Direct Write Technology as a Tool to Rapidly Prototype Patterns of Biological and Electronic Systems

B.R. Ringeisen*, D.B. Chrisey*, A. Piqué*, D. Krizman¥, M. Brooks*, B. Spargo*

*Naval Research Laboratory, 4555 Overlook Ave. SW, Code 6372 and 6115, Washington, DC

¥National Cancer Institute, Advanced Technology Center, 8717 Grovemont Circle, 134F, Gaithersburg, MD. 20877

ABSTRACT

The full potential of modeling and simulations is only realized when it is based on experimental results under varied conditions and systems. A single tool to rapidly prototype electronic and biological circuits would enhance the understanding and application of many systems. We have developed a novel laser-based direct write technique which has sub-10 micron resolution and is capable of rapidly prototyping micron-scale passive electronic devices, patterning active biological materials, and micro-machining conformal, flexible, and planar substrates. This manuscript presents our recent efforts to fabricate a living microfluidic device comprised of patterned and differentially cultured pluripotent cells, and to construct an improved antibody microarray capable of rapid and efficient protein identification (proteomics) from malignant and healthy cells.

Keywords: direct write, microfluidic device, pluripotent cells, protein microarray, rapid prototyping.

1 INTRODUCTION

Methods to generate mesoscopic patterns of viable cells and active biomaterials are required to fabricate next generation cell, protein, or antibody-based microfluidic biosensors as well as gene and protein recognition microarrays [1-8]. These applications also often require biomaterials to be placed onto electronic circuits or other detection devices, producing interfaces between abiotic and biotic materials. We believe that in order to fully understand these devices and improve the fabrication process, a combined effort including both experimental testing and theoretical modeling is needed. This approach should improve the overall performance of these devices, help make their fabrication more efficient, and provide valuable information about biomaterial-electronic interfaces.

At present there are several technologies capable of writing adjacent patterns or three-dimensional structures of different biomaterials, but fewer techniques exist that produce patterns that are software generated, have micron resolution, and are written at sub-millisecond times. These

qualifications are especially important when rapid prototyping is used in conjunction with modeling methods to improve microfluidic devices or the circuits used for actuation or detection. We have developed a laser-based transfer process that is capable of forming patterns and 3D structures of living cells and active biomaterials with resolution of less than 10 microns [9, 10]. This laser transfer technique is also capable of forming interfaces between biological and electronic materials by forming patterns of biotic (living microorganisms, active proteins, enzymes, DNA, antibodies, etc.) and abiotic (passive electronic devices and other inorganics) material adjacently on the same substrate or in multilayers [11-13]. This technology also eliminates the need for masks or moulds when fabricating surface patterns of biomaterials, can be driven by computer aided design/computer aided machining (CAD/CAM), allows rapid prototyping without the use of mask patterning and/or photolithography steps, and is compatible with most any substrate.

This manuscript describes the formation of a protein microarray and cell patterns using this laser transfer technique. These experiments provide the framework necessary for the production of antibody microarrays and the fabrication of living machines grown from pluripotent cell patterns.

2 EXPERIMENTAL METHODS

2.1 Laser Transfer Approach

As shown in Figure 1, the laser transfer apparatus consists of a quartz support coated with a matrix and biomaterials (see sample preparation section below for details), a substrate spaced 25 to 100 microns from the support that can be cooled or kept at room temperature during the transfer, and a Lambda Physik ArF excimer laser. By removing the support, laser pulses can be used to (subtractively) machine three-dimensional features in the substrate via micro-machining and drilling. With the support in place, laser pulses are focused at the quartz-matrix interface to a spot size of 10^6 to 10^2 μm^2 and a fluence of 0.2 J/cm^2 to direct write (additively) three-dimensional features of biomaterials including *E. coli*, Chinese hamster ovaries (CHO), and biotinylated fetal

bovine serum. Pulse frequency ranges from 1 to 20 Hz, resulting in variable feed and processing rates. Deposition speeds for electronic material patterns have been demonstrated to 200 mm/sec using higher pulse frequencies. The fluence used for the biomaterial transfers was 0.2 J/cm^2 or lower to limit the depth of material affected by the laser energy.
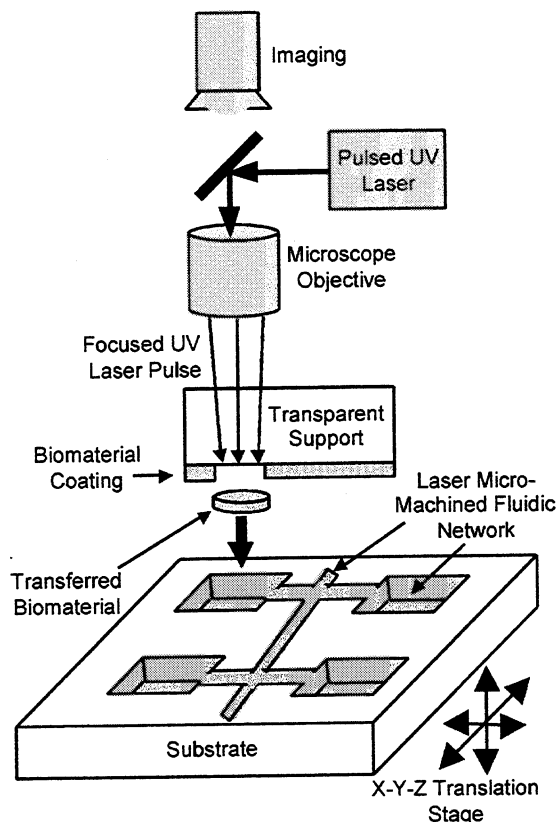

Figure 1: Schematic of our laser transfer technique capable of rapidly prototyping micron-scale patterns of passive electronic devices, micro-machining fluidic networks, and forming patterns of polymers, active proteins, and living cells.

Most biological species, such as cells or proteins, are active in aqueous buffer solutions or media, and in order to attain sufficient absorption of laser energy by water, an ArF excimer laser emitting 193 nm pulses is used. We use several matrices to stabilize these biomaterials on the quartz support. One method is to freeze a 2 to 10 micron layer of an aqueous biomaterial solution onto the transparent disk. Room temperature transfers can also be performed by uniformly spreading a composite containing the biological and a biocompatible material such as nutrient agar, collagen gel, a hydro-gel, a polymer, or an inert ceramic onto the disk. To perform the transfer, we focus the laser pulses at the matrix-disk interface. After the pulse passes through the transparent support, the laser energy induces sublimation of the matrix via electronic and vibrational excitation. The vaporized interfacial layers then propel the

remaining biomaterial and matrix towards the substrate. Depending on the matrix, this release transfers frozen solution or solid portions of composite material to the substrate. The observation that frozen material is transferred for the water-based experiments is an indication that there is little to no laser-induced heating to the vast majority of matrix.

2.2 Sample Preparation

For the *E. Coli* transfers, the jellyfish *Aeoquorea victoria* green fluorescent protein (GFP) was isolated from the pGFPuv plasmid (Clontech, Paolo Alto, CA), cloned into the TA vector (Invitrogen, Carslbad, CA) and then cloned into an 11.9 kb mobilizable broad-host range plasmid pKT230. *E. coli* containing pKT230::gfp was grown overnight at 37°C in Luria-Bertani (LB) broth with kanamycin (50 µg/ml). Before direct write experiments were performed, the cell concentration was increased to approximately 10^7 cells/ml by centrifuge and reconstitution. For frozen transfers, 20 µL of LB containing the concentrated *E. coli* was pipetted onto a sanitized two-inch quartz disk. The liquid was evenly distributed on the support and frozen in liquid nitrogen, resulting in an approximately 10-micron thick ice film. Each frozen transfer at a laser spot size of 0.09 cm^2 resulted in the placement of approximately 90 nL of ice, or 9×10^2 *E. coli* (average of 1 *E. coli* per 1×10^4 µm^2).

CHO cells were transferred at room temperature only, using a support layered with MATRIGEL® (Becton Dickinson Labware) and then viable CHO cells. This layering forms a viable CHO cell culture over the entire quartz disk. Prior to transferring living cells, we observe the area of the culture that we desire to transfer using the optical microscope objective discussed in Section 2.1. Other room temperature cell transfers were performed by screen-printing a homogenous mixture of cells, cell media, nutrient agar or inert ceramic nanopowders onto the quartz support.

Patterns of biotinylated bovine serum albumin were formed by using a quartz support coated with a 40% solution of glycerol and protein. We find that this is a universal method to transfer most biomolecules of interest.

3 RESULTS AND DISCUSSION

3.1 Bovine Serum Albumin

We have successfully used this laser transfer technique to directly write patterns of living *Escherichia coli*, living Chinese hamster ovaries (CHO), and bovine serum albumin onto various substrates ranging from nutrient culture plates to semiconductors and functionalized glass slides. Figure 2 is an optical micrograph of an array of active biotinylated bovine serum albumin deposited onto an aldehyde-treated
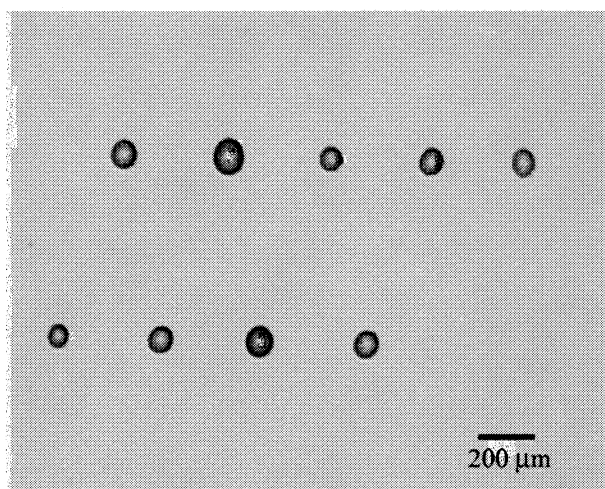
Figure 2: Microarray of biotinylated bovine serum albumin fabricated by our laser transfer technique. Spot sizes range from 50 to 100 μm.

glass slide by our laser-transfer technique. The spot size of deposited protein by our method ranges from 50 to 100 μm, whereas current available techniques produce spots with a minimum diameter of several hundred microns. This test experiment demonstrates that our technique is capable of fabricating a high-density microarray, where each spot uses less than 100 pL of total protein solution. Future experiments using the same procedure will deposit microarrays of antibodies specific to a variety of classes of cancer related proteins including intracellular and extracellular matrix proteins, signaling proteins, cell cycle proteins, growth factors, and growth factor receptors.

3.2   *E. Coli* Bacteria

Figure 3a shows an optical micrograph of an *E. coli* pattern deposited by laser transfer. The line width of the pattern is approximately 100 microns and demonstrates the ability of our approach to accurately place biomaterials on a glass substrate. *E. coli* cells containing the jellyfish *Aequorea victoria* green fluorescent protein (GFP) have been used to assess cell viability and to positively identify the transferred microorganisms from possible contaminants. Figures 3b and 3c show micrographs of a ceramic/*E. coli* composite patterned to write "NRL" (portion of "R" shown) under white and UV light, respectively. The characteristic fluorescence of the GFP is emitted only in the areas where *E. coli* was written. This relatively large pattern was written in order to transfer enough bacteria to observe the green fluorescence shown in Figure 3c. When the pattern was submerged in Luria-Bertani (LB) broth, fluorescence remained over a period of several days, indicating the bacteria were viable after transfer and that the composite material used as a matrix acted to immobilize the transferred cells.

Patterns of cells were also formed using a variety of different materials as stabilizing agents for the transfers. Mixing *E. coli* and cell media with nutrient agar or other composite materials provided a stable platform for room temperature experiments, and successful transfers were also performed without adding solidifying materials by freezing a thin film of cells and cell media to the support. This versatility in the types of materials that can be transferred by this technique is an advantage over current approaches that are only capable of patterning cells alone. Our method, for example, could be used to transfer cells with the nutrients, proteins, or amino acids needed to grow, adhere to the substrate, multiply, or maintain functionality. By
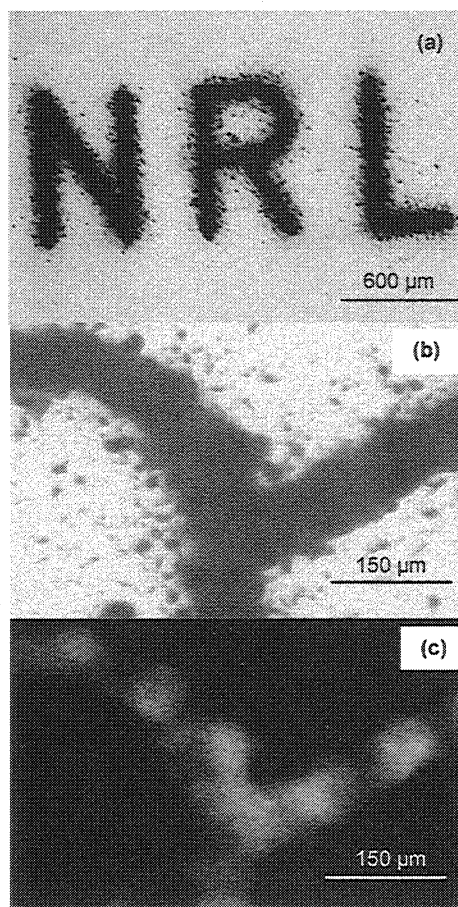

Figure 3: (a) Optical micrograph of laser-transferred ceramic/*E. coli* composite pattern. Scale equals 600 microns. (b-c) Transferred *E. coli* under white light (b) and 365 nm UV exposure (c). Green fluorescence is observed from viable cells expressing the green fluorescent protein (GFP).

using a rigid, biocompatible matrix such as collagen or sol-gels, our method could also form three-dimensional cellular structures that encapsulate the transferred cells and maintain the desired structure in various environments. In addition, the serial nature of this technique enables multiple

layers to be constructed step-by-step from various biomaterials.

3.3 Chinese Hamster Ovaries (CHO)

The next step in complexity for transferring living cells is to form patterns of mammalian cells. These cells are generally larger and more fragile than bacteria, and would therefore be more susceptible to the shear forces present during the laser transfer. Figure 4a is a micrograph of native (pre-transfer) CHO cells, while figure 4b is an optical micrograph of several CHO cells after laser transfer (dotted circle outlines the 200 µm spot of transferred cells). Figure 4b shows the growth and reproduction of the transferred CHO cells after three days in growth media. We observe no damage to the plasma membrane post-transfer, and the transferred cells appear very similar to the native cells in panel (a). The increased size and stretched appearance (attachment to the substrate) verify the viability of the transferred cells.
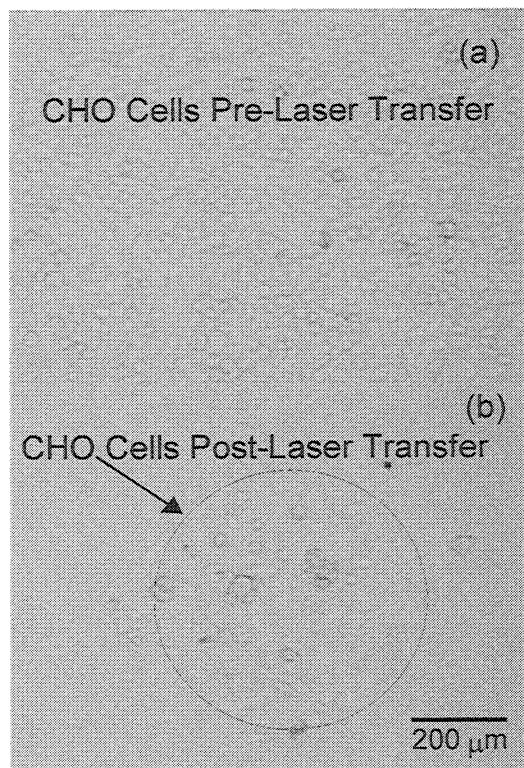

Figure 4: (a) Micrograph of living Chinese hamster ovaries (CHO) before laser transfer. (b) Laser-transferred 400 µm diameter pattern of living CHO cells after three days of culturing (transferred pattern before culturing was 200 µm).

Because the hydro-gel that we used as a matrix in these experiments is also compatible with many species of mammalian cells, we believe this approach is a general method to form cell patterns. We are now performing other

experiments with the intent to pattern individual pluripotent cells and cell structures. By differentially culturing these cell patterns into heart, muscle, and neural tissue, we will attempt to fabricate living microfluidic components such as pumps, valves, and electrodes, respectively.

4 SUMMARY

We have demonstrated the formation of novel patterns of living cells and active proteins by a new laser transfer technique. Previous results have demonstrated how this laser-based technique can also be used to rapidly prototype mesoscopic passive electronic devices and laser micro-machine conformal, flexible, and planar substrates [11-13]. In the future, we will use this approach to produce improved microfluidic biosensor arrays, to fabricate protein identification antibody arrays, to electronically probe intercellular signaling, to control the transfer and placement of pluripotent mammalian cells for differential culturing, and to even form three dimensional biological structures not found in nature (e.g., combinations of unique cells or cell arrays). We also hope to improve biological microfluidic devices by joining theoretical modeling of microfluidic networks and biological-electronic interfaces with our rapid prototyping capabilities.

5 REFERENCES

[1] Ptitsyn, L.R., et al. *App. Environ. Microb.* **1997**, 63, 4377-4384.

[2] Pancrazio, J.J., Whelan, J.P., Borkholder, D.A., Ma, W., Stenger, D.A. *Ann. Biomed. Eng.* **1999**, 27, 697-711.

[3] Corbisier, P. *Res. Microb.* **1997**, 148, 534-536.

[4] Yershov, G., Barsky, V., Belgovskiy, A., Kirillov, E., Kreindlin, E., Ivanov, I., Parinov, S., Guschin, D., Drobishev, A., Dubiley, S.; Mirzabekov, A. *Proc. Nat. Acad. Sci. USA* **1996**, 93, 4913.

[5] Guschin, D., Yershov, G., Zaslavsky, A., Gemmell, A., Shick, V., Proudnikov, D., Arenkov, P., Mirzabekov, A. *Anal. Biochem.* **1997**, 250, 203.

[6] Graves, D. J., Su, H. J., McKenzie, S. E., Surrey, S., Fortina, P. *Anal. Chem.* **1998**, 70, 5085.

[7] Miyachi, H., Hiratsuka, A., Ikebukuro, K., Yano, K., Muguruma, H.,Karube, I. *Biotech. Bioeng.* **2000**, 69, 323.

[8] Borman, S. *Chem. Eng. News* **2000**, 78, 31.

[9] Ringeisen, B.R., Chrisey, D.B., Piqué, A., McGill, R.A. "Generation of Living Cell and Active Biomaterial Patterns by Laser Transfer", Patent Pending, Navy Case# 82,621.

[10] Ringeisen, B.R., et al. "Generation of Mesoscopic Patterns of Viable *Escherichia coli* by Ambient Laser Transfer", submitted to *Biomaterials*, 2000.

[11] Piqué, A., et al. *App. Phys. A- Mat. Sci. Proc.* **1999**, 69, S279-S284.

[12] Chrisey, D.B., et al. *App. Surf. Sci.* **2000**, 154, 593-600.

[13] Fitz-Gerald, J.M., et al. *App. Phys. Lett.* **2000**, 76, 1386-1388.