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OPTIMAL TUBE LENGTH FOR THE SUBMERGED PRINTING OF CELLS

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ABSTRACT

Ovarian cancer is the fifth most common cancer affecting US women, killing more women each year than all other gynecologic cancers combined. Treatment of ovarian cancer is challenging with an overall 5-year survival rates of only 28-46% based on the metastatic state of the disease. While overall survival has improved with modern chemotherapy, poor outcomes have persisted. One of the greatest challenges in cancer therapeutic research remains that late-stage drug development trials for drug candidates have high attrition rates, up to 70% in Phase II and 59% in Phase III trials. The development of *in vitro*, high-throughput, cell based assays could provide a tool to overcome the challenges associated high attrition rates by allowing for controlled cell deposition with a defined, controlled phenotype. Submerged, three-dimensional (3D) microfluidic printing technology is uniquely capable of controlling cell deposition without sacrificing the viability of cells for cell-based assays. Here, we investigate the phenotypic effects of tube length during printing on the cells. We observe that the length of the tube has minimal effects on the viability and density of A2780 ovarian cancer cells different cell lines. This study details foundational information for developing a high-throughput cell-based assays (CBA) for screening effective cancer drug candidates.

Keywords:

INTRODUCTION

For women in the United States, ovarian cancer is the fifth leading cause of cancer-related death [1]. The numbers are staggering with approximately 22,000 women diagnosed and 14,000 dying from ovarian cancer this year alone [4] (Figure 1). As with most cancers, an increased cure rate heavily relies on early diagnosis; stage III or IV ovarian cancer has a long-term (5 to 10 year) survival rate of less than 30%; whereas, diagnosis in stage I has a rate up to 90% [2], [3]. Unfortunately, ovarian cancer is most commonly diagnosed in Stage III or IV of the disease, where the disease is already widely metastatic, difficult to treat, and likely to reoccur [5]. The result is a 35% five-year survival rate for patients diagnosed in the latter stages [5]. 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 [2].

Present therapeutics for late-stage ovarian cancer treatment are often ineffective [6]. Monoclonal antibodies, currently the most advanced

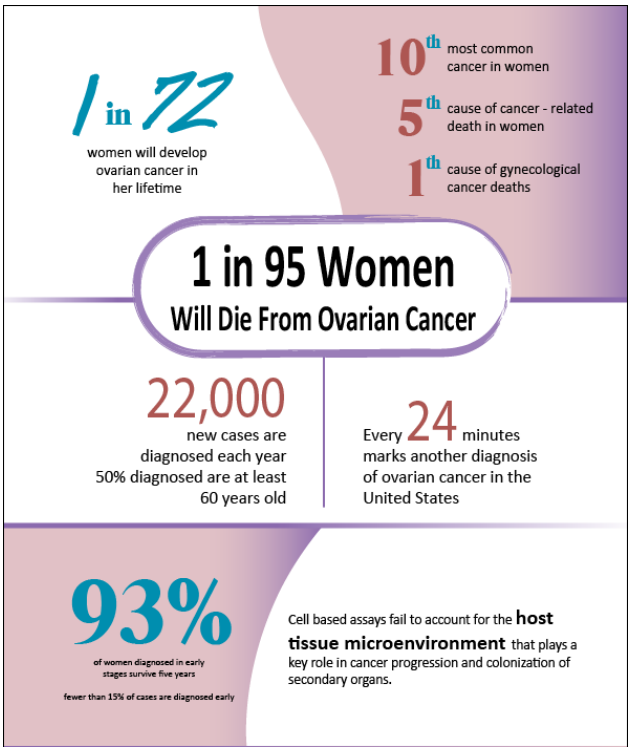

Figure 1. Ovarian cancer statistics underlie the therapeutic potential for cell- based assays. Statistics obtained from the Ovarian Cancer National Alliance [1].

and promising treatment used to target and attack cancer cells, are only able to interact with specific types of cells that display their targeted antigen [7]. Not only are the failure rates high, but the overall cost of a single treatment for ovarian cancer can run up to $100,000 [8]. Several factors play into the high failure rate including drug resistance, poor specificity, and disease heterogeneity [6].

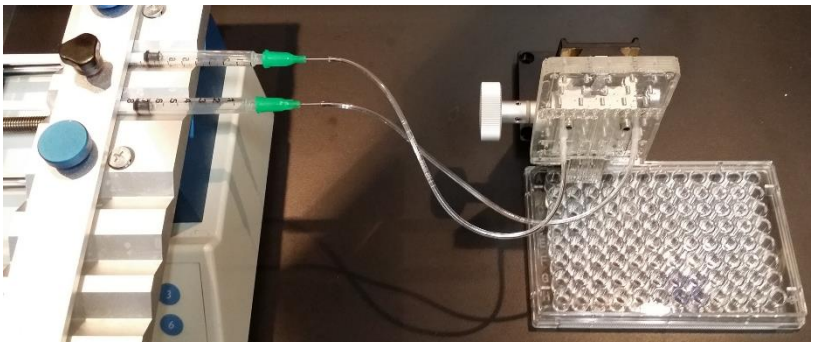

Figure 2. Experimental set up with the syringe pump, 12 channel print head, and well plate for cell collection.

Contributing to the high attrition rate, multiple phases of screening are required for a new cancer therapeutic to reach the market. Unfortunately, modern drug screening techniques, are currently insufficient due to low throughput and unnecessarily large sample volumes required for accurate diagnostic and therapeutic assays [6]. Individualized screening models would allow for a variety of cancers to be tested with current cancer treatments saving money, time, and ultimately patient lives [9]. In the end, the potential to decrease the health care costs and increase in the number of women who are effectively treated for ovarian cancer is possible if better therapeutics could be screened more efficiently. Microfluidics offers a gateway to creating high throughput cell screening assays to test new cancer treatments while consuming less reagents and requiring less time for assay completion.

Here, we apply a proven, 3D printing technology, the Microfluidic Cell Array (MFCA) for biomolecules [10]–[12] to the submerged printing of ovarian cancer cells. Current cell printing technology allows for cells to be printed on Petri dishes, glass slides, or in multi-well plates. However, all of these techniques use 2D microfluidics, which limit density, multiplexing, and microscope integration for highly parallel analyses. These limitations can be overcome using 3D printing techniques. This would allow for the creation of more efficient accurate drug screening methodologies. An important first step in the characterization of in vitro model development based on cell printing for drug screening applications is determining the effect of the length of the tubing on the density and viability of printed cells. Tubing length may affect cell density and viability based on the adhesion and death of the cells in the tubing during the printing process. Four different tubing lengths were compared during the course of this study.

METHODS

Cell Culture: A2780 ovarian cancer cells (Cat# 93112519, Sigma-Aldrich, USA) were cultured in RPMI 1640 media (Cat# 11875-085, 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 on a haemocytometer. Cells were printed as previously described with modifications [14]. Briefly, prior to printing, the print head was primed with the RPMI 1640 media with supplements as described above 5 minutes at 50 µL/min. Cells (200 µL) were loaded into a 1 mL syringe at a density of 50,000/mL. Cells were then printed using a Chemyx Fusion 200 (Chemyx, USA) syringe pump at 20 µL/min through 250 µm tubing of varying length (42, 21, 10.5, and 5.25 inches) on a 12 channel print head and collected in a tissue culture treated well plate (Figure 2). The syringes were then exchanged for empty syringes and a 200 µL plug of air, at 20 µL/min was pushed behind the cells to empty the entire 200 µL cell bolus out of

the print head. After printing the print head was positioned over new wells and 200 µL of media was flowed through the system at 100 µL/min to flush any cells that may have remained in the tubing. Again, a 200 µL plug of air was pushed behind the media to empty the tubing of media. Once printing was completed, the well plate was placed in the incubator for either 2 or 24 hours prior to imaging. Cells (200 µL) at a density of 50,000 cells/mL were pipetted into a well and allowed to incubate for 2 to 24 hours as controls. Printed and flushed cells were collected into a 96 well plate and placed in the incubator for 2 and 24 hours to allow time to adhere and grow.

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. Cells were kept in suspension and 10 µL was removed for counting. Trypan blue (10 µL) (Cat#, 15250-061, Invitrogen, USA) was added to determine cell viability. Cells were mixed and 10 µL was then loaded into a Haemocytometer and counted for viability prior to printing. Post printing, 20 µL of trypan blue was added to the 200 µL of media and cells collected in each well.

Microscopy: A Leica DMIL LED Fluo (Leica, USA) was used for viewing the cells at 10X magnification for determining cell density and viability 2 hours and 24 hours post-print of the cells that were collected in a 96 well plate.

Statistical Analysis: A 2-tailed, unpaired, unequal variance student's t-test was performed to compare the cell densities of each of the tubing lengths to the controls.

RESULTS

Moving cells through tubing is a common occurrence through such technologies as flow cytometry and even several forms of microscopy. We hypothesized that shorter length of tubing lengths would increase cell viability or reduce cell phenotype changes. At 2 hours, 5.25, 10.5, and 42 inch length tubing exhibited a significantly lower cell density than the controls ($p=0.013$, $p=0.006$, and $p=0.001$, respectively). Neither cellular density nor viability produced from 21 inch length tubing was significantly different when compared to the unprinted control at 2 hours ($p=0.89$ and $p=0.43$ respectively) (Figure 3). After 24 hours, all tubing lengths (5.25, 10.5, 21, and 42 inches) showed a significantly lower cell density than the controls at 24 hours ($p=0.0008$, $p=0.0003$, $p=0.00004$, and $p=0.00003$, respectively). (Figure 3). Cell viability, in all tested cases, was above 98% viability. However, the viability of the printed cells was significantly lower than the controls at 2 hours for 5.25, 10.5, and 42

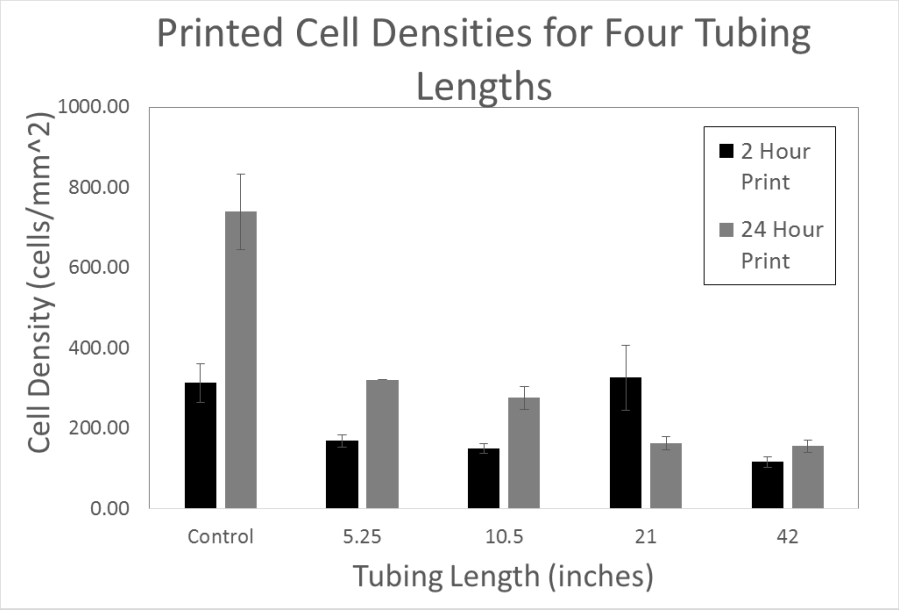

Figure 3. Graph showing cell density for various length tubing and the associated cell densities after printing.

inch length tubing (p=0.028, p=0.017, and p=0.006, respectively). The viability of cells at 24 hours was not significantly different for any of the four tubing lengths when compared to the controls.

DISCUSSION

Previous studies explored the use of a peristaltic pump as part of a Continuous Flow Microspotter (CFM); however, the flow tended to pulse as the tubing passed over each roller within the pump [14], potentially eliciting a negative cellular response due to the printing process. Additionally, two pieces of tubing must be used in a peristaltic pump. One set of tubing is specific to the pump, and the other is Tygon ® tubing that connects the pump to the print head. This extra tubing for the pump unnecessarily increases the length of the tubing. A syringe pump was chosen for this study because of its more consistent flow rate and ease of use as it requires only 1 piece of tubing. The CFM contains a standard tubing length of 21 inches, which was one of the lengths chosen for this study. Lengths that were ¼, ½, and 2 times the length of the standard CFM tubing were selected to study the effect of tubing length on the cells. It was hypothesized that a longer tubing length would result in a lower cell viability and cell density as cells would have an increased surface area in the tubing to attach or potentially shear while shorter tubing may enhance cellular print density and viability. In addition, concerns about the printhead design including square channels and 90° turns were raised and tested in this study, which data indicates are not concerns for viability. Further studies need to address phenotype changes.

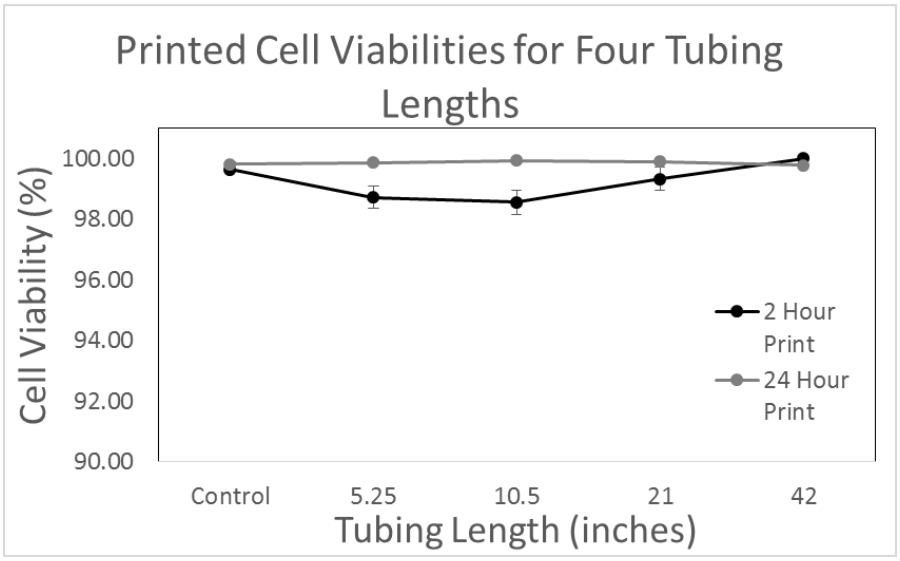

Figure 4. Graph showing cell viability for various length after printing.

Printing the cells through 21 inch length tubing had the most minimal effect on the cell density and viability, when viewed within 2 hours of the print. While the cell densities were not significantly different between the various lengths of tubing and the controls, there was a difference in the amount of cells that were printed versus those that were flushed out post-print when observing the ratio of the average density of printed cells to the average density of cells that were flushed out of the tubing at a higher flow rate and collected into a 96 well plate (Table 1). 5.25 inch length tubing had the highest ratio of cell density in the print to that from the flush, indicating that less cells remained in the tubing. Conversely, more cells remained in the tubing for the longest length, 42 inches. Although this intuitively makes sense, a linear relationship was not observed between the

Table 1. Ratio of the cell densities (cells/mm^2) of printed to flushed cells through four tubing lengths.

Tubing Length (inches)	2 Hours	24 Hours
5.25	10.9	15.0
10.5	7.6	25.3
21	17.3	8.7
42	3.7	4.5

length of the tubing and the amount of cells that were flushed out of the tubing after printing.

Despite the length of the tubing, a lower cell density was observed for all lengths when compared to the unprinted control. This indicates that while the printing process does decrease the number of cells that attach to the surface, the length of the tubing is not the cause of the change in density. Surprisingly, the longer tubing did not have a negative effect on the cells as initially expected. Tubing material may prove a greater factor in increasing cell density. Tygon ® tubing was chosen previously by Wasatch Microfluidics as the standard tubing for the CFM as it has low protein binding. This low protein binding capability may decrease cellular attachment to the tubing, making the surface area less important.

By understanding that the tubing length appears to have a minimal effect on the cells, a new generation of the CFM can begin to be constructed that is optimized for the complexity of cell printing rather than single biomolecule systems. The CFM has multiple components such as tubing material, flow rate, and print head dimensions, that may prove to have a larger effect on the cell density and viability and can be engineered and optimized for cell printing.

CONCLUSIONS

Screening drug candidates remains a tricky proposition. Currently, the industry uses cell-based assays (CBA) as both a primary and secondary screening tools. However, methods for cell-based assays typically are performed in Petri dishes (sometimes with dividers to increase density), on glass slides, or in multi-well plates. These techniques suffer from a variety of disadvantages for cell assays including: abnormal cell context, typically static conditions, low density assays, difficulty or impossibility of multiplexing or combinatorial assays, consumption of large amounts of samples and/or reagents, and relatively large size making compact, spatially consistent results difficult to obtain. Microfluidics has been used to help address several of these disadvantages, especially those related to size and volume. Unfortunately, research using microfluidics for CBAs commonly use 2D microfluidics, which limits density, multiplexing, and microscope integration for highly parallel analyses [15]–[19]. Digital microfluidics and droplet-based microfluidics have been proposed for these types of applications and can operate in highly-parallel, multiplexed configurations [20]–[22], but they are limited to single or small cell groups and lose all 3-D, multi-cell type tissue structure during sample preparation. Co-cultures also suffer from lack of true tissue representation and can be difficult to manage properly [23]. In contrast, the 3D MFCA technology enables fully automated deposition of biomolecules and delivery of reagent to cell spots or intact tissue slices in a densely packed array (Figure 1). The proposed system would not only be able to generate arrays, but would do so in a "flowing" environment that allows for studies of shear stresses, delivery of different compounds, and growth conditions on these arrays and will lead to more predictive *in vitro* drug screening tools. A variety of applications can be envisioned that are only limited by the imagination of the investigator, allowing innovation to continue indefinitely.

As a fundamental experiment, we explored the use of different tube lengths on the density and viability of printed cells. The data indicates that the printing process does decrease the number of cells that reach and attach to the surface, but the length of the tubing is irrelevant. Future work in this area will expand on the printing of different types of cells for diverse types of CBAs with the potential for multiplexing various cell types. With independent flow cells a different cell type with its appropriate media could be flowed through each of the 12 channels. Envisioned as an outcome for future work is the ability to identify promising drug candidates in a personalized, format for preclinical testing phases of anticancer drug candidates.

This novel platform will assist the pharmaceutical industry in the timely translation of bench discoveries to bedside, personal cures, in particular for ovarian cancer. Ovarian cancer remains one of the

most devastating cancers, and with this research, we have begun to develop a tool for pharma to use against this tragic disease.

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