

Effect of Laser Fluence in Laser-Assisted Direct Writing of Human Colon Cell

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ABSTRACT

Matrix-Assisted Pulsed-Laser Evaporation Direct-Write (MAPLE DW) has been emerging as a promising biological construct fabrication technique. The post-transfer cell viability in MAPLE DW depends on various operation conditions such as the applied laser fluence. For wide implementation of MAPLE DW-based biofabrication, the effect of laser fluence on the post-transfer cell viability should be first carefully understood. This study investigates the effect of laser fluence on the post-transfer cell viability in transferring of human colon cell HT-29. It has been observed that: 1) the HT-29 cell viability decreases from 95% to 78% as the laser fluence increases from 258 to 1,500 mJ/cm²; and 2) cell injury in this study is mainly from the process-induced mechanical stress during the cell droplet formation and cell landing processes while the effects of thermal influence and ultraviolet radiation are below the level of detection.

1. INTRODUCTION

Recently, various additive direct write technologies including laser guidance direct writing [1], modified laser-induced forward transfer (LIFT) (including Matrix-Assisted Pulsed-Laser Evaporation Direct-Write (MAPLE DW) [2,3] and its modifications [4,5]), ink-jetting [6], electro-hydrodynamic jetting [7] and biopolymer deposition [8], have been investigated for living cell-based pattern and construct fabrication. Among all these technologies, MAPLE DW, a non-contact laser-based direct-write technique, has emerged as one of the most promising surface deposition and additive manufacturing techniques [9]. As shown in Fig. 1, a typical, MAPLE DW setup includes three basic components: an ultraviolet (UV) pulsed laser source; a laser transparent quartz disk coated with materials to be transferred, known as a “ribbon”; and a computer-controlled receiving substrate for the transferred material. In next generation system the ribbon is also computer-controlled.

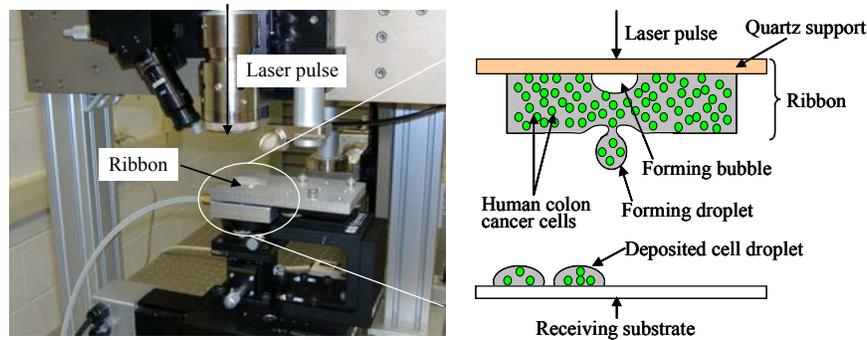


Fig.1. MAPLE DW apparatus and mechanistic schematic

Especially enabling biofabrication processes like the aforementioned may introduce excessive thermal and/or mechanical stresses to biomaterials including living cells. If this process-induced stress exceeds the adaptive capacity of a cell, irreversible damage may occur. The cell injury/damage can be simply classified as the thermal and/or mechanical cell damage and the biochemical damage [10]. Generally, cell injury is reversible up to a certain point; however, exposure of a cell to a high magnitude and/or lasting external stress may cause irreversible cell injury even cell death. Indeed, cell death due to process-induced cell injury is common in biofabrication processes [2, 11-13], and the post-transfer cell viability is a key index to evaluate the feasibility and efficiency of a biofabrication technique.

Some studies [2, 12-13] have been performed to investigate biofabrication process-induced cell injury. For MAPLE DW, there have been some interesting contributions, which include the experimental work on the effect of Matrigel coating thickness of the receiving substrate on the post-transfer mammalian cell viability [2] and the effect of laser fluence on the post-transfer yeast cell viability [13] as well as some modeling attempts regarding the MAPLE DW process-induced mechanical stress profile during bubble expansion [14] and landing [15]. However, the effects of laser fluence on mammalian cell injury have not been documented so far as mammalian cells may be more sensitive to external stress that is largely related to the laser fluence.

This study investigates the effects of laser fluence on the post-transfer HT-29 cell viability during MAPLE DW. The rest of the paper is organized as follows: First, the experimental materials and methods are illustrated in detail. The effects of laser fluence on the HT-29 cell viability are presented. Finally, some conclusions on the effects of laser fluence on cell injury during MAPLE DW are drawn.

2. MATERIALS AND METHODS

2.1 Cell culture

Human colon cancer cells such as HT-29 have been widely used as a research cell source to

study various normal and neoplastic processes [16-18]. As the HT-29 cell has retained certain characteristics of a normal tissue such as hormone receptors [19], it has been selected as a model cell to investigate the effects of laser fluence on post-transfer cell injury.

The HT-29 cells (ATCC, Manassas, VA) were cultured to 90% confluency in McCoy's 5A modified medium with L-Glutamine (ATCC, Manassas, VA), 10% (v/v) fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) and 1% (v/v) Penicillin-Streptomycin (ATCC, Manassas, VA) in a 37°C, 5% CO₂, humidified incubator. Then the cultured cells were washed twice with 10 ml of phosphate buffered saline (PBS) followed by the addition of 4 ml Trypsin-EDTA (0.25% Trypsin/0.53 mM EDTA in HBSS, ATCC, Manassas, VA) and incubated for 5 minutes. The trypsinized cell suspension was transferred to a 15ml centrifuge tube and vortexed with 1ml of calf serum (Biowhitaker, Walkersville, MD). The suspension was centrifuged at 454 g for 5 minutes, and the resulting cell pellet was resuspended in the same medium to a final density of 1×10^7 cells/ml for use in subsequent laser printing experiments.

2.2 Laser-assisted direct writing system

In order to better understand the post-transfer cell viability during MAPLE DW, direct writing of HT-29 cells have been studied using an ArF excimer laser (Coherent ExciStar, 193 nm, 12 ns FWHM duration). The MAPLE DW experimental process schematic is illustrated in Fig. 1. An Edmund optics quartz flat (Edmund optics, Barrington, NJ, 85% transmittance at 193 nm) was used as a quartz disk to make the ribbon. The substrate position was computer-controlled using an Aerotech XYZ translation stage.

2.3 Laser-assisted cell transfer process

The supporting quartz discs and receiving substrates were washed with 70% ethanol (Fisher scientific, IL) and then rinsed with deionized water (Fisher scientific, IL). When quartz discs were dried, a 3M Scotch tape was used to make a 1.5×1.5 cm well with a 100 μ m depth on the quartz disc. Then, 20 μ l of HT-29 cell suspension was pipetted into the well, resulting in an approximately 80 ~ 100 μ m thick coating. A freshly coated quartz disc was prepared for each cell transfer process.

The laser spot size was focused to a 120×280 μ m rectangle and the laser repetition rate was held at 50 Hz. Since the effect of the laser fluence on the cell viability is of interest, the laser fluence was varied from about 250 to 1,500 mJ/cm² during the experiment, and the actual laser fluence during each printing was determined based on the average of 100 laser pulses which were individually measured using a FieldMax laser power/energy meter (Coherent, Santa Clara, CA). The measured laser fluences were 258 ± 6 , 458 ± 6 , 669 ± 10 , 869 ± 13 , 1063 ± 13 , 1262 ± 15 , and 1482 ± 15 mJ/cm².

The receiving substrate was a 24-well flat bottom microliter plate and each well was filled with 1 ml McCoy's 5A modified medium with L-Glutamine supplemented with 10% (v/v) fetal bovine

serum and 1% (v/v) Penicillin-Streptomycin, resulting in a 5 mm thick cell medium coating inside each well. The transferred cells were directly printed into the liquid media inside one of 24 wells. The cell medium in each well not only provided an impact cushion for the transferred HT-29 cells during landing, but also provided a growth medium for the transferred HT-29 cells. Three control wells each with 20 μ L HT-29 cell suspension were also prepared to provide the benchmark information about the HT-29 cell viability under a nominal condition.

Under each fluence level, 1000 laser pulses as a cell transfer process were applied at different locations of a newly-coated quartz disk to transfer the trypsinized cell suspension to a well. The cell transfer process was repeated three times to deposit cells into three different wells of a multiwell plate for each laser fluence. Transferred cells were tested for cell viability (as described below) immediately after printing.

2.4 Cell viability test

Trypan blue is a traditional and widely employed cell viability assay for HT-29 cells [20-22]. The viability of HT-29 cells was evaluated using 0.4% trypan blue stain (Biowhittaker, Walkersville, MD). Post-transfer cell suspension and trypan blue dye were mixed first at a 50%-50% volume ratio. Then the samples were viewed using an optical microscope, and the live/dead cell assay was performed using a hemocytometer. During the counting process, transparent cells were considered live while blue cells were dead since viable cells with intact cellular membrane will exclude the blue dye. Each well sample was counted twice to get an average cell viability value.

3. CELL TRANSFER EXPERIMENTAL RESULT

As discussed in the previous sections, the process-induced cell injury is present in different methods used in biofabrication [2,12,13]. Generally speaking, any biologically important molecule in a cell can be the target of injury producing stress, and four biochemical systems are particularly vulnerable: cell membrane and cytoskeleton system, energy metabolism, protein synthesis, and nucleic acids representing the integrity of the genetic apparatus of a cell. Since many biochemical systems of the cell are inter-dependent, injury at one site typically causes a secondary injury to other cellular processes.

Cell injury in biochemical systems is usually due to cellular stresses caused by different physical changes, and the following lists the main negative changes: 1) mechanical deformation and rupture due to crush/blast injury, fractures, lacerations, hemorrhage, osmotic shock, and noise trauma; 2) physical changes due to thermal (such as burns, heat stroke, heat exhaustion, frostbite, and hypothermia), electrical (such as electric shock), and light (such as x-ray, radioactive element, and ultraviolet radiation) damage; 3) chemical changes due to caustic agents, poisons, genotoxic and proteotoxic compounds; 4) nutritive variations due to deficiency of oxygen, vitamins and basic nutrients; and 5) biological changes due to viruses, microorganisms, protozoan and metazoan parasites) [13]. During biofabrication processes, negative changes are

mainly process-induced thermomechanical stresses [13-15].

There usually is a time lag between the stress and the morphologic changes during cell injury or death, and this time lag varies with the sensitivity of methods used to detect these changes [23]. Cell injury can be simply classified as the mechanical cell damage and the biochemical damage. The biochemical damage is not immediately obvious and takes a time scale of the order of a cell-generation time before changes can be detected; and the mechanical cell injury takes place instantaneously and is immediately detectable [10]. Fortunately, cells have the ability to compensate some reversible injuries through normal physiologic adaptive mechanisms. If the injury is reversible, the damaged cells can recover to their normal functions. Otherwise, the cell death happens. This study has investigated the effects of laser fluence on the post-transfer cell viability and the cell injury reversibility, which may be mechanical and/or biochemical damage-based, respectively.

The membrane of transferred HT-29 cells may be: lysis (broken), permeable, and intact. For lysis cells, their cell membranes are seriously damage and dissolved in the cell suspension, so they cannot be easily detected using trypan blue. Only cells with permeable and intact membranes are considered here. For intact cell membrane HT-29 cells, the blue indicator turned colorless in the presence of active enzymes, thus indicating living cells, as shown in Fig. 2. For dead/damaged cells with a cell membrane structure, their cell membranes are permeable, resulting in a blue stain inside the cells as shown in Fig. 2.

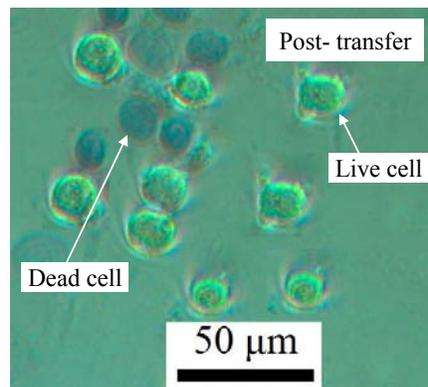


Fig. 2. Post-transfer HT-29 cells after trypan blue treatment

4. EFFECT OF LASER FLUENCE ON POST-TRANSFER CELL VIABILITY

As shown in Fig. 3, as the laser fluence increased from about 250 to 1500 mJ/cm², the HT-29 cell viability decreased from 95% to 78% (after considering the nominal apoptosis based on the control group). This relationship is mainly attributed to the process-induced mechanical stress during the cell droplet formation and cell landing processes, the process-induced thermal influence, and any residual UV radiation penetrating the cells. To improve the cell viability during MAPLE DW, the applied laser fluence should be carefully selected.

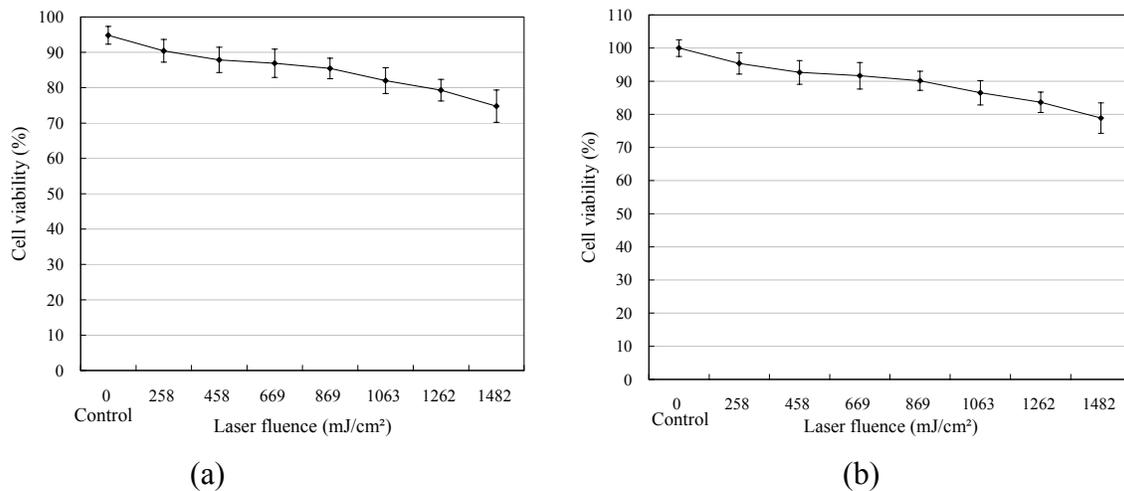


Fig. 3. Post-transfer cell viability as a function of laser fluence: a) the control effect is not considered and b) the control effect is considered

The process-induced mechanical stress during MAPLE DW may come from two different processes: the cell droplet formation (acceleration) and the cell droplet landing on the receiving substrate (deceleration). During the droplet formation, the HT-29 cell droplet is generated due to the expansion of forming the bubble, which is the result of the laser-matrix material interaction. The rapid expansion due to the high pressure bubble accelerates the forming cell droplet, and such an acceleration can be as high as $10^5 \sim 10^9$ g and the resulting droplet velocity can be as high as 50~1000 m/s during laser-assisted cell transfer [24,25]; the higher the laser fluence, the higher pressure, resulting in a higher acceleration/velocity. The impact during the cell droplet landing process also brings a significantly higher deceleration to HT-29 cells as simulated [15,26], and the cell viability was found to be closely related to the coating thickness of receiving substrate [2]. Higher laser fluences introduce higher cell droplet accelerations during the cell droplet formation process and higher cell droplet decelerations during the cell landing process [15]. As recognized, the process-induced high acceleration (or deceleration) and velocity can easily lead to severe cell injury or death as observed in the centrifugal force-induced cell damage studies [27,28]. In this study, the dependence of the cell viability on the laser fluence has proved the existence of mechanical damage as the higher laser fluence led to the higher cell acceleration (or deceleration) and velocity, resulting in the lower cell viability. It should be pointed out it is acceleration (or deceleration)-induced normal/shear stress, strain, and/or strain rate that damage cells being transferred over less than 5 microseconds [15].

The process-induced thermal injury to cells or tissues might be of concern as the laser energy absorbed causes the deactivation of enzymes, denaturation of proteins, and vaporization/carbonization of cells. Depending on the laser energy-induced temperature rise and its duration, thermal injury can be classified into four stages: hyperthermia, coagulation (denaturation), vaporization and carbonization [29]. In fact, thermal damage to biological

materials is always a temperature-time governed process [30]. If a laser pulse duration is longer than 10 μs , an Arrhenius-type activation process is considered as the dominant thermal damage mechanism [31,32]. For a pulse duration less than 1 μs , the bubble formation-induced pressure is the dominant mechanism for damage [33,34]. During the 12 ns laser pulse duration in this study, the thermal penetration depth due to the Fourier heat conduction is a few micrometers as the whole cell droplet formation usually happens within a few microseconds. Compared with the typical coating thickness (around 100 μm), the possible heat affected zone and the resulting thermal damage is considered negligible.

UV radiation from laser pulses can also effectively kill living cells; fortunately, the UV light in MAPLE DW is well constrained inside a confined volume at the quartz and the coating interface as discussed below. The pulsed laser fluence threshold for photochemical DNA damage was reported as small as 4 mJ/cm^2 in studying the DNA damage of normal human fibroblasts (NHF) and Chinese hamster ovary cells using 193-nm radiation [35]. Using the Beer–Lambert law, the damage threshold depth is estimated just around 5 μm under a 1,000 mJ/cm^2 using the 4 mJ/cm^2 threshold laser fluence and the 10^4cm^{-1} absorption coefficient of water [36,37]. For a 100 μm thick coating in this study, it means that only 5 % of coating is affected by UV radiation (193 nm), and this should result in very limited UV radiation-related injury to cells. As a matter of fact, comet assay of DNA damage showed that UV damage was not detected within the limits of the assay for MAPLE DW [2], and laser micro-dissection and laser pressure catapulting [38]. Even under high laser fluences, comet assay of DNA damage indicated that more than 97% of the specimen was not at all affected by UV radiation in laser micro-dissection and laser pressure catapulting [38]. Therefore, it is considered UV radiation-induced injury to be negligible.

In this study, it is considered cell injury to be from the process-induced mechanical stress during the cell droplet formation and cell landing processes. As seen from Fig. 3, a higher the laser fluence leads to a higher pressure-induced acceleration/velocity and a concomitant lower cell viability. Moreover, it was also found that the post-transferred cells can proliferate through confluency as shown in Fig. 4 when incubated at 37°C, 5% CO_2 , humidified incubator. Future work will use more sensitive bioindicators to focus on the effects of laser fluence on post-transfer cell proliferation and cell injury reversibility of HT-29 cells during MAPLE DW.

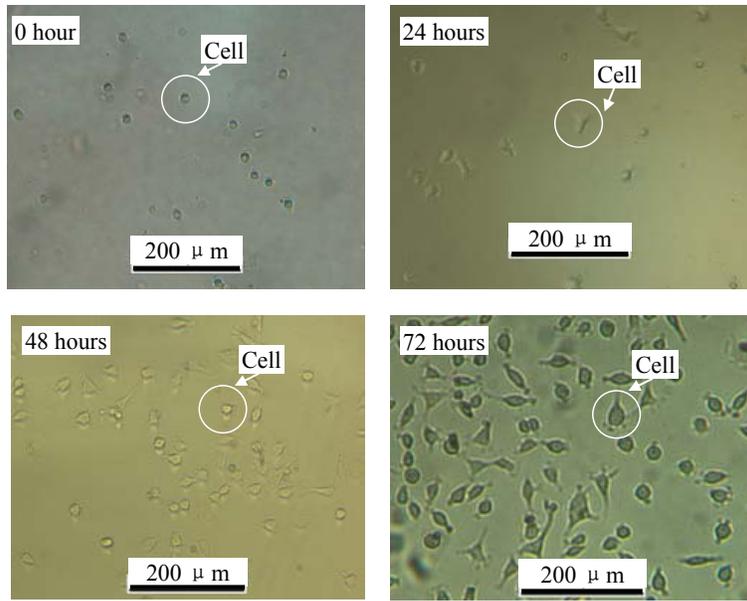


Fig. 4. Cell proliferation under a 258 mJ/cm² laser fluence using a 25 laser-pulse transfer (0 to 72 hours after transfer)

5. CONCLUSIONS

The HT-29 cell transfer using MAPLE DW has been studied to elucidate the effects of laser fluence on the post-transfer cell viability. While MAPLE DW is of interest in this study, the resulting observations are expected to be applicable to other laser-induced forward transfer studies. Some conclusions are drawn: 1) the HT-29 cell viability decreases from 95% to 78% as the laser fluence increases from 258 to 1,500 mJ/cm²; and 2) cell injury is mainly from the process-induced mechanical stress during the cell droplet formation and cell landing processes while the effects of thermal influence and ultraviolet radiation might be negligible. While this study reveals some interesting effects of laser fluence on the HT-29 cell viability, the future work will address the effects of laser fluence on post-transfer cell proliferation and cell injury reversibility of HT-29 cells using more precise bioindicators.

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