fresh 2% isopropanol was added. The media bottle was placed on an orbital shaker overnight at 150 RPM to remove any excess solvent. The following day, the microspheres were dried and stored under house vacuum until use. Using a Z2 Coulter® particle counter and size analyzer (Beckman Coulter; Coulter Electronics Limited, England), the microspheres were analyzed to ensure proper microsphere dimensions needed for patterning.

2.2 Neuron Dissection and Culturing[20]

A day-7 white leghorn chick embryo (Clemson Poultry Farm; Clemson, SC) was removed from an egg and placed into a 100 mm petri dish containing Hank's Balanced Salt Solution (HBSS; Invitrogen; Carlsbad, CA)) without $Ca^{++}$ and $Mg^{++}$. The head was removed and placed into a 35mm petri dish containing fresh HBSS. With the aid of a Fisher Scientific Stereomaster Zoom microscope (Fisher Scientific; Suwanee, GA) and using two Dumont #5 forceps (Fine Science Tools; San Francisco, CA), the skin covering the brain was removed and the brain tissue exposed. The meninges and blood vessels were removed from the brain tissue. The cleaned tissue of the forebrain was removed from the chick and placed into a new 35 mm petri dish containing fresh HBSS. Any remaining blood vessels were removed to prepare the tissue for further treatment.

The clean forebrain tissue was placed into a 2 mL centrifuge tube with 1 mL of 0.1% (w/v) trypsin (1:250; Sigma; St. Louis, MO) solution. The centrifuge tube was placed into a 37°C incubator with a 5% $CO_2$ atmosphere for 10 minutes. Following the incubation period, the trypsin solution was removed and 2 mL of neuron medium (medium 199 (Sigma; St. Louis, MO) supplemented with 10% fetal bovine serum (FBS; Invitrogen; Carlsbad, CA) and 100 ng/mL 2.5S NGF (Harlan Bioproducts; Indianapolis, IN) was added to the centrifuge tube. Using a 3 mL syringe with a 22 gauge needle, the tissue and medium was triturated approximately 12 times to form a cell suspension. The cell suspension was then counted using a hemacytometer and partitioned into the necessary concentrations.

2.3 Glial Dissection and Culture[21]

The same dissection procedures were used as described previously, with the one exception being the use of embryonic day-14 chick forebrain tissue.

After forebrain isolation the tissue was chopped into small pieces and placed into a 2 mL centrifuge tube with 2 mL of 0.1% trypsin solution. The centrifuge tube was placed into a 37 $^0$C incubator with a 5% $CO_2$ atmosphere for 10 minutes. After incubation the trypsin was removed and 2 mL of glial medium (medium 199 complimented with 10% FBS) was added to the centrifuge tube. The tissue and medium was triturated approximately 12 times. The 2mL of cell suspension was then transferred to a 15 mL centrifuge tube and 8 mL of fresh media was added and the suspension triturated approximately 3-5 times. The cells were then counted using a hemacytometer and seeded at a density of 250,000 cells/well on a 6-well nonsurface treated multi-well plate. The cells were then passaged several times to purify the glial cell population into an astrocyte culture.

Glial cells approaching confluence were removed from the surface of the 6-well plate by first rinsing each dish with warm phosphate buffer solution (PBS; Sigma; St. Louis, MO). Approximately 1 mL of 1x trypsin EDTA (Sigma; St. Louis, MO) was pipetted into each well and placed into a 37 $^0$C incubator with a 5% $CO_2$ atmosphere for 2-3 minutes. After incubation, the wells were rinsed with 3 mL of glial media. The cells were then counted and partitioned for use in patterning.

2.4 Surface Treatment

Fifteen minutes prior to dissection, a thin coating of 0.1% poly-L-lysine hydrobromide (PLL, $M_w$ = 150,000; Sigma; St. Louis, MO) was applied to cleaned gridded coverslips. After thirty minutes, the PLL solution was removed and the chamber rinsed three times with distilled water and allowed to air dry.

2.5 Laser Cell Micropatterning

The output from a $TEM_{00}$ mode, tunable (750nm – 950nm), CW (continuous wave), Nd:$YVO_4$ pumped, solid state Ti:Sapphire laser (3900S; Spectra Physics; Mount pleasant, CA) was expanded to achieve the required numerical aperture (0.3) and focused into a custom made laser cell deposition chamber. The required cell/particle suspension was injected into the chamber that was mounted onto a three-axis translation stage (FA-90; Aerotech; Pittsburgh, PA). The patterning progress was monitored in real time using a 20X objective lens connected to a SONY XC-ST 70 CCD camera (Edmund Optics; Barrington, NJ) that projected images simultaneously on a Sony PVM 137 black and white video monitor (Sony; New York, NY) and the adjoining computer monitor. The images were transferred to the computer using a Matrox Meteor II Multichannel Frame Grabber.

Labview 7.0 (National Instruments; Austin, Texas) was used for image processing and to control the translation stage.

Post pattern formation, the patterns were incubated in a 37 $^0$C incubator with a 5% $CO_2$ atmosphere. To form a copattern, the initial patterned cells were incubated for one hour to allow for cell attachment. A second cell suspension was then injected into the chamber and the copattern was formed.

Individual patterns of neurons and polymer microspheres were observed using either the 20X objective in the micropatterning system, a Nikon Eclipse microscope (TS100; Nikon; Melville, NY) with 20X or 40X objective, or a Zeiss Axiovert inverted microscope (200M; Carl Zeiss; Thornwood, NY) with 40X or 63X oil emersion objective.

## 3. Results

3.1 Polymer Microsphere Characterization

To ensure the proper microsphere size to be used in laser micropatterning, the size and size distribution for the polymer samples were measured using a Coulter® counter. Targeting a size range of 10 – 20 microns, the generated PLLA microspheres had an average diameter of 16.08 microns with a standard deviation of 3.56 microns.

3.2 Formation of Micropatterns

Various patterns of microspheres were created, as shown in Figure 1, to optimize the laser guidance procedure. In these patterns, polystyrene microspheres (Magsphere Inc; Pasadena, CA) with a diameter of 10.2 microns, similar to a neuron diameter, were used.

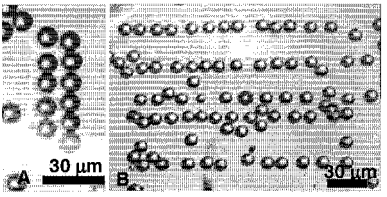


Figure 1. Different micropatterns (20X objective) created using 10.2 micron polystyrene microspheres.

After optimization of the guidance procedures, neuron patterns were created, as shown in Figure 2, ranging from a simple 2 by 2 matrix to a 3 by 8 matrix.

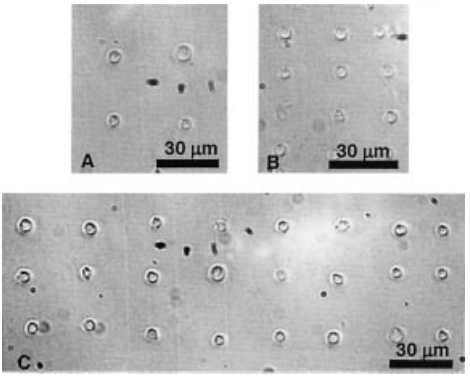


Figure 2. Neurons patterned into different matrix using the laser micropatterning system: A) 2 by 2 matrix; B) 4 by 3 matrix; and C) 3 by 8 matrix.

3.3 Neuron Viability

Once the patterns were formed, it was necessary to demonstrate that these cells were viable. After cell patterning, the chamber was placed into a 37 $^{0}$C incubator with a 5% $CO_2$ atmosphere for one hour to allow cell attachment; following incubation the pattern was observed using a Nikon phase microscope with 20X or 40X objective, as well as an Axiovert 200m Zeiss microscope with an oil emersion 40X objective and time-lapse imaging facility. From these images, we were able to observe dynamic neurite extensions, as depicted in the white circles shown in Figure 3.

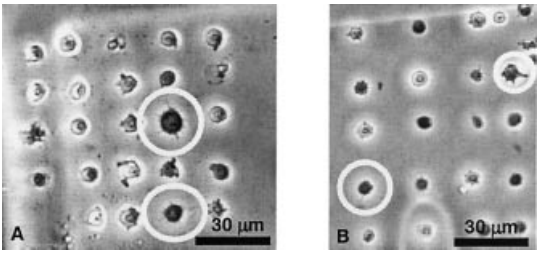


Figure 3. Two different neuron patterns observed A) 1 hour and B) 3 hours after cell patterning; neurite outgrowth can be seen from the neuronal cell bodies highlighted by white circles; images were recorded with the Nikon microscope.

Patterned neurons were viable for up to 5 days post laser deposition; extensive neurite extension and elongation was observed 1, 3, 6, and 12 hours after patterning.

3.4 Neuron, Glial, and Polymer Copatterns

Copatterning was achieved by laser depositing the neurons into designed positions and patterning the glial cells around the neurons. After an hour of incubation, the polymer microspheres were patterned. Before patterning the microspheres, the glial cells started to spread and grow in the pattern. Figure 4 shows a copattern completed with the placement of four polymer microspheres in the corners of a 3 by 3 neuronal-glial matrix.

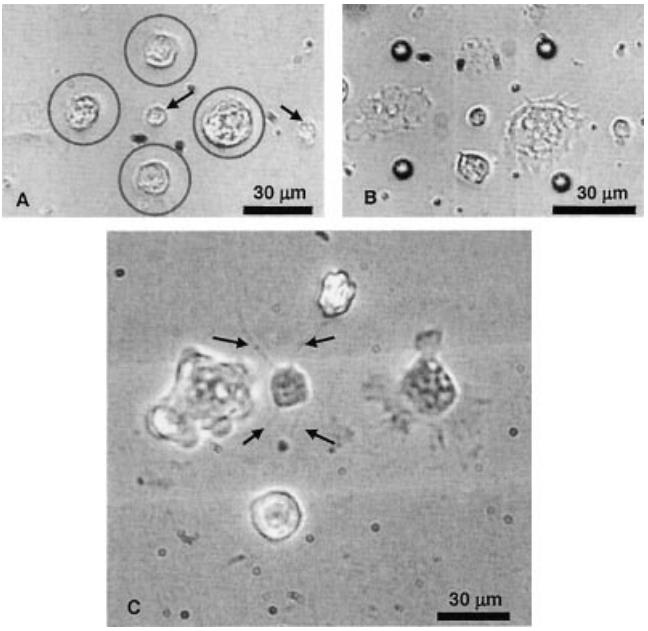


Figure 4. A) Initially neurons were placed into a pattern (highlighted by the black arrows); later, glial cells (highlighted with grey circles) were deposited around a neuron; B) After one hour of incubation, polymer microspheres were positioned into the pattern, completing the 3 by 3 matrix; C) Neurites (highlighted by the black arrows) were observed growing in four distinct directions from the neuron in the center.

## 4. Discussion

Unlike conventional micropatterning techniques where the surface is patterned with a combination of permissive and inhibitory substrates that restrict where and how cells grow or move[16,17], laser micropatterning does not depend on surface treatment. This unique feature allows cells to react to various environmental cues as they naturally would *in vivo*. Using laser guidance, both biological and nonbiological particles can be

copatterned with a spatial resolution of less then 1 micron (determined by the thermal motions of the cells or particles within the radial trap) for the systematic study of the responses of a single cell to its neighboring cells as well as to different agents released from polymer particles. For example, in Figure 4C, neurites can be seen extending from the body of the neuron in four different and distinct directions. This extension of neurites can be contributed to an array of factors surrounding the neuron. Further experiments must be conducted to identify if the neurite extensions are regulated by the glial cells patterned around and/or a specific way of releasing the NGF from patterned microspheres.

## 5. Conclusions

We have successfully demonstrated the ability to study the specific cell-cell-polymer interactions that are associated with neurons, glial cells, and PLLA microspheres using a laser micropatterning system. We have formed patterns using single particles or cells, as well as copatterns of various cell types and microspheres. These patterns lend the ability to study individual interactions between cells and polymer particles. In the future, we will encapsulate NGF into the PLLA microspheres and study the influence that local concentration gradients have on guiding axon regeneration either in a pure neuron culture or a coculture of glial cells and neurons.

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[1] "Spinal cord Injury." *The Gale Encyclopedia of Medicine. 2nd Ed.* Jacqueline L. Longe, Editor. 5 vols. Farmington Hills, MI: Gale Group, 2001.
[2] Schmidt, C. and Leach, B. (2003). "Neural tissue engineering: strategies for repair and regeneration." *Annu Rev of Biomed Eng.* 5:293-347.
[3] Grados-Munro, E. and Fournier, A. (2003). "Myelin-associated inhibitors of axon regeneration" *J of Neurosci Res.* 74: 479-485.
[4]Rhodes, K.E., Moon, L.D., Fawcett, J.W. (2003). "Inhibiting cell proliferation during formation of the glial scar: effects on axon regeneration in the CNS." *Neurosci.* 120: 41-56.
[5] Cajal S.R.Y. (1928) *Degeneration and Regeneration of the Nervous System* (trans. May R.M.) Oxford University Press, London.
[6] Kackzmarek, L.K. and Levitan, I.B. (2002) *The Neuron: Cell and Molecular Biology 3rd ed.* Oxford University Press, New York
[7] Novikov, L.N., Novikova, L.N., Mosahebi, A., Wiberg, M., Terenghi G., Kellerth, J.O. (2002). "A novel biodegradable implant for neuronal rescue and regeneration after spinal cord injury." *Biomaterials.* 16: 3369-76.
[8] Schmidt, C.E., Shastri, V.R., Vacanti, J.P., Langer, R. (1997). "Stimulation of neurite outgrowth using an electrically conducting polymer." *Proc Natl Acad Sci U.S.A..* 94: 8948-53.
[9] Oudega, M., Gautier, S.E., Chapon, P., Fragoso, M., Bates, M.L. Parel, J.M., Bunge, M.B. (2001). "Axonal regeneration into Schwann cell grafts within resorbable poly (α-hydroxyacid) guidance channels in the adult rat spinal cord." *Biomaterials.* 22: 1125-1136.
[10] Rosner B.I., Siegel R.A., Grosberg A., Tranquillo R.T. (2003). "Rational design of contact guiding, neurotrophic matrices for peripheral nerve regeneration." *Ann of Biomed Eng.* 11: 1383-401
[11] Dowling, J. (1992) *Neurons and Networks: An Introduction to Neuroscience* Harvard University Press, Cambridge.
[12] Cao, X. and Shoichet, M.S. (2001). "Defining the concentration gradient of nerve growth factor for guided neurite outgrowth." *Neurosci.* 103: 831-840.
[13] Cao, X. and Shoicet M.S. (1999). "Delivering neuroactive molecules from biodegradable microspheres for application in central nervous system disorders." *Biomaterials.* 20:329-339.
[14] Lam, X., Duenas, E.T., Cleland, J.L. (2001) "Encapsulation and Stabilization of Nerve Growth Factor into poly (lactide-co-glycolic) acid microspheres." *J of Pharm Sci.* 90:1356-65.
[15] Benoit, J.P., Faisant, N., Venier-Julienne, M.C., Menei, P. (2000). "Development of microspheres for neurological disorders: From basics to clinical applications." *J of Control Release.* 65: 285-296.
[16] Tien, J. and C.S. Chen, (2002). "Patterning the cellular microenvironment." *IEEE Eng in Med and Biol Mag.* 21(1):95-98.
[17] Folch, A. and Toner M., (2000). "Microengineering of cellular interactions." *Annu Rev of Biomed Eng.* 2:227-256.
[18] Odde, D.J. and Renn, M.J., (1999). "Laser-guided direct writing for applications in biotechnology." *Trends Biotechnol.* 17(10): p. 385-389.
[19] Jain, R., Shah, N.H., Malick, A.W., Rhodes, C.T. (1998). "Controlled drug delivery by degradable poly(ester) devices: different preparative approaches." *Drug Dev Ind Pharm.* 8: 703-27.
[20] Heidemann, S.R., Reynolds, M., Ngo, K., Lamoureux, P. (2003). "The culture of chick forebrain neurons." *Methods of Cell Biol.* 71: 51-65.
[21] Meberg, P.J. and Miller, M.W. (2003). "Culturing hippocampal and cortical neurons." *Methods of Cell Biol.* 71: 111-27.