Fabrication of Miniature Drug Screening Platform Using Low Cost Bioprinting Technology

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Abstract

In the pharmaceutical industry, new chemicals and substances are being tested to find appropriate compounds or mix for treating a specific disease. The demand for screening large compound collections against and increasing number of therapeutic targets has stimulated technology development in the areas of assay automation and miniaturization. Unfortunately, some of these new compounds are rather hard to obtain, which causes an expensive research and limited material availability; therefore, increasing the time development for future cures.

We have developed a new and low-cost deposition method to fabricate miniature drug screening platform that can realistically and inexpensively evaluate biochemical reactions up to 4 substances per trial in a picoliter-scale volume. This paper describes the development of the controls for a deposition method (inkjet printing technology) which will simultaneously place therapeutic drugs and cells onto target sites to fabricate cell/drug chips for drug screening application. Using a modified HP 5360 CD printer, droplets of GFP expressing Escherichia coli have been deposited in an agar coated coverslip chip as small reliable volume of 180 picoliters per each colony dot, along with this bacteria it has been patterned different antibiotics in such a way that we evaluated the growth of the bacteria under antibiotics presence. The viability and function of the printed cells were evaluated by the live/dead and plasmid gene transfection experiments resulting in more than 98% viability and maintaining DNA function. Moreover, it has been recorded as high throughput process printing 213 assays per second. Due to the reduction of volume, this method will increase the effectiveness of the resources utilized for emerging drug screening processes. The results show promising usage of resources for future drug screening through new biochemicals.

Introduction

Drug discovery and development is a long process and costly endeavor that requires heavy investment in financial and human resources. Many surveys that have been conducted in USA have indicated that drug discovery and development costs have been rising. Although there are no fixed cost estimates, the most recent estimates stands at US$ 802 million [1] spread over 12 years or US$ 880 million distributed over 15 years [2]. The suggested probable reason for the rising in costs for drug discovery and development processes are associated with the fact that the new drugs are rather hard to obtain, which causes expensive research and limited material availability; therefore, increasing the time of the development and consequently the amount of investment required. Recent information have revealed that pharmaceutical research and development of new drugs in the period of 25 years (1975-1999), have provided a total of 1,393 new drugs that entered the market [3]. Moreover, a strong increase in the number of chemical compounds for testing and the concomitant increase in the number of molecular targets for lead finding can be accommodated only via substantial miniaturization of High Throughput Screening (HTS) assays.[4]. The current throughput of the screening processes is limited to 200,000 assays per day [4]. A proposed solution is described within the following manuscript for these current limitations.

On the other hand, majority of developing countries still rely on drugs developed by pharmaceutical firms in the developed countries. However, the driving force for drug discovery and development by these pharmaceutical firms has been the foreseeable profit from drug sells. Since infectious diseases prevail more in developing rather than developed countries, and the fact that people living in the former countries have poor purchasing power, make the market for anti-infective drugs unattractive to the pharmaceutical firms. The firms have therefore been reluctant in investigating and developing new drugs for the diseases that mainly affect developing countries. As a result, very few drugs for tropical diseases are coming into market [5]. The drug screening through inkjet technology demonstrated within this manuscript represents a low cost and low volume potential solution for developing new drugs in these countries.

It is hypothesized that modified commercial inkjet printers can directly deliver small volumetric amounts of given biochemical substances to evaluate the behavior of living organisms in the presence of different drugs (drug screening).

Materials and Methods

Agar Films Preparation

Print substrates were made from soy agar. Prewarm sterilized BBL™ Trypticase™ Soy Agar solution (Becton Dickinson & Co, Cockeysville, MD) were poured into 100 mm Petri dishes, containing three 24x40mm precleaned sterilized premium cover glasses (Fisher Scientific, Pittsburgh, PA). The solution was allowed to cool at room temperature; when cooled, a thin gel layer was formed on the substrates. This substrate provides the sufficient nutrients for bacterial proliferation.

Bacterial Strain and Suspension

Escherichia coli Efficiency™ DH5α cells (Invitrogen, Stockholm, Sweden) were grown overnight at 37ºC on a Trypticase™ Soy Agar plate (Becton Dickinson & Co, Cockeysville, MD). Two loopfuls of organisms (representing approximately two large colonies) were transferred into a centrifuge tube containing 5 ml sterilized water. This formed the original print suspension of bacteria. The cell concentration in the *E. coli* solution was determined by the standard plate count

method [6]. The tubes containing bacterial suspensions were forcefully shaken before printing, to break up clumps and ensure good distribution of the bacteria. The movement of the cartridge during printing allowed the cells to be maintained in suspension.

Viability Evaluation of the Bacterial Array

The viability of the Efficiency™ DH5α cells (Invitrogen, Stockholm, Sweden) contained in the printed agar films was evaluated by a two-color fluorescence live/dead assay using a solution consisting of 3.34 mM SYTO 9 in anhydrous DMSO 4.67 mM hexidium iodide in anhydrous DMSO (Invitrogen, Stockholm, Sweden).

Cell Functionality

The host *E. coli* strain library Efficiency™ DH5α (Invitrogen) was used for plasmid production. The pEGFP-C1 plasmid was produced and purified in small-scale using QIAGEN plasmid mini kit (Qiagen, Venlon, Netherlands) according to company plasmid purification handbook [7], and then it was transfected using FuGENE® HD Transfection Reagent (Roche, Basel, Switzerland).

Controls were defined as negative when no plasmid was used to human kidney 293 cells transfection, and as positive when cells were transfected with pEGFP-C1. Two samples groups were evaluated, the first was bacteria dispensed by the inkjet printing process and the second was bacteria dispensed manually by micropipettes.

High Throughput

An array of points was design using PowerPoint software (Microsoft Inc., Redmond, WA), formatted under the smallest font size allowed by the software (font size 1), and time was measured from the start of delivery of ink until completion of printing. Results were recorded in dots per seconds and the diameter size of the dispensed points was recorded. Typically 41 dots about 420 μm spaced were dispensed in sequential lines.

Volume Determination

In determining the amount of volume being dispensed by the inkjet printer an evaluation test was design to allow calculating the delivered volume by knowing the concentration of the solution and the mass being dispensed by the inkjet printer. A sodium chloride (Acros Organics, Geel, Belgium) and calcium chloride (Acros Organics, Geel, Belgium) were dissolve in distilled water (Millipore, Billerica, MA) in the closest ratio to full saturation, 1:3 and 1:6 respectively. Both solutions were inkjet printed with a black and color cartridge, results were recorded.

The procedure for determining the volume was as follow.

1. Mass of the glass slides utilized to print any given solution was measured with laboratory balance model ALF 204 (Fisher Scientific, Pittsburgh, PA).
2. Different patterns were printed utilizing both solutions onto clean glass slides to correlate the dot size with the volume dispensed.
3. Glass slides were exposed to 100 ± 15 °C at isotemp oven (Fisher Scientific, Pittsburgh, PA) for at least 10 minutes to allow all the water to evaporate.
4. Mass of the glass slides with the remaining salt was measured with laboratory balance.
5. Knowing the solution concentration and dot size being print, volume was determined.

As control, a controlled volume was dispensed over a glass slide under a conventional micropipette deposition method (100 μl). Percentile error was obtained from this test to quantify the tolerance error of the printed parameters under both substances being subjected to the test.

Smallest Reliable Volume Dispensed of E. coli

Microsoft PowerPoint software was used to edit a colony array pattern with a 3 dots in sequential lines under different font sizes (16, 8, 3, 2, and 1). A black cartridge were emptied of its contents, thoroughly washed, rinsed with a 70% ethanol solution, and distilled water and dried in a sterilized Labculture® Class II, Type A2 Biological Safety Cabinet (ESCO, Hatboro, PA) before being filled with 1 ml of bacterial suspension. *E. coli* concentration was measured through absorbance reading (Eppendorf, Hamburg, Germany) and volume calculated. The *E. coli* suspension was ejected onto an agar coated coverslip and bacterial deposition was read under fluorescent microscope for each dot. Results were plotted finding the smallest reliable volume being dispensed by the inkjet printing technology. Understanding reliable as the ability to repeatable dispense a given amount of *E.coli* with a variance below to 50%.

Drug Screening

To build a single capsule drug screening test, and avoid migration by diffusion of the antibiotics towards the rest of the deposition dots, three consecutive layers were printed using as layer 1 a solution of sterilized Broth BBL™ Trypticase™ Soy Agar (Becton Dickinson & Co, Cockeysville, MD), as layer 2 a 0.3% alginic acid solution (Acros Organics, Geel, Belgium), liquid that is known to cross-link under mild conditions to form a biodegradable hydrogel scaffold [8], and as layer 3 an alternating solution of three different antibiotics mixed in a 1:1 ratio to 1.4% $CaCl_2$ (Acros Organics, Geel, Belgium), which is known to promote the cross-linking of the individual alginate chains resulting in an encapsulated environment. Black and color cartridges were used as appropiate to take advantage of the four compartments provided.

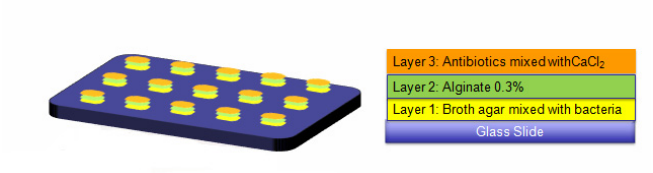

Figure 1. *Schematic illustration of capsules dispensed on glass slides*

Image Processing – Photoshop

The technique of selection of similar features on digitized images has previously been utilized on previous bioengineering research projects [9-11]. This method has been utilized for the quantification of bacteria in the cell viability, functionality, smallest reliable volume dispensing and drug screening tests.

Results

Cell Viability

Survival rate of the printed Efficiency™ DH5α cells within the particles was analyzed by a commercial cell survival assay and compared to the controls (n=3), which were prepared by manually placing the cells onto standard Trypticase™ Soy Agar plate. The live/dead assay showed that more than 98% of printed cells remained viable within the microparticles (dots) measured immediately after printing; moreover, bacteria growth exponentially until the point that colonies were distinguished by simple eye.

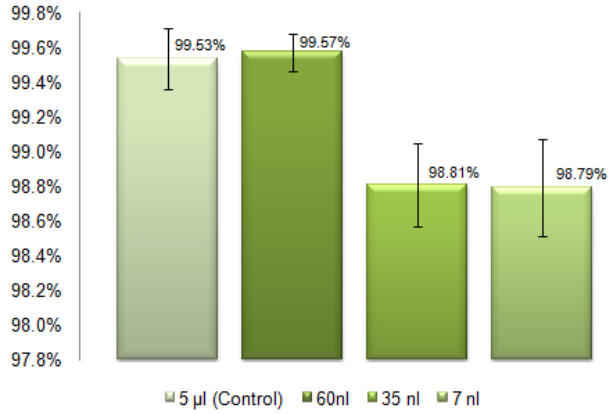

Figure 2. *Viability of the bacteria-loaded microparticles at different volume rates (n=3) read immediately after printing. The samples rated in nanoliters were printed, and the sample rated in microliters was manually dispensed onto standard agar culture plates. All samples were well above 98% viability.*

Cell Functionality

After transfection test was performed, it was found that animal cells expressed GFP plasmids collected from both samples groups (printed *E. coli* and control sample <manually dispensed>). The fluorescent expression was evaluated by image processing method described above finding that both samples groups were significantly equal (p>0.05).

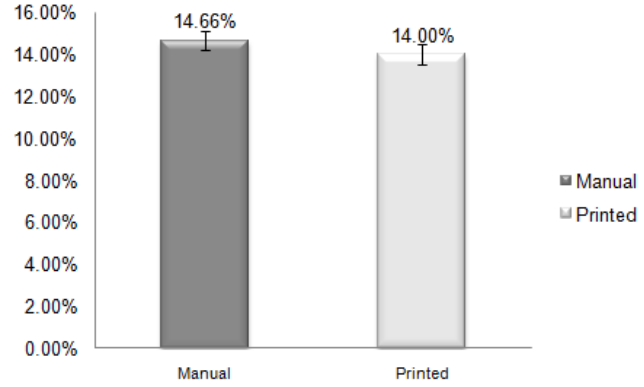

Figure 3. *Percentage of GFP expressed on cells collected from manual and print dispensed samples. Samples are significantly equal (p>0.05) (n=3).*

High Throughput

This technology has been recorded as an ultra high throughput process printing spots of 150–240 µm in diameter at a rate of 213 assays per second (18 million assays per day).

Volume Determination

Sodium Chloride (NaCl) solution was found to be the most reliable substance to be use as control for the volume determination test; as its percentile error remained more than 5 points below the Calcium Chloride ($CaCl_2$). Using Sodium Chloride it was determined the correlation of the area of the dot size and the volume being dispensed obtaining equation 1.

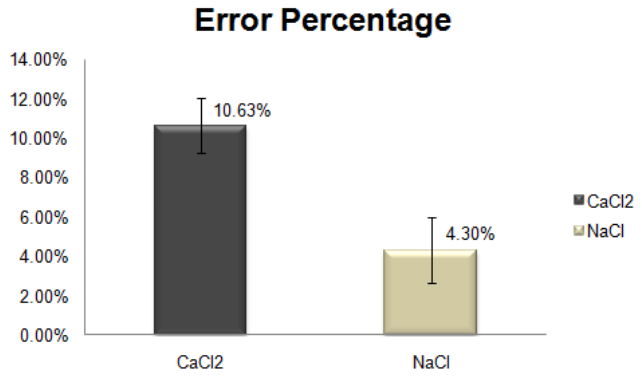

Figure 4. *Percentage error of $CaCl_2$ and NaCl solutions. From this it was decided to plot the volume-area curve using NaCl solution.*

$$\text{volume } (y) = 3.0789\ [\text{area}(x)]^3 - 20.746\ [\text{area}(x)]^2 + 7643.5\ \text{area}(x) + 3\text{x}10^{-7} \quad \text{.....Equation (1)}$$

Smallest Reliable Volume Dispensed of E. coli

It is desirable that every assay the amount of bacteria cells delivered in each dot remain as constant as possible. It was found that the smallest reliable volume was under font size number 3, which dispended 180 picoliters per each colony dot, containing in average 22.3 ± 34.3% (standard deviation) bacteria cells.

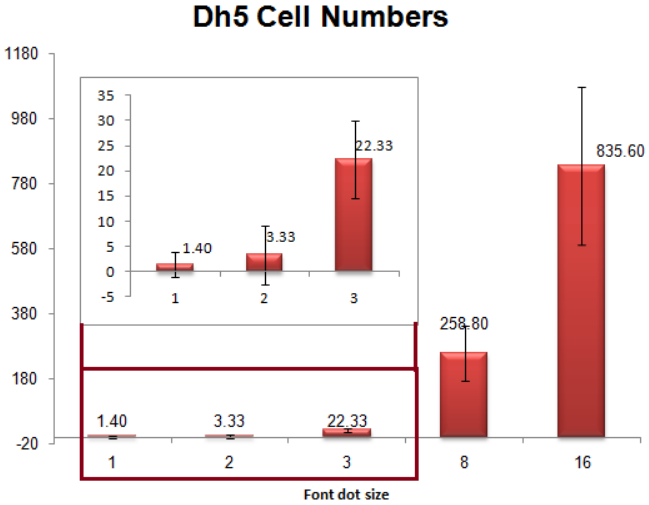

Figure 5*. Number of E. coli strain library Efficiency™ DH5α celsl at different font sizes (n=3).*

Drug Screening

It was found that, in the printing process, the three antibiotics restricted the viability of the cell below the 50% of the control sample test with no antibiotic presence, which was found to have a direct correlation with the data obtained from the manually micropipetted samples.

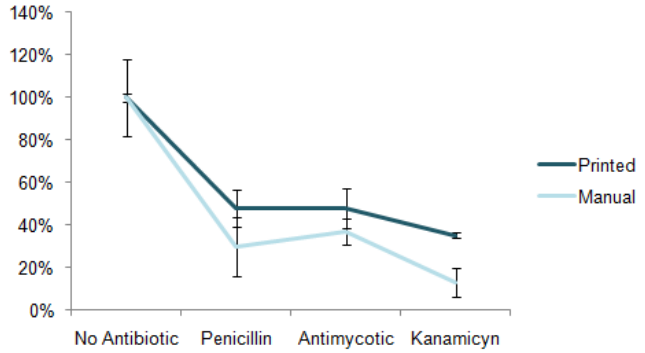

Figure 6. *Normalized graph comparing behavior of E. coli under different antibiotics presence by printing and manual samples dispensed (n=3).*

Discussion

In this study, living organisms and antibiotics patterns were produced using the inkjet technology printing technique. The viability test proves that this method does not significantly affect the number of viable cells, which support the hypothesis that this method can be used for effectively deliver living organisms. Moreover, genetic material (GFP plasmid) in the bacteria was not affected significantly by the printing process.

Regarding high throughput, the 18 million assays per day represent a well above solution for the current screening limitation of 200,000 assays per day.

In the determination of the volume being dispensed by the inkjet technology, sodium chloride (NaCl) solution was found to be the most reliable substance to be use over the calcium chloride ($CaCl_2$); which can be explained by the fact that the second solution can create strongest bonds with water molecules.

The fact of dispensing 180 picoliters per colony dot represent the main advantage of this research project demonstrating that the inkjet technology can reliable deliver living organisms and substances under a small volumetric constrain for drug screening purposes in a chip (plane) design basis

More importantly, in regards to the drug screening results, it can be appreciated in Figure 6 that both methods have the similar behavior along the samples, proving that the inkjet printing methodology can effectively mimic the typical drug screening test. For the penicillin and antimycotic antibiotics it was found that even the variance overlapped, indicating the close behavior that they had; however, in the case of the kanamicyn sulfate there was more difference in their percentage value. A possible explanation is that this antibiotic could be affected by the heat occurred during the inkjet printing process

Conclusions

In this research project, it has been demonstrated that this method can effectively deliver reliable cell volume at level of hundreds of picoliters Due to the reduction of volume, this method will increase the effectiveness of the resources utilized for emerging drug screening processes. Moreover, it was proved that bacteria maintained viability and function after the printing process. Furthermore, different antibiotics can be screened at ultra-high throughput. The results show promising usage of resources for future ultra high throughput drug screening through new biochemicals at low cost. The present drug screening method represents a low cost possible solution to stimulate the drug discovering process for developing countries.

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