

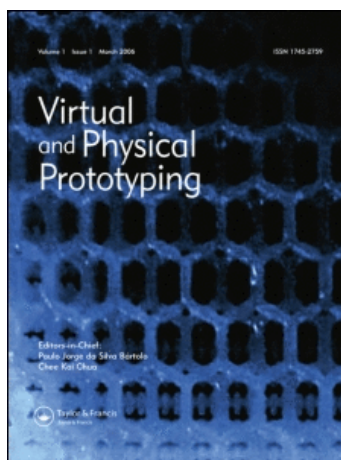
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# Electrowetting-based multi-microfluidics array printing of high resolution tissue construct with embedded cells and growth factors

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To overcome limitations of current tissue fabrication methods, explore new biocompatible materials, and develop new biomimetic manufacturing process for soft tissue, we are developing a new technology, electrowetting-based microfluidics array printing, which will be used in a solid freeform fabrication (SFF) system to meet the challenges of high resolution manufacture of hydrogel scaffolds with dimension scale less than 10  $\mu\text{m}$ , cell placement, growth factor delivery, and vascularization for soft tissue engineering. Preliminary work has been done on the biomaterials research, electrowetting printing, biomimetic modeling, scaffold fabrication, and system integration and control. This research is the first application of electrowetting on dielectric to tissue engineering and dispensing hydrogels.

**Keywords:** Tissue engineering; Solid freeform fabrication; Biomaterials; Electrowetting; Hydrogel scaffold

## 1. Introduction

### 1.1 Tissue engineering and required characteristics of a scaffold

Tissue engineering (TE) is evolving as a potential solution for repair and reconstruction of diseased or damaged tissues (Langer and Vacanti 1993). TE has recently been used successfully to replace a blood vessel in pediatric surgery (Yuji 2001), but TE faces major manufacturing challenges: (1) manufacturing techniques are needed to mimic tissue and extra cellular matrix (ECM) architecture, with high resolution (less than 10  $\mu\text{m}$ ) for tissues such as myocardium (heart muscle), blood vessels, bone or nerves; (2) innovative methods are needed for delivery of cells and growth factors into scaffolds; and (3) manufacturing techniques are needed to create vascular structure in tissue

construct; lack of nutrient transport currently limits the size and cellular content of implants (Griffith and Naughton 2002).

For engineering soft tissues, ideal scaffolds are made of synthetic or natural biopolymers providing porous (up to 90%) support structure, thus mimicking the natural ECM environment in which cells attach, multiply, migrate and function (Zeltinger *et al.* 2001). At the cell scale (order 10–100  $\mu\text{m}$ ), the microarchitecture of the scaffold pores or fibers can control cell orientation and migration, and ultimately affect cell function. At a slightly larger scale (order 100  $\mu\text{m}$ ), most tissues require a microvascular network to function once implanted, which ideally should be available at the time of implant (Yin *et al.* 2004). At the tissue scale, the overall size should match the exact grafted size.

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## 1.2 Limitations of the current art in SFF tissue engineering manufacturing

Solid freeform fabrication (SFF) methods have promise to allow CAD model-based manufacturing of 3D scaffolds with embedded cells/growth factors for soft tissue engineering model enabling unprecedented control of structural and biological properties. SFF methods for soft TE are at an early stage of development and face many limitations. Inkjet printing methods depend on commercial jets designed to dispense ink. These systems function in a narrow low viscosity range, which limits the type and strength of solutions that can be printed. In addition inkjets are not well suited to dispense cells, based on the 25% cell death reported (Wilson and Boland 2003). Inkjets have problems with cells clogging the jets and have resolution limit around 200  $\mu\text{m}$ . This process also includes an extra step of using an intermediate substrate 'bio paper' for supporting cells, because it cannot jet out hydrogel along with cells, which limits the prospects of this method for 3D printing. Extrusion-based SFF methods (Khalil *et al.* 2005) produce a limited range of scaffold architectures (parallel linear elements stacked in layers), at a resolution of around 100  $\mu\text{m}$ , and do not enable heterogeneous cell patterning (precise arrangement of multiple cell types). Laser-based SFF methods (Barron *et al.* 2005) expose cells to high stress, UV light, and heat, which must be carefully controlled to avoid damaging cells. The current method also may not scale up easily to 3D manufacturing because cells are delivered from 2D arrays.

A new system can overcome the existing limitations to fabricate bioactive soft tissue scaffold, by combining the advantages of SFF method, a new application of electro-wetting-based microstructure printing, and various biomaterials into one innovative tissue manufacturing system. The system works on the principle of Electro Wetting on Di-Electric multiple microfluidics array printing that can create droplets of sizes less than 10  $\mu\text{m}$ , work with a variety of hydrogels, and include cells and growth factors during scaffold fabrication.

## 1.3 Electrowetting on di-electric (EWOD)

Liquid handling and actuation by controlling surface tension (ST) has many advantages in microscale applications because of the dominance and effectiveness of the ST force, as the liquid handling system becomes smaller. One of the most promising tools for manipulating tiny amounts of liquids on surfaces based on surface tension manipulation, EWOD, has never been used in TE. EWOD applications range from 'lab-on-a-chip' system for applications such as DNA and protein analysis, and biomedical diagnostics, to adjustable lenses and new kinds of electronic displays (Cho *et al.* 2003, Fair *et al.* 2003). High resolution,

multi-microarray printing capability, programmable control and compact size, make EWOD a promising technique for tissue fabrication. EWOD microarrays within a printing mechanism would enable control of droplet size as well as precise deposition of the droplets, creating a unique combination of high precision and flexibility of materials compared to existing micro-droplet technology.

**1.3.1. Principle of EWOD.** EWOD uses the electrocapillary principle: ST is a function of electric potential across an interface, and the change in liquid–solid ST,  $\gamma_{\text{SL}}$ , changes the contact angle at the liquid–solid–gas interfaces (figure 1). When voltage  $V$  is applied between the liquid and electrode, surface energy is balanced by electrical energy, and  $\gamma_{\text{SL}}$  is lowered, as expressed in Lippmann's equation (table 1, equation 1). Young's equation (2) relates contact angle and ST, and Lippman–Young's equation (3) relates contact angle to voltage (Cho *et al.* 2002). In EWOD, a droplet of liquid rests on a surface or in a channel coated with a hydrophobic and dielectric material. Charge accumulates at the solid–liquid interface, and the surface wettability is modified from hydrophobic to hydrophilic by applying a voltage between the liquid and an electrode under the dielectric layer. EWOD uses the change in contact angle direction to induce liquid motions. Droplets are ejected from reservoirs and programmed to move to specific locations where they can be merged and cut. By applying a sequence of voltage to electrodes patterned under the dielectric layer, four fundamental droplet manipulation mechanisms can be established (figure 2): (1) creating, (2) cutting, (3) joining, and (4) transporting of droplets from a reservoir and in the fluid path.

## 2. Objectives

The main objective of the project is to develop an innovative manufacturing system for 3D, bioactive soft tissue scaffold fabrication with embedded cells and growth factors using biomimetic structure modeling, solid freeform fabrication, biocompatible hydrogel material, and electro-wetting on dielectric (EWOD)-based multi-micro array printing.

## 3. System design

### 3.1 Development of multi-microfluidic array printing system

The system can integrate a new EWOD array design with a computer-controlled motion system to position the EWOD chip for hydrogel, crosslinker, growth factor and cell delivery based on CAD models.

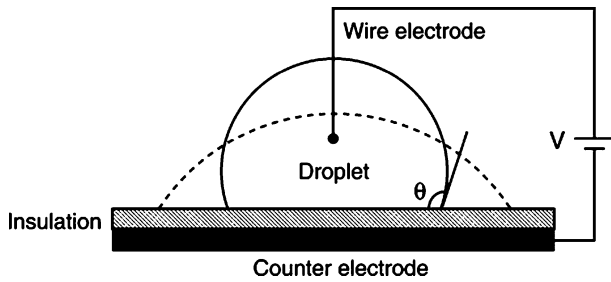


Figure 1. Principle of EWOD.

Table 1. Design equations.

$$\gamma_{SL} = \gamma_0 - \frac{1}{2} c V^2 \quad (1)$$

$$\gamma_{SL} = \gamma_{SG} - \gamma_{LG} \cos \theta \quad (2)$$

$$\cos \theta_v = \cos \theta_0 + \frac{1}{2} \frac{c}{\gamma_{LG}} V^2 \quad (3)$$

where  $V$  is the voltage between liquid and electrode;  $c$  is the capacitance of dielectric layer;  $\gamma_{SL}$  is the surface tension (ST) at solid and liquid interface;  $\gamma_{SG}$  is the ST at solid and gas interface;  $\gamma_{LG}$  is the ST between liquid and gas interface;  $\gamma_0$  is  $\gamma_{SL}$  when  $V=0$ ;  $\theta_0$  is the contact angle when  $V=0$ ; and  $\theta_v$  is the contact angle when voltage applied.

**3.1.1. Development of EWOD array chip.** The proposed EWOD printing system will be based on self-aligned, non-contact sub-nanoliter array printing using droplet-based microfluidics (figure 3). The microfluidic chip has microliter reservoir pads appropriately treated to hold various solutions on the left and right sides of the chip. These solutions will be loaded onto the microliter reservoir pads by robotic means. Then from the reservoirs, sub-nanoliter solution droplets will be automatically formed and transported on parallel buses to an electrode array. Subsequently, a desired number of droplets will be transported rapidly onto the array electrodes. Once the droplets are positioned on the electrode array, wire traction electrodes above the insulator surface of the electrode array will electrically ground the droplets.

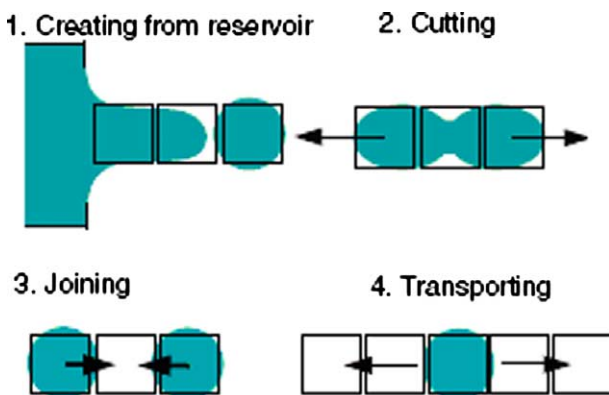


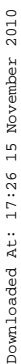
Figure 2. Fundamental droplet operations.

Upon the application of a modest voltage to a bottom substrate, all of the droplets in the array will be actuated vertically to print in a self-aligned manner onto the corresponding receiving sites on the substrate. The surface characteristics of the plate will be controlled to allow the droplet to adhere to the plate. Once the droplet printing is completed, the array can be moved from the printing system.

Currently, nanoliter droplets have been dispensed using our EWOD chips yielding droplets on the order of 100  $\mu\text{m}$  diameter (Kolar and Fair 2001). We expect to deliver 100 droplets per minute at a single location, so the anticipated built rate will be around 1 cm/min for a nanoliter droplet and the total size of the structure that can be built will obviously depend on the size of microfluidic array. For picoliter-volume droplets, diameter will be on the order of 10  $\mu\text{m}$  and build rate will be approximately 0.1 mm/min.

**3.1.2. CAD modeling and complete system design, control and integration.** *Representation of heterogeneous tissue construct.* In our EWOD multi-microarray printing system, four different kinds of materials will be combined to make soft tissue constructs: hydrogel, crosslinker, cells and growth factor. We will treat this multiple material scaffold as a heterogeneous object in the modeling process. To represent the heterogeneous tissue construct we will not only design tissue geometry, but also the material distributions of the object. The CAD model is thus enriched with useful material information. The continuously varying material composition produces gradation in material properties, often known as functionally gradient materials (FGM) (Cheng and Lin 2005). In the scheme of heterogeneous tissue construct modeling we will use voxel-based volumetric datasets to represent the material variations. The core issue of heterogeneous tissue representation is to design a scheme to represent the geometry and material information in order to integrate with CAD system and material design. First, we define geometric space  $S$  having the macroscopic shape of the tissue. We define a heterogeneous solid  $H$  on geometric space  $S$ , such that  $H$  consists of different kinds of materials having different material composition functions within the interior, specified by the material designer. For material composition we define the material space as a vector space, whose components are the material primitives. A FGM of the heterogeneous class can thus be represented; and a distance field is referred to as a distance map of all inner points to the selected features. The FGM takes the distance from inner object point to the selected feature(s) as arguments, and must satisfy  $0 < \text{FGM} < 1$  in the material gradient range.

*Design of an automatic and intelligent soft tissue manufacturing system.* The proposed hydrogel printing system for freeform fabrication of tissue constructs will be able to deposit living cells and other biological compounds at room

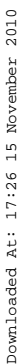


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dispense the crosslinker solution genipin immediately to crosslink the deposited hydrogel solution on the substrate. The third microarray is designed to dispense growth factor suspended in de-ionized water. The fourth microarray will be used to dispense living cells such as cardiomyoblasts or endothelial cells suspended in complete culture medium. Under the control of the computer, the moving table moves up one layer height in Z direction after finishing each layer. In this manner, the hydrogel scaffold is stacked up layer by layer; a three-dimensional hydrogel scaffold will be fabricated. The multiple microarray system allows a simultaneous deposition of cells, growth factor, and scaffold materials, thus enabling the fabrication of heterogeneous tissue scaffolds which will contain living cells and bioactive compounds.

**3.1.3. Support structure.** In most SFF systems there is a support structure build mechanism to deal with overhang features on the built part. The hydrogel tissue fabrication system does not include this mechanism for three reasons: (1) most scaffolds have self-supported internal tissue matrix, and can be oriented to eliminate support structures; (2) the deposited hydrogel is self-adhesive, and easily sustained and interconnected; and (3) hydrogel and cell densities are similar to water, and a buffer solution may be used to support the weight of the deposited hydrogel and cells. Eliminating the support structure simplifies the system and reduces build time.

**3.1.4. Bio-mimetic CAD modeling and reverse engineering.** The major advantage of the EWOD system is that it can take advantage of micro and macro scale 3D CAD models. The CAD model may be created directly using CAD software (such as Pro/E, Ideas, Solidworks, or AutoCAD). Or the model may be created using reverse engineering, which can reconstruct the 3D model from surface coordinates or multi-planar images of cross-sections of the model using image-to-model software such as AMIRA (TGS) or Mimics (Materialize). The 3D model of the imaged parts will be reconstructed from these high-resolution multi-planar images through biomimetic model software.

## 4. Initial studies

### 4.1 Dispensing and actuation of hydrogels and cells

**4.1.1. Hydrogel dispensing.** The capability of EWOD for handling hydrogels and cells has been demonstrated in collaborative efforts between Drexel and Duke. Several solutions were mixed, filtered, and then used: a 1% (w/v) sodium alginate, a 2% (w/v) sodium alginate and a 1% (w/v) calcium chloride solution. The 2% alginate solution has a viscosity of 250 cp at 25°C. These experiments were

performed on a glass chip with patterned chrome electrodes, having a pitch of 0.75 mm, and a gasket to maintain top-plate height above the electrodes. The chip was first coated with Parylene C, which functions as a dielectric and chemical insulator, and then was coated with a thin layer of Teflon AF for hydrophobicity. The top plate consisted of a sputtered indium-tin-oxide (ITO) coated film, which was later coated with Teflon AF for hydrophobicity. ITO is a transparent conductor, allowing the top plate to remain grounded during operation. All of the experiments were performed at room temperature.

The first experiments performed were to determine whether the alginate and crosslinking solutions could be actuated, dispensed, combined, and split (figure 2). For each test, the glass chip and top plate were washed and dried, then the top plate was aligned and placed on top of the glass chip and the space between them was filled with 2cSt silicone oil, which prevents evaporation and reduces the actuation voltage. The first test was performed by filling a reservoir with 650 nl of 1% alginate. A 55-V actuation voltage was chosen through experimentation, ensuring a reasonable velocity without causing dielectric breakdown. The solution was dispensed by extending a 'finger' of the solution from the reservoir through electrowetting, then by applying voltage only to the last electrode in the 'finger' and the reservoir, causing the finger to narrow and eventually split between the two electrodes. This solution appeared to require a longer 'finger' before splitting than other solutions due to the higher viscosity. The first droplet was then moved away via actuation and a second droplet was dispensed. The two droplets were combined by actuating them into each other and then split using a method similar to dispensing. Dispensing, transport, combining, and splitting were demonstrated on the 1% crosslinking solution and the 2% alginate solution (figure 5). Two percent alginate required a higher actuation voltage and had slower velocity due to its higher viscosity ( $\sim 250$  cp).

EWOD may also be used to create crosslinked hydrogel on-chip. A total of 650 nl of 1% alginate and 1% crosslinking solution were inserted into their own adjacent reservoirs. The crosslinker was dispensed and moved to a holding electrode, and then the alginate was dispensed and combined with the crosslinker. The combined droplet rapidly became immobile and resistant to manipulation with a pipette.

**4.1.2. Cell manipulation on EWOD chip.** A second study was performed to demonstrate the viability of the EWOD process for handling, dispensing and actuating cell suspensions. Secondary objectives include finding the maximum voltage that can be applied without damaging the cells, and the effect of the silicone oil on the cells. Initial tests were conducted on the EWOD chip with human fetal osteoblast cell line (hFOB 1.19, ATCC passage 11 to 13). Cells were



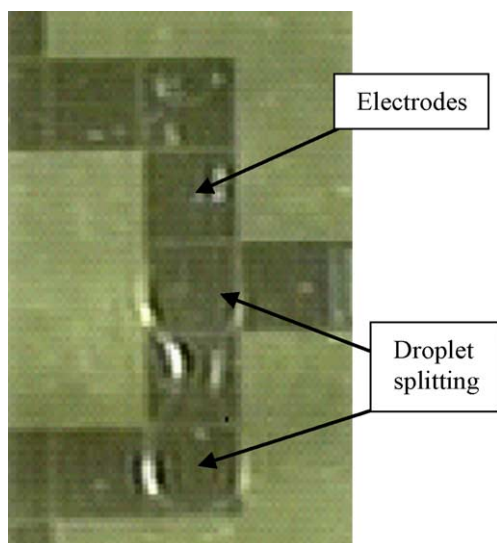


Figure 5. High-viscosity (250cP) droplet manipulation.

cultured in Dulbecco's Modified Eagles Medium (10%FBS, 1%Pen-Strep) prior to the experiment. Cultured cells were initially trypsinised, diluted with PBS and separated by centrifuging. The separated cells were treated with a Live Dead Assay (Molecular Probes) reagent solution (6  $\mu$ M ethidium homodimer-1 and -2 micromolar calcein in PBS). The test cell suspension was loaded on chips, which were actuated with voltages ranging from 40 to 60 V. After actuation the EWOD chips were observed under fluorescent microscope to quantify live and dead cells. The fraction of live cells is about 94% (figure 6), comparable to the live fraction in the solution before loading into the chip. Even after 2 h with 60 V applied to the switching electrodes, there was no noticeable change in the ratio of live-to-dead cells in the immersed droplet. Dead cells were observed at locations where hydrolysis takes place. Hydrolysis can be avoided with careful chip manufacture and limited voltage application. Overall the experiments demonstrate that the proposed method of EWOD is viable for dispensing cells.

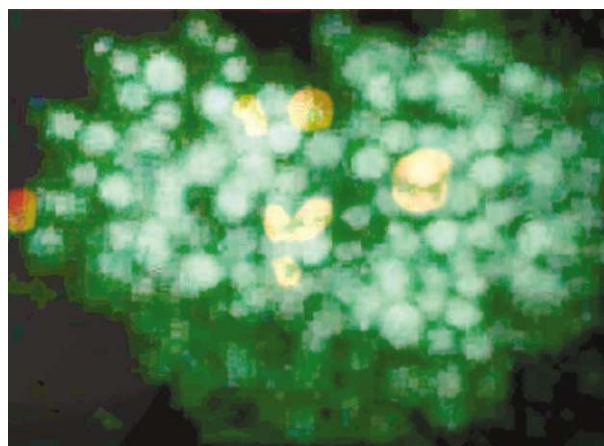


Figure 6. Cells after EWOD actuation (Live-Green/Blue, Dead-Orange/Red.)

#### 4.2 EWOD-based vertical actuation of nanoliter-microliter droplets

Vertical actuation of nanoliter to microliter droplets has been demonstrated in our laboratory (figure 7). A Teflon-coated platinum wire acted as a grounding connection to the droplet, allowing  $x-y$  actuation in the plane of an electrode array. The entire system was immersed in silicone oil which prevented chemicals from adsorbing on the Teflon-coated wire or other transport surfaces. The top piece consisted of an ITO-coated slide attached to a microarray slide. The microarray slide was placed at the bottom of the top layer and faced the drop (figure 7). The bottom plate was fabricated and inserted into a spacer block. The platinum wire strung across the spacer block formed the ground electrode, and the space between was filled with silicone oil. In operation, a voltage was applied to the ITO layer on the top piece, relative to the ground wire. The ground wire 'focused' the electric field, allowing for a significant charge to build up in the droplet. This caused the droplet to be attracted (actuated) upward in the  $z$ -direction to the top microarray plate. The drop was propelled to the

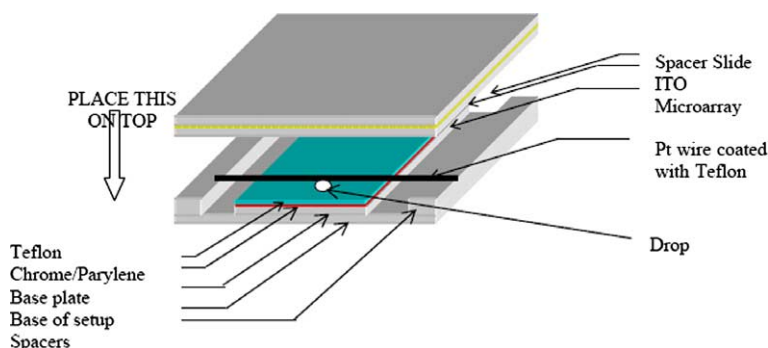


Figure 7. Droplet vertical actuation configuration.

top plate where it adhered. The performance of the printing system in figure 7 has been demonstrated over a wide range of droplet compositions, volume ranges over hundreds of nanoliters, variable system dimensions, and different oil and droplet viscosities. A minimum  $z$ -axis actuation voltage of 40–50 V was used for this system. By contrast, aperture-based systems required upwards of 1000 V for  $z$ -axis actuation. The actuation voltage for silicone oil droplets and a gap height of 590  $\mu\text{m}$  ranged from 40 to 50 V was used, which was almost independent of the drop volume in the 250–700-nl range, and for oil viscosity from 2 to 50 Cst. Also, the actuation voltage was the same for droplets of  $20 \times \text{SSC}$  buffer containing 3 M NaCl and 0.3 M sodium citrate, over a range of dilutions, and also for deionized water droplets.

A successful demonstration has been made in our lab of  $z$ -axis actuation of DNA and subsequent binding on derivatized glass substrates using an actuation voltage of 40 V. DNA was tagged with Cy5 dye in  $1 \times \text{SSC}$  buffer solution, and 500 nl of this fluid was stamped using the vertical electrostatic method.

## 5. Conclusion

The theory and first feasibility studies of EWOD micro array printing system for tissue engineering have been presented. An EWOD chip has been developed to dispense droplets of size less than 100  $\mu\text{m}$ . A complete EWOD-based multi-microarray printing system has been designed and its related CAD programming and control have been studied. Hydrogel and crosslinker fundamental droplet operations, on chip crosslinking and cell manipulation on a EWOD chip were demonstrated, which shows the system is capable to make micro array printing for tissue construct.

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