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Excimer laser forward transfer of mammalian cells using a novel triazene absorbing layer

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Abstract

We present a novel laser-based approach for developing tissue engineered constructs and other cell-based assembly's. We have deposited mesoscopic patterns of viable B35 neuroblasts using a soft direct approach of the matrix assisted pulsed laser evaporation direct write (MAPLE DW) process. As a development of the conventional direct write process, an intermediate layer of absorbing triazene polymer is used to provide gentler and efficient transfers. Transferred cells were examined for viability and proliferation and compared with that of as-seeded cells to determine the efficacy of the process. Results suggest that successful transfers can be achieved at lower fluences than usual by the incorporation of the intermediate absorbing layer thus avoiding any damage to cells and other delicate materials. MAPLE DW offers rapid computer-controlled deposition of mesoscopic voxels at high spatial resolutions, with extreme versatility in depositing combinations of natural/synthetic, living/non-living, organic/inorganic and hard/soft materials. Our approach offers a gentle and efficient transfer of viable cells which when combined with a variety of matrix materials allows development of constructs and bioactive systems in bioengineering.

Keywords: Laser forward transfer; MAPLE DW; Triazene polymer

1. Introduction

Tissue engineering integrates biology and materials engineering in developing next-generation medical technologies. Controlling cell adhesion/location, proliferation and patterning is critical in developing next-generation sensing devices and advanced tissue engineered constructs. Two-dimensional patterning of a single cell-type has been demonstrated previously by various surface recognition techniques. In a biomimetic approach Garcia et al. [1] demonstrate that the cell adhesion, proliferation and differentiation can be controlled by functionalizing the surface using RGD peptides. In another approach, laminar flow of the media in capillary systems has been used to create simple patterns of different types of ''cells and proteins'' within the same substrate [2]. Laborious steps are used to create elastomeric stencils using photoresists that are then used as masks to pattern cells and proteins [3]. Soft photolithographic methods such as microcontact printing, which involve the fabrication of stamps using masking techniques, have been demonstrated to create cell and protein patterns [4,5]. Rapid patterning techniques were proposed by using dielectrophoretic force to transfer cells from the electrode to a glass substrate, however with relatively low resolutions and accuracy [6]. Ink-jet technology, termed organ-printing, has been used to demonstrate patterning of proteins and cells [7–9]. The technology uses a CAD set-up with cell transfer through

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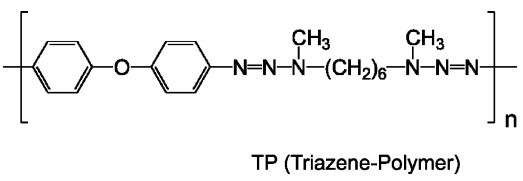
jet-based cartridge. Laser induced forward transfer (LIFT) has been shown to successfully transfer various dry films with good resolutions [10].

In this study, we present a novel laser-based approach of depositing viable cells into mesoscopic patterns that allow integration of cell to the matrix while the construct is being created. Our direct write approach termed matrix assisted pulsed laser evaporation direct write (MAPLE DW) [11] was originally developed to satisfy the need for conformal and mesoscopic passive electronic components. This process has recently been extended to soft and biological materials including a variety of polymers, biomolecules, and viable cells. Using this process various lines of viable cells [12,13] have been generated into mesoscopic patterns onto a variety of receiving substrates. In a recent study [14] we have demonstrated the co-deposition of osteoblast cells and ceramics as a composite mixture to develop novel heterogeneous scaffolds. MAPLE DW is recognized as a highly versatile tool for developing patterns of variety of materials with relativistic ease and short times, involving no masks, screens or preparation of stamps.

In a conventional approach, MAPLE DW utilizes a UV source light to evaporate the solvent to drive the solute or suspended particles forward to a receiving substrate. In depositing sensitive materials such as biomolecules and live cells, the absorption coefficient of the solvent might not be entirely preclude absorption by the biomolecules. To by pass this, an intermediate absorbing layer was developed in this study. Decomposing polymers with high absorption coefficient near the UV-spectrum were thought to be promising. The ablation characteristics for various polymers have been studied earlier [15] to determine the appropriate polymer for the required absorption coefficient and resonant frequency. For the current study, triazene polymer (TP) (structure shown in Fig. 1) was thought to be most appropriate given the source light and threshold fluence for ablation.

Triazene polymer was incorporated in our technique as an absorbing layer in the ribbon. As illustrated in Fig. 2, the ribbon includes a glass support coated with a layer of the explosive triazene polymer and a layer of the material to be transferred (viable cells). According to the suggested mechanism, the material and solvent to be transferred would absorb lower incident fluence when incorporated with an intermediate explosive layer than without. This would allow gentle and safe transfer of viable cells. The resolution and pattern dimension can be controlled by the distance of separation between the ribbon/substrate, the fluence, the spot-size and stage transition. MAPLE DW transfer process has clear advantages over other patterning processes in its providing a



TP (Triazene-Polymer)

Fig. 1. Chemical structure of triazene polymer (TP).

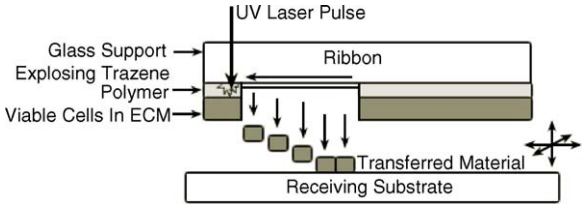


Fig. 2. Schematic of MAPLE DW, a laser-based technique for patterning organic, inorganic and living materials. A novel variation in using absorbing layer such as triazene polymer allows a softer transfer.

versatile environment of transferring a wide range of materials, with great accuracy, resolution, speed and efficiency (in minimal resources utilized and the cost for set-up).

The goal of this effort was to demonstrate efficient transfer of viable cells, by utilizing an absorbing layer of triazene polymer film on the ribbon. This would be achieved at a lower fluence when compared with the conventional MAPLE DW approach. The process would be extended for co-deposition of viable cells and biomaterials as a heterogeneous layer-by-layer approach.

2. Experimental

2.1. Matrix assisted pulsed laser evaporation direct write (MAPLE DW)

The MAPLE DW process can be used to transfer materials onto the substrate surface and micromachine channels or other features into the substrate. Fig. 1 shows a schematic of the matrix assisted pulsed laser evaporation direct write system process with a novel absorbing layer to minimize the energy required for deposition. An ArF pulsed excimer laser (λ = 193 nm, f = 10 Hz, τ = 30 ns) was focused onto the ribbon plane using a lens. A biomaterial-coated 1 in. diameter quartz disc is used as a ribbon, which is placed directly above the substrate. The gap between the ribbon and substrate was controlled using a Z-translation stage and kept constant at 150 μm s. Patterns were created using a joystick- or computer program-controlled X–Y translation stage. The diameter of the laser spot on the ribbon was maintained at 50 μm. Laser transfer was observed using an inverted lens camera that was focused on the receiving substrate. These experiments were performed under ambient air and at room temperature (25 °C).

2.2. Cell culturing

The B35 neuroblast clonal cell line isolated from the rat central nervous system (American Type Culture Collection, Manassas, VA, USA) were sub-cultured in a Dulbecco's modified Eagle's medium (DMEM) media, which contained 2 mM L-glutamine, 1.5 g/l sodium bicarbonate, 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, 10% heat-inactivated fetal bovine serum and 1% antibiotic syrup. Sub-confluent cells were trypsinized and split 1:5 to maintain division of the cell line and avoid differentiation. The cells were stored in a 37 °C, 5% CO_2 culture incubator.

2.3. Nuclear and cytoskeletal staining

Cells growing at time intervals of 3, 6, 12, 24 and 48 h post-transfer were washed with 1% PBS before fixation in formaldehyde at room temperature. For cytoskeletal staining, cells were first incubated in 0.2% Triton X-100 (Sigma–Aldrich, St. Louis, MO) for 1 min at room temperature followed by three washes with PBS. Cells were then incubated with phalloidin (Molecular Probes, Eugene, OR) in PBS (diluted 1:150) for 20 min at room temperature. After washing three times with PBS, cells were subjected to nuclear staining by incubating in a 10 μM solution of DAPI (Molecular Probes) for 5 min at room temperature. After the incubation time, the samples were washed twice with PBS and mounted on glass microscope slides with Fluoromount-G (Southern Biotechnology Associates, Inc., Birmingham, AL). Fluorescent-viable cells were then examined with a Zeiss LSM 510 confocal microscope.

2.4. Transfer of viable B35 neuroblast cells

The ribbons were prepared by spin coating triazene polymer on clean 1 in. glass discs to a thickness of 90, 110 and 140 nm. Each of these discs and the receiving glass substrates were spun coat at 1000 rpm for 10 s with 0.5 ml ECM (Extracellular Matrix, American Type Culture Collection). The ribbon was left in the incubator for 30 min to allow complete cross-linking of the ECM. Meanwhile, sub-confluent B35 neuroblast cells were counted prior to removal from the culture flask. The cells were then trypsinized and centrifuged at 5000 rpm for 3 min. The concentrated cell pellet at the bottom of the tube was reconstituted with 0.25 ml media to a concentration of approximately 10^7 cells/ml and pipetted onto the prepared ribbon containing the ECM. The ribbon containing the cells was then placed in the incubator for 10 min to allow cells to attach the ECM, prior to transfer. The gap between the ribbon and substrate was set at 150 μm. The fluence was varied to determine the threshold level for the polymer to start absorbing the incident light and rupture. For the cell transfer to occur the threshold frequency was determined to be 0.05 J/cm^2, monitored with a capture device and confirmed with optical micrograph. Fluence slightly above the threshold of approximately 0.07 J/cm^2 was used for the time profile study. The CAD/CAM program was used to generate the desired pattern. After the transfer, both the ribbon and the substrate were stored in separate Petri dishes containing pre-warmed media. The cells on the substrate are subsequently observed viability and proliferation at various time points of 3, 6, 12, 24, 48 h, using an inverted optical microscope.

3. Results and discussion

The preliminary step of the study was to determine the biocompatibility of the triazene polymer material. Various cells including osteoblasts, neuroblasts, myoblasts and endothelial cells were studied for adhesion, viability and growth on a triazene polymer substrate. Results indicated good adhesion and growth compared with other known compatible surfaces such as polystyrene. The subsequent step was to use the triazene coated ribbons for transferring viable cells onto ECM receiving substrates and culture them for growth profile study.

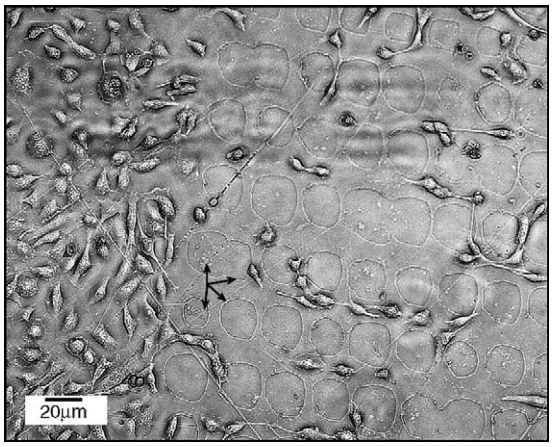


Fig. 3. Optical micrograph of the ribbon used for MAPLE DW transfer of B35 neuroblasts. Arrows indicate circular regions of desorbed polymer due to the absorption of incident light causing forward transfer of viable cells. Scale equals 20 μm.

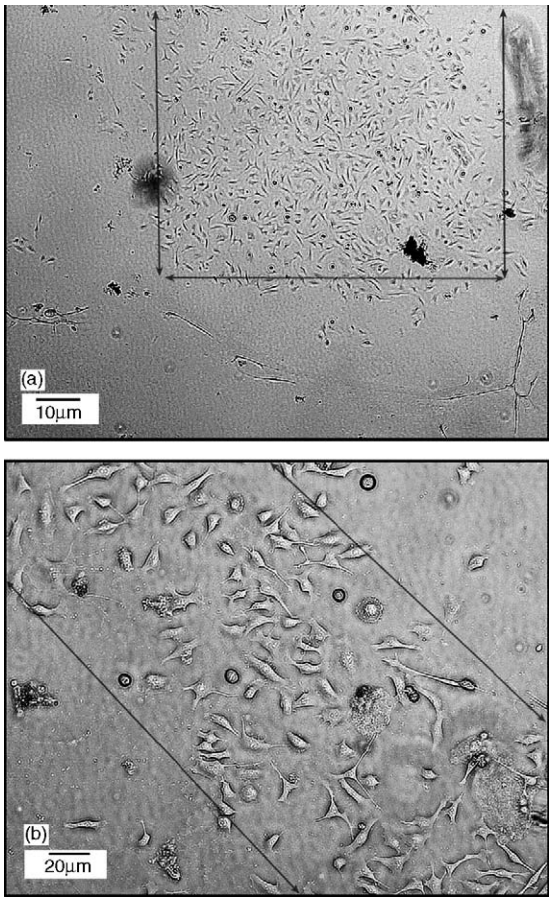


Fig. 4. (a) Optical micrograph of MAPLE DW transferred B35 neuroblasts into pre-defined patterns such as a box-like pattern. Scale equals 100 μm. (b) Line-like cell patterns. Scale equals 20 μm. Arrows are drawn to guide the eye.

In order to determine the efficacy of B35 neuroblasts deposition, a capture device was set-up and the transfer monitored for various fluences. Cell transfer was found to occur at fluences above 0.05 J/cm^2. Various cell-patterns were generated by using different algorithms for the stage movement. The cells on the ribbon were cultured in parallel as shown in Fig. 3, an optical micrograph of the ribbon at 48 h. The arrows shown indicate the explosions or ablations of the triazene layer within the laser spot size. The remaining few cells on the transfer region can be seen to form around the hole created by the ablation. The cells are removed from the ribbon in a well-defined pattern due to the ablation of the triazene layer. To determine if the cells were actually captured alive in the substrate beneath, the optical micrographs of the substrate are studied.

The transferred cells were to be cultured for at least 3 h before the pattern was clearly observed. Fig. 4a shows an optical micrograph of the MAPLE DW transferred B35 cells into a box-like pattern. Arrows are shown at the edges to guide the eye. The boundaries are fairly well-defined, considering cell locomotion post-transfer. In another pattern, line width (Fig. 4b) of approximately 100 μm is demonstrated, with arrows shown to guide the eye. In Fig. 4a and b, the small approximately 10 μm spheres are dead cells. We have earlier shown that spot sizes can be focused to under 10 μm to achieve high-resolution patterns [11]. However, in this case our primary goal was to establish the non-involvement of the explosive absorbing polymer film in the viability and proliferation of deposited cells.

Optical micrograph of MAPLE DW transferred cells at 6 and 48 h is shown in Fig. 5a and b, respectively. The micrograph at 6 h shows few neuroblasts cells at the initial stage of growth with short axons. The image at 48 h on the other hand illustrates a higher cell-density with longer extensions, suggesting viability and proliferation post-deposition. In Fig. 5a and b, the small approximately 10 μm spheres are dead cells. The optical micrographs at selective time points are compared to determine the growth rate based on the average number of cells per unit area. The growth profile of the B35 neuroblast cell transferred using the triazene absorbing layer is comparable to the growth rate of B35 cells deposited without the intermediate layer and that of the pipetted cells.

In a further step to determine viability of cells and possible quantification of axonal extensions, confocal images of stained nucleis and cytoskeleton are compared. Fig. 6a and b are confocal images of MAPLE DW transferred B35 cells at 24 and 48 h. At 24 h the nuclei of the transferred cells appear intact, while the development of axons cannot be clearly determined. The cells at 48 h post-transfer show intact preservation of nuclei with well-developed axonal extensions. These results demonstrate that the technique is competent in creating patterns of viable cells at low fluences.

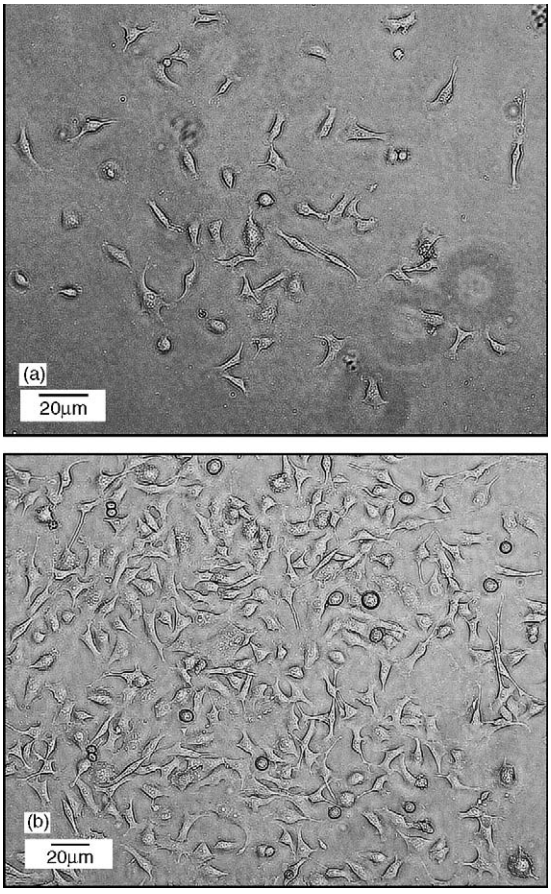


Fig. 5. (a) Optical micrograph of MAPLE DW transferred B35 rat neuroblast cells at 6 h post-transfer and (b) at 48 h post-transfer. Scales for both equal 20 μm.

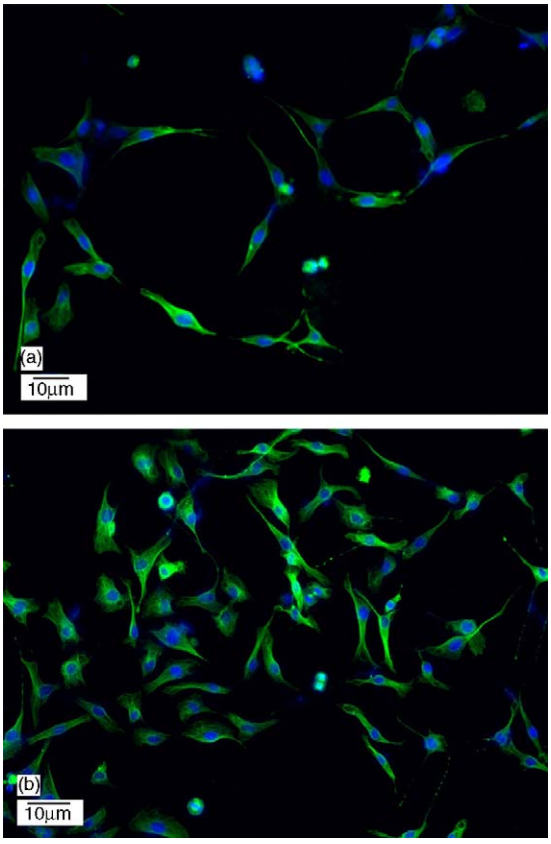


Fig. 6. (a) Confocal micrograph of stained B35 neuroblasts shows viable nucleus and cytoskeleton at 24 h post-transfer and (b) at 48 h post-transfer. Scale for both equals 10 μm.

Our current approach is able to provide successful transfers at approximately 0.07 J/cm^2, while our previous efforts without the absorbing layer were achieved at approximately 0.15 J/cm^2. This shows a reduction of more than 50% incident fluence required for transfer. This is particularly important when transferring viable cells and soft biomolecules. Ideally materials to be deposited should have a low minimum laser interaction. A near-zero absorption by soft materials is more likely in our approach by using an intermediate absorbing layer. In such as case, not only is the required fluence lower but the incorporation of the intermediate layer (with a high absorption coefficient at the operating wavelength) allows a safer transfer of biomolecules. At present, we are studying the effects of transfer on other cell lines with various cytotoxicity assays.

4. Conclusions

We have demonstrated that patterns of viable B35 neuroblasts can be achieved at low fluences than earlier by incorporating absorbing layers of polymers such as triazene polymer with a high linear absorption at the operating wavelength. MAPLE DW is a unique process that involves no masks, stamps, etching and other lithographic tools. It is arguably one of the most versatile tools for generating mesoscopic patterns in terms of materials selection with a wide range from organic to inorganic, living to non-living, ceramics to composites and various biomolecules. These demonstrate the potential of MAPLE DW in developing cell-based biosensors, bio-interfaces, three-dimensional heterogeneous tissue constructs and advanced tissue-based approaches.

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