COMPARISON OF SUBMERGED AND UNSUBMERGED PRINTING OF OVARIAN CANCER CELLS

Sherry N. Davidoff[1*], David Au[1,2*], Samuel Smith[1*], Amanda E. Brooks[3], and Benjamin D. Brooks[1].
[1]Wasatch Microfluidics, Salt Lake City UT 84103 USA
[2]Department of Bioengineering, University of Utah, Salt Lake City UT 84112-5820 USA
[3]Department of Pharmaceutics, North Dakota State University, Fargo ND USA
*These authors contributed equally to this work

ABSTRACT

A high-throughput cell based assay would greatly aid in the development and screening of ovarian cancer drug candidates. Wasatch Microfluidics has developed a three-dimensional microfluidic printer that is capable of controlling cell deposition location, while maintaining a liquid, nutrient rich environment that helps maintain cell phenotypes. In this study we investigated the phenotypic effects of submerging or not submerging the print head in cell media. It was determined that not submerging the print head in cell media maintained a higher cell density and greater cell viability. This article details an in depth study detailing the impact of one of the foundational components of a high-throughput microfluidic cell based assay for creating an *in vitro* biocompatibility screening tool that mimics the *in vivo* cell environment.

Keywords: ovarian cancer, cell printing, microfluidic cell assay, high throughput, drug discovery

INTRODUCTION

Some of the first biomedical microfluidic devices were designed to pattern surfaces with specific biomolecules and were developed for biosensor platforms such as Surface Plasmon Resonance (SPR) ***[1]***. Subsequently, arrayed microfluidic devices composed of two dimensional (2-D) isolated microfluidic channels and flow cells were created to deposit biomolecules in specific micropatterns on the surface ***[2]–[5]***. These advances, in addition the ability lift the flow cell, rotating it 90° and flowing the analyte solution back across the patterned lanes, allows dense microarrays to be created for complex *in vivo* translatable bioassays ***[2], [6]***. Unfortunately, microfluidic array devices still remain limited in throughput because of the large substrate surface area of the microchannel reaction zones and the SPR biosensor active sensing region ***[7]***. More recent attempts have focused on 3-D microfluidic networks (continuous flow microspotter) to confine deposition to specific locations on the substrate to minimize sample depletion and increase reaction zone density ***[8], [9]***. In fact, printing biomolecules with the novel 3-D Continuous Flow Microspotter (CFM^{TM}) demonstrated a dramatic increase in spot uniformity and quality when compared with standard pin or piezoelectric spotting techniques ***[10]–[13]***. By printing with flow, biomolecules are maintained in a liquid environment throughout the printing process, enabling the printing of sensitive biomolecules or cells without exposure to air. The Microfluidic Cell Array (MFCA) is unique in its ability to dock to a surface that has been covered with a liquid, such as cell media, and create distinct flow cells in which cells can be flowed across the surface (Figure 1).

In conjunction with the unique engineering of the MFCA, 3-D flowcells can be utilized when printing cells to maintain elements of *in vivo* complexity ***[14]***. By printing cells in a 3D environment under the more natural mechanical forces imposed by flow, cellular phenotype and hence cellular function may be maintained. Conserving *in vitro* cellular phenotype may prove critical for developing *in vivo* relevant assays for biomedical materials testing, drug discovery screening, tissue engineering ***[15], [16]***, and disease state modeling, etc. We hypothesize that printing cells submerged will provide more native cell-cell contacts and enhance the ability to maintain cell phenotypes, a particularly critical

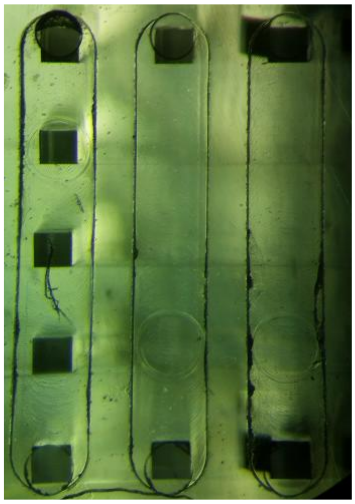

Figure 1. The MFCA 12 channel print head in which 3 of the parallel channels are visible.

feature to any disease state cell-based drug screening model. Recapitulating the complexity of cancer cell phenotype in any *in vitro* model would represent a significant advancement in cancer therapeutics and personalized cancer treatment. Using 3D flow cells with MFCA may be such an advancement.

In the United States, ovarian cancer is the fifth leading cause of female cancer-related deaths ***[17]***. Despite recent advances in the treatment of ovarian cancer, including cytoreductive surgery and combination chemotherapy, increased median survival has failed to translate to an increased cure rate ***[18]***. A distinctly new approach is needed. Such an approach necessitates a new model and a paradigm shift to personalized designer therapeutics based on individualized cancer cell drug screening. Here, we perform a preliminary investigation printing the ovarian cancer cell line A2780 3D CFM to investigate the impact of submerged printing on cell density, cell viability, and cellular phenotype as foundational studies toward the development of a drug screening platform.

METHODS

Cell Culture: A2780 ovarian cancer cells (Cat# 93112519, Sigma-Aldrich, USA) were cultured in RPMI 1640 media (Cat# ,Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS, Cat# 16000-036 Invitrogen, USA) and 1% penicillin-streptomycin (Cat# 15070063, Invitrogen, USA).

Cell Printing: Cells were cultured to 80% confluency prior to use in the microfluidic cell printer. Once cells reached 80% confluency they were removed from the surface using a cell scraper and counted using a haemocytometer. Prior to printing the print head was primed with the appropriate media for 10 minutes at 50 µL/min. Cells (200 µL) were loaded into a one mL syringe at a concentration of 500,000 cells/mL. The entire 200 µL of cells were then printed using a Chemyx (Chemyx, USA) syringe pump at 50 µL/min through 250 µm tubing through a 12 channel print head onto the surface of a 30 mm tissue culture treated petri dish (Figure 2). Printed cells were then placed in the incubator, with the print head docked for one hour to allow cells time to adhere. For submerged cell printing, two mL of warmed media was pipetted into the petri dish before the print head was removed after the print, while in an unsubmerged print the media was added after the print head was removed post-print (Figure 2).

Cell Viability: The viability of each cell line was determined prior to and post printing to determine the effect the printing process has on the viability of the cells. Prior to printing, cells were kept in suspension and 10 µL was removed of the cell suspension and added to 10 µL of trypan blue (Cat#, 15250-061, Invitrogen, USA). Cells were mixed and 10 µL was then loaded into a Haemocytometer and counted for viability. Post printing 200 µL of trypan blue stain was added to the two mL of cell culture media in the petri dish.

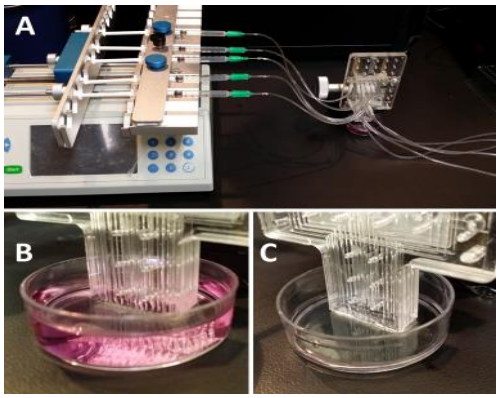

Figure 2. A) The MFCA printing set up with 5 channels connected. B) Print head tip submersed in cell media while docked for submerged printing and C) print head docked to the petri dish surface without cell media present for unsubmerged printing.

Microscopy: A Leica DMIL LED Fluo (North Central Instruments, USA) was used to view the cells at 2.5X and 10X magnification for determining cell density and viability. Cells were imaged with the print head docked and with it removed to determine the effect removing the print head had on the cell adherence.

Statistical Analysis: A two-tailed, unpaired, unequal variance student's t-test was performed to compare the cell densities of the submerged and unsubmerged prints. Each print was performed 10 times (n=10).

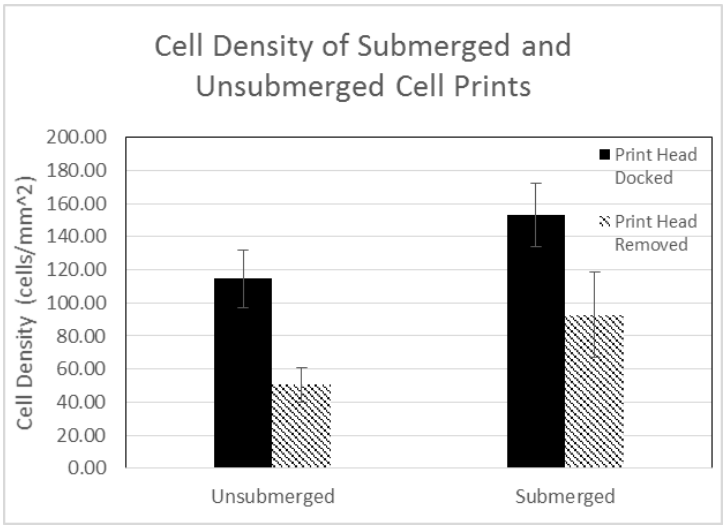

Figure 3. Comparison of cell densities for unsubmerged and submerged cell printing before and after the print head was removed.

RESULTS

Submerged cell printing averaged 153 ± 19 cells/mm^2 prior to print head removal and 93 ± 26 cells/mm^2 once the print head was removed (Figure 3). There was a 64.4% difference in cell densities once the print head was removed, with media already present in the petri dish. Unsubmerged cell prints averaged 115 ± 17 cells/mm^2 prior to print head removal and 50 ± 19 cells/mm^2 once the print head was removed (Figure 3). There was a 44.4% difference in cell densities once the print head was removed and then media added. The cell density was not significantly different (p=0.156) between submerged and unsubmerged cell printing. Both submerged and unsubmerged cell printing had a 100% cell viability once the print head was removed. Prior to the print head being removed, in both unsubmerged and submerged cell printing, cells clustered at high densities near the inlets and outlets. Once the print head was removed, less cells were present; however, a higher density of cells remained near the inlets and outlets, independent of the print type (Figure 4).

Independent of the any differences in the cell density, the cellular phenotype of the submerge printed cells seemed

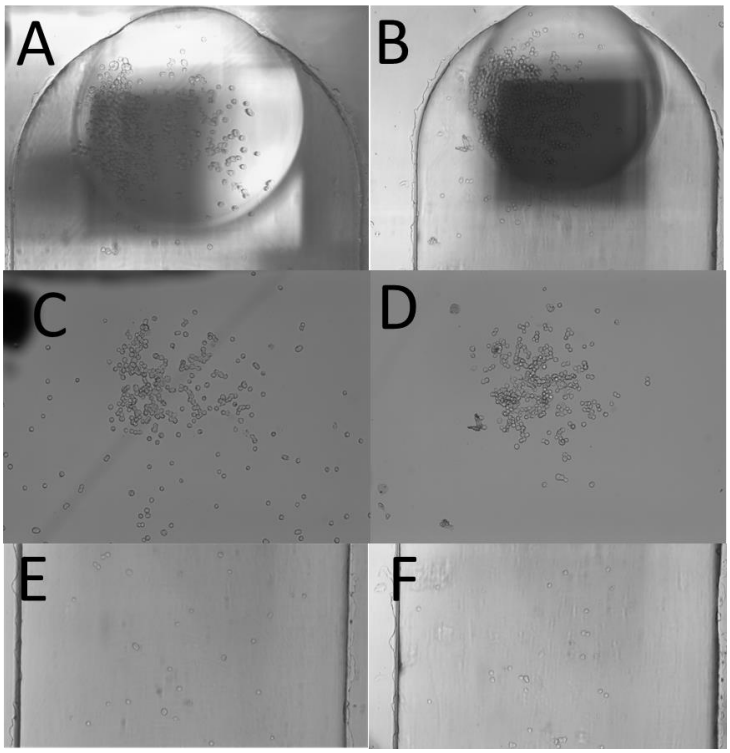

Figure 4. Representative images of submerged (A, C, E) and unsubmerged (B, D, F) cell prints. (A,B) The inlet region of the print head after printing. (C,D) The same region as A and B, but with the print head removed. (E,F) A region in the channel that is not an inlet or outlet. Note the high cell density near the inlet, but low cell density in the body of the channel.

more similar to other published images from this cell line [19]. Additionally, cells from submerged prints appeared slightly more spread and had an increased number of dividing cells evident in the images (23 shown in B and only 7 shown in A) when compared to unsubmerged cells that appeared more rounded (Figure 5).

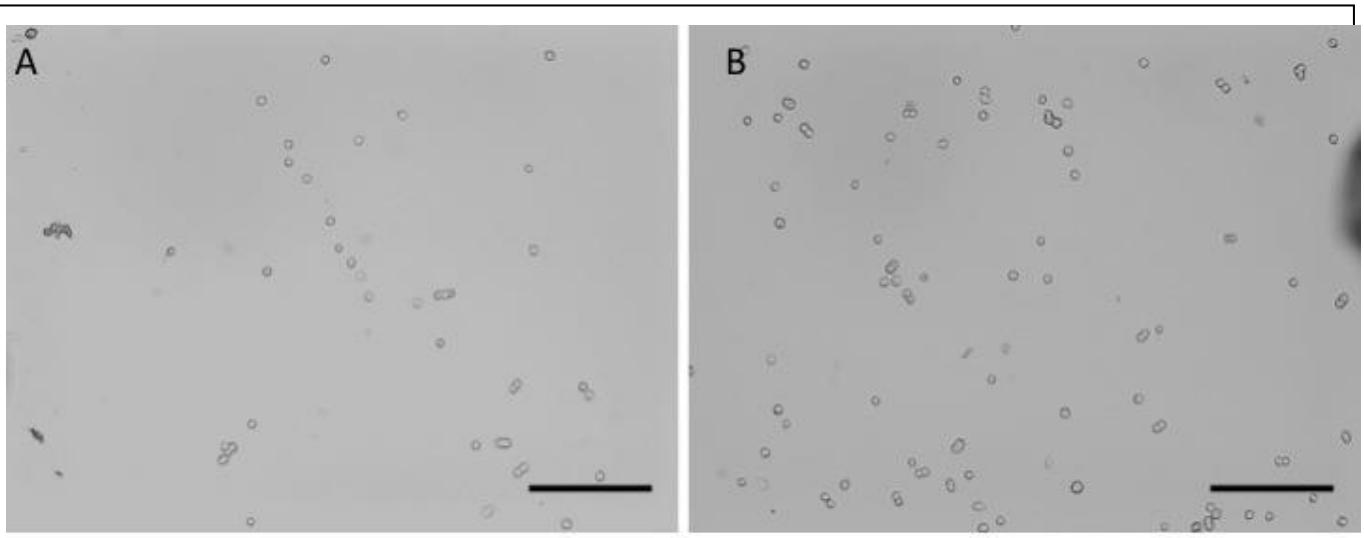

Figure 5. Microscopic images (10x) showing cellular phenotypes of unsubmerged (A) and submerged (B) printed cells. Scale bars are 200 μm.

DISCUSSION

Submerged cell printing resulted in a 44.4% difference in cell density once the print head was removed, while unsubmerged cell printing resulted in a 64.4% difference. While the submersion of the print head into cell media did not appear to have a significant effect on the cell density, the effect submersed printing had on cell viability remains undetermined. Cells were viewed for viability by the entire petri dish being stained with trypan blue. The viability of the cells at 100% can be misleading as dead cells tend to not remain on the surface. Imaging of the cells prior to print head removal revealed a higher cell density than once the print head was removed. This could be a result of cells not being fully attached, and therefore not able to withstand the force exerted on them by the removal of the print head. Optimizing print head docking times is critical to determining the effect of submerged versus unsubmerged printing. Print head undocking may have also dispersed any dead cells that were only slightly or not adhered for each channel since the flow cells were keeping any floating cells contained within the channel. This effect also warrants additional study and should be coupled with determining dependencies on cell type and engineering surface coatings.

Although there appeared to be some moderate differences in cell appearance between submerged and unsubmerged prints, phenotypic appearance is not always indicative of cellular function [20]. Nevertheless, cells printed under submerged conditions appeared to be more mitotically active, indicating a more cancer like function. Such function would certainly be necessary for any *in vitro* cancer model to be useful for drug screening.

CONCLUSIONS

While virtually no difference was observed in the viability or density of the cells in the submerged or unsubmerged cell printing of A2780 ovarian cancer cell, indications of phenotypic or functional changes in the cells were observed including attachment and motility. These observations warrant additional studies to prove, quantify, and identify. In addition, other cell lines, both ovarian and general cell lines, may be affected by the submerged or unsubmerged printing and thus warrant further study.

Future work will look into further characterizing cell phenotype including quantifying cytokine amounts in the milieu, membrane protein characterization (cell surface markers), mitotic markers, protein upregulation through microarray studies, and long term cell viability using alternative staining techniques, such as fluorescent microscopy. In addition, an expanded study to determine the long-term viability of the cells for multiple unsubmerged or submerged conditions should be considered. Earlier studies have also shown mixed results with fibroblasts warranting further study (data in press).

The desired outcome of these studies is the development of cell-based assays for screening drug candidates for ovarian cancer. This study along with others lay the foundation for the important work of treating ovarian cancer through the engineering of a cell-based assays for the screening of drug candidates.

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