

Generation of Mesoscopic Patterns of Viable *Escherichia coli* by Ambient Laser Transfer

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Abstract

We have generated mesoscopic patterns of viable *Escherichia coli* on Si(111), glass, and nutrient agar plates by using a novel laser-based transfer process termed matrix assisted pulsed laser evaporation direct write (MAPLE DW). We observe no alterations to the *E. coli* induced by the laser-material interaction or the shear forces during the transfer.

Transferred *E. coli* patterns were observed by optical and electron microscopes, and cell viability was shown through green fluorescent protein (GFP) expression and cell culturing experiments. The transfer mechanism for our approach appears remarkably gentle and suggests that active biomaterials such as proteins, DNA and antibodies could be serially deposited adjacent to viable cells. Furthermore, this technique is a direct write technology and therefore does not involve the use of masks, etching, or other lithographic tools.

KEYWORDS: direct write, laser processing, viable cell patterns, biomaterials, MAPLE DW

Introduction

Methods to generate mesoscopic patterns and arrays of viable cells are required to fabricate next generation tissue-based sensing devices [1-5], to build three-dimensional cellular structures for advanced tissue engineering [6-8], and to selectively separate and differentially culture microorganisms for a variety of basic and applied research applications [9-11]. Because of the broad nature of these applications as well as their technological importance, there are currently several methods to pattern or produce patterns of active biomaterials and viable cells. Cells can be patterned through various lithographic techniques utilizing self-assembled monolayers, microcontact printing, and photolithography to generate surfaces with cell-specific adhesive patches that capture cells from solution [12-15]. This approach can be modified to form networks of microfluidic channels or micromachined stencils that have been used to successfully pattern two different cell types adjacently [16-18]. Laser capture microdissection (LCM) is capable of transferring and separating specific cells from a large sample, but has not been used to "capture", or transfer, viable microorganisms [19,20]. Most laser-based patterning techniques use UV photoablation to micromachine biological substrates [21-23], although one approach uses laser guidance to deposit patterns of certain biomaterials [24,25]. This manuscript describes the formation of viable *E. coli* patterns by a novel laser-based direct write method that forms cell patterns without the use of masks, stamps, etching, or other lithographic tools.

Matrix assisted pulsed laser evaporation direct write, or MAPLE DW, is a laser-based processing technique that was originally designed to fabricate and rapidly prototype mesoscopic electronic devices from composite materials [26-28]. We also find

that this process is gentle enough to successfully transfer a wide variety of organics including polymers and active proteins. Figure 1 shows a schematic of the MAPLE DW technique. The material to be transferred is mixed in a UV-absorbent matrix and coated onto a quartz disk that is UV transparent. A focused UV laser pulse is directed through the backside of the quartz support so that the laser energy first interacts with the matrix at the quartz interface. The laser pulse is focused at the matrix-support interface by a UV microscope objective that also serves as an optical guide to determine the area of the matrix to transfer. Layers of matrix near the support interface evaporate due to localized heating from electronic and vibrational excitation. This sublimation releases the remaining material further from the interface by gently and uniformly propelling it away from the quartz support to a substrate positioned 25 to 100 microns away. MAPLE DW is capable of producing passive electronic devices (i.e., interconnects, resistors, capacitors) with line widths under 10 microns [26,28]. By removing the quartz support and allowing the laser pulse to interact with the substrate, this approach is also able to micromachine channels and through vias into polymer, semiconductor, and metal surfaces as well as trim passive devices to meet design specifications. All micromachining and material transfer can be controlled by computer (CAD/CAM), which enables this tool to rapidly fabricate complex structures without the aid of masks or moulds.

We have successfully used a novel variation of this technique to directly write patterns of viable *Escherichia coli* JM109 containing pKT230::gfp (green fluorescent protein reporter plasmid) onto Si(111), glass slides, and nutrient agar culture plates [29,30]. Most biological species are active in aqueous buffer solutions, and in order to attain sufficient absorption of laser energy by water, an ArF excimer laser emitting 193 nm pulses is used. In order to stabilize the bacteria on the quartz support, a 2 to 10

micron layer of cell media is frozen onto the transparent disk, or a room temperature composite containing cell media and a biocompatible material such as nutrient agar, collagen gel, a polymer, or an inert ceramic is screen-printed onto the disk³¹⁻³⁴. The mechanism for direct write of viable cells is based on the same principal as the electronic material transfers. The focused laser pulse passes through the transparent support and induces sublimation of the matrix via electronic and vibrational excitation. The vaporized interfacial layers then propel the remaining cells and matrix towards the substrate. Depending on the matrix, this release transfers frozen cells and growth media 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. Therefore, we believe that the optical processing tool described here may be capable of not only forming mesoscopic patterns of *E. coli* but also of other biological materials.

Materials and Methods

Laser Transfer Approach

As shown in Figure 1, the MAPLE DW apparatus consists of a quartz support coated with a matrix of nutrient and *E. coli* (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 micromachining 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 \AA^2 and a fluence of 0.2 J/cm^2 to direct write (additively) three-dimensional features of *E. coli*. 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 *E. coli* transfers was 0.2 J/cm² or lower to limit the depth of cell material affected by the laser energy. All experiments were performed in air, although future experiments performed at sub-freezing temperatures may need the addition of a dry box to reduce condensation on the quartz support.

Sample Preparation

The jellyfish *Aequorea victoria* green fluorescent protein (GFP) was isolated from the pGFPuv plasmid (Clontech, Palo Alto, CA), cloned into the TA vector (Invitrogen, Carlsbad, 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 10⁷ 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 cm² resulted in the placement of approximately 90 nL of ice, or 9x10² *E. coli* (average of 1 *E. coli* per 1x10⁴ µm²).

Room temperature transfers were performed by screen-printing a homogenous mixture of LB broth, *E. coli*, and nutrient agar or inert barium titanate nanoparticles onto the quartz support. These additive materials were used to form a paste that is adherent to the quartz support at room temperature. These materials also maintained the *E. coli* patterns in an aqueous environment post-transfer.

Results

Optical Microscopy

In order to determine whether viable *E. coli* JM109 were successfully transferred, cell patterns were first observed with an optical microscope. Figure 2a shows an optical micrograph of an *E. coli*/barium titanate nanoparticle/glycerol composite pattern transferred using MAPLE DW. The line width of the pattern is approximately 100 microns and demonstrates the ability of our approach to accurately place biological material 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 [35]. Figures 2b and 2c show micrographs of the *E. coli* pattern (portion of "R" shown) under white light and 365 nm 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 2c. 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.

The pattern shown in Figure 2a was obtained using a support coated with a composite mixture of *E. coli*, LB broth, glycerin and a ceramic powder. Other experiments using supports coated with frozen cells and cells mixed with nutrient agar resulted in similar patterns of viable *E. coli* that also emitted green fluorescence upon exposure to a black light. These results demonstrate that this technique is capable of transferring patterns of viable *E. coli* from a variety of matrices either frozen or at room temperature. This experiment was designed to demonstrate our ability to transfer living

organisms and was not an attempt to reach the ultimate resolution of the apparatus.

Previous work in our group shows that the resolution of direct write patterns is limited by the laser spot size, which can be focused to under 10 microns [26,28], and the material-substrate interaction.

Electron Microscopy

Scanning electron microscopy (SEM) was used to determine if there was any laser-induced damage to the transferred *E. coli*. Figures 3a and 3b show SEM micrographs of dried *E. coli* that was pipetted onto a Si(111) substrate. Figure 3a shows that the cell density is approximately $100 E. coli/10^4 \mu m^2$, and Figure 3b demonstrates the size, shape, and structure of *E. coli* not exposed to a laser pulse. Figures 3c and 3d show micrographs of dried *E. coli* after the laser-based transfer from a quartz support with frozen cell media to a Si(111) substrate. The areal density of bacteria is over 10 times smaller due to the small volume of frozen cell media transferred (on the order of 90 nL over an area of 0.09 cm^2) by the direct write process. The number of cells transferred to the substrate can be altered over orders of magnitude by concentration prior to forming the film on the transparent support. The novelty in the MAPLE DW process is the unique interaction of the laser with the coating on the support and is thus a quality that needs further optimization for applications where dense transfers are needed. Figure 3d shows that transferred bacteria are undamaged by the laser energy and are identical in shape and size to the cells transferred via pipette. The external cell membrane appears intact, and there is no evidence of laser heating or other destructive processes induced by the transfer.

Culturing of Transferred Cells

Two separate experiments were performed to determine the viability of the laser-transferred *E. coli*. First, frozen *E. coli* patterns were directly written onto nutrient agar culture plates in order to determine if specific *E. coli* growth could be observed around the transferred spots. The transferred crystals did not appear to have undergone a freeze-thaw cycle during the transfer process. If the laser transfer process induced the frozen cell media to melt, we would have observed a droplet of media on the substrate. Contrary to this, we observed several small crystals that were carried to the substrate and resulted in an *E. coli* pattern in the shape of the laser beam spot. Cell growth was observed around the transferred patterns on the agar plates after 24 hours incubation post-transfer, and the viability of transferred *E. coli* was determined by GFP emissions under black light. Secondly, six 0.09 cm² patches of frozen *E. coli* were written onto two sterilized glass slides that were immediately submerged in LB broth. Assessed at 40 hours, the transferred cells placed in liquid media showed significant growth and expression of GFP. Both culture experiments indicate that MAPLE DW successfully transferred viable *E. coli*.

Discussion and Conclusions

Our results present evidence that a laser-based processing technique can be used to directly write patterns of viable *E. coli* with no observable damage incurred from the laser treatment or the transfer process. Cell viability was determined by observing growth and GFP expression of *E. coli* that were directly transferred to nutrient agar culture plates. The observation of GFP expression from the transferred *E. coli* also indicates that the cell functionality as measured by biochemical reactions is maintained. SEM micrographs of the transferred cells demonstrate that any sheer forces present

during the transfer process were not strong enough to rupture the external membrane of the *E. coli*. We also find that during transfers performed with a frozen support, the *E. coli* and cell media that are carried from the support to the substrate remain frozen, indicating that little or no photon-induced heating occurs to the transferred material. This observation demonstrates that the laser energy used to release the matrix is not deposited into the majority of material transferred. Future experiments will determine whether larger, more fragile eucaryotic cells and other biomaterials, such as DNA or antibodies, can withstand the laser-matrix interaction and shear forces involved in the transfer process.

Patterns of *E. coli* 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 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 [36]. In addition, the serial nature of this technique enables multiple layers to be constructed step-by-step from various biomaterials.

We have also demonstrated that patterns of viable *E. coli* can be deposited on a variety of substrates including silicon, glass, and gels. Other techniques that use microfabricated stamps or microfluidic channels form well-defined cell patterns, but these approaches rely on specific cell-substrate interactions to anchor viable cells to a substrate from solution [14, 17, 18]. These methods are limited to certain substrates that can be chemically functionalized or that can withstand exposure to aqueous solutions. Therefore, these techniques may not be compatible with all technologically relevant surfaces or potentially complex sensing devices that juxtapose electronic and biomaterial platforms. On the other hand, our approach is compatible with miniature electronic devices and could potentially pattern biomaterials adjacent to many different functional materials [26, 37].

Directly transferring patterns of viable *E. coli* bacteria onto various substrates with a laser-based technique is a significant advance in biomaterial processing and shows progress in our understanding and manipulation of natural systems. Because the cell patterns are formed using a direct write approach, the process does not involve masks, etching, or other lithographic procedures. Our technique is also able to micromachine substrates as well as sequentially deposit passive electronic devices adjacent to viable cells. It therefore possesses all the tools necessary to rapidly fabricate unique cell-based biosensors and bio-electronic interfaces. In the future, we will use this approach to produce improved microfluidic biosensor 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).

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Figure Legends

Figure 1. MAPLE DW is a laser-based technique that transfers patterns of inorganic materials. A novel variation of this laser transfer technique is not only capable of optically imaging and directly writing micron-scale patterns of passive electronic devices, but is also capable of forming patterns of polymers, active proteins, and living cells.

Figure 2. (a) Optical micrograph of MAPLE DW transferred *E. coli* 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. Scale is equal to 150 microns.

Figure 3. (a-b) SEM photos of pipette transferred *E. coli* showing undamaged cell features (not exposed to laser energy). (c-d) SEM photos of MAPLE DW transferred *E. coli*. The crystals around the cells are due to dried LB broth that is used as the growth medium and matrix for the *E. coli*. This broth is transferred along with the cells and when dried, shows the perimeter of the *E. coli*. Comparison of panels (d) and (b) indicate that the MAPLE DW transfer process does not alter the shape, size, or viability of *E. coli*. Scales in panels (a), (b), (c), and (d) are 10, 10, 1, and 1 micron, respectively.

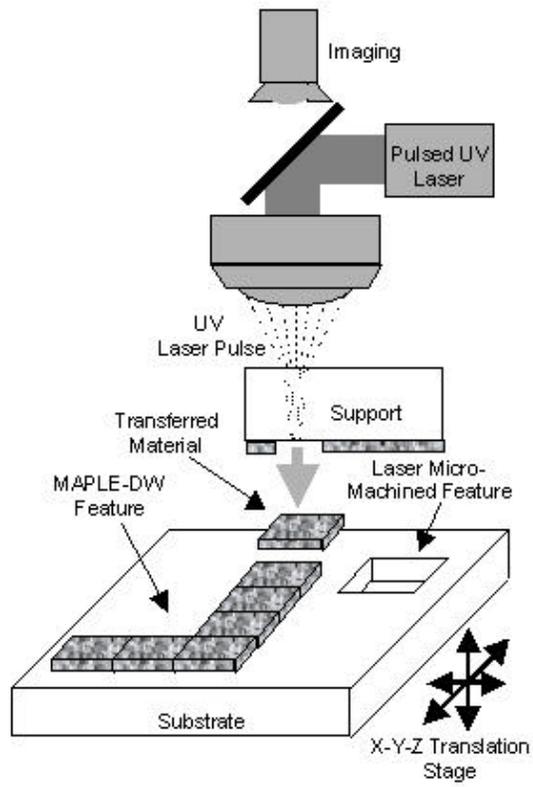


Figure 1

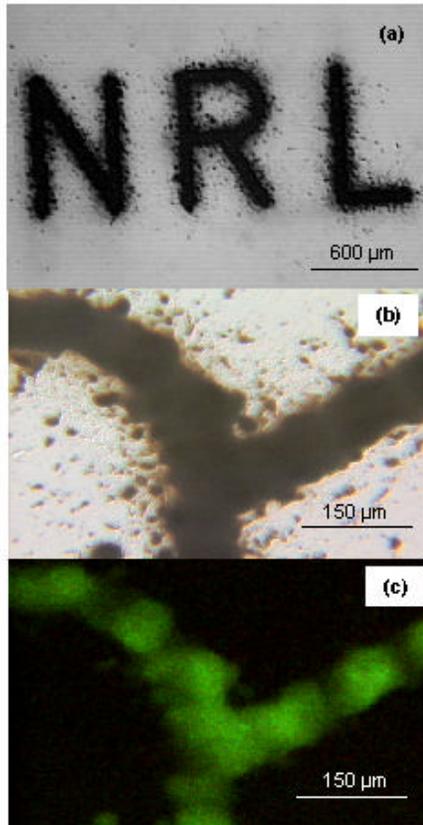


Figure 2

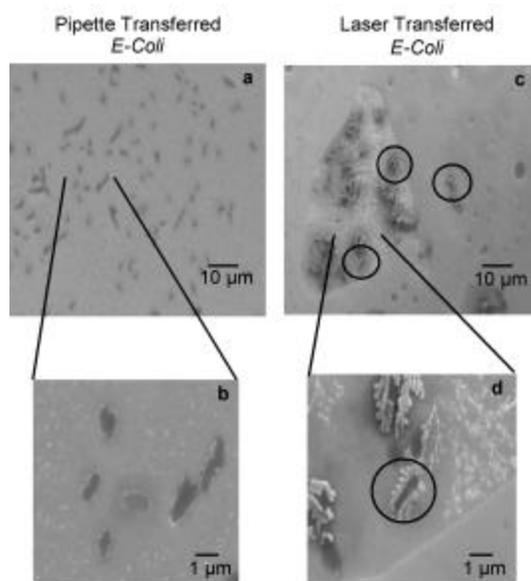


Figure 3