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Fabrication Of Miniature Drug Screening Platform Using Low Cost Bioprinting Technology

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FABRICATION OF MINIATURE DRUG SCREENING PLATFORM USING LOW COST BIOPRINTING TECHNOLOGY

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To my lovely wife, Daisy.

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by

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Fabrication of Miniature Drug Screening Platform Using

Low Cost Bioprinting Technology

ABSTRACT

by

JORGE IVÁN RODRÍGUEZ DÉVORA

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. Current methods for evaluating the reactions of cells use a volume in the range of microliters. 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 simultaneously place therapeutic drugs and cells onto target sites to fabricate cell/drug chips for drug screening application. Using a modified HP D5360 CD printer, droplets of GFP expressing *Escherichia coli* have been deposited in an agar coated coverslip chip as small repeatable 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 98% viability and maintaining DNA functionality. Moreover, it has been recorded as a high throughput process printing 213 assays/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.

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1. 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].

The realization of high-speed, miniaturized, low-cost, and high throughput biological devices in the microarray format is also very important issue in the other fields as biosensing, environmental monitoring, forensic investigation, military defense, and so forth [4].

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.[5]

2. LITERATURE REVIEW

2.1. Drug Screening

Current drug discovery relies on massive screening of chemical libraries against various extracellular and intracellular molecular targets to find novel chemotypes with the desired mode of action. In recent years, high-throughput technologies for combinatorial and multi-parallel chemical synthesis, automation technologies for the isolation of natural products, and also availability of large compound collections from commercial sources, have substantially increased the size and diversity of compound collections among most Pharma and Biotech companies, in some cases exceeding one million distinct chemical entities.[5]

The strong increase in both the number of available compounds as well as molecular targets has caused a fundamental change in the drug discovery process applied at Pharma and Biotech companies during the past two decades. Various technologies for assay miniaturization, lab automation and robotics enable testing of chemical compounds in biological systems by means of high-throughput screening (HTS) and ultra-highthroughput screening (uHTS). Whereas HTS is defined by the number of compounds tested to be in the range of 10 000–100 000 per day, uHTS is defined by screening numbers in excess of 100 000 data points generated per day. Taken together, the technologies of HTS and uHTS are seen as key elements for filling the drug discovery pipeline in industry with new chemical compounds and new modes of action [5].

As a base model for this project the behavior of *E.coli* in the presence of different antibiotics has been evaluated, that is why in the following section the *Escherichia Coli* and Antibiotics are introduced.

2.1.1. Escherichia Coli

According to Oxford Dictionary of Biochemistry and Molecular Biology [6], *Escherichia coli* or *colon bacillus* abbreviated *E. coli*; is the sole member of the bacterial genus Escherichia and arguably the most widely used experimental cell system in biochemistry and molecular biology. The cells are straight, round-ended rods, commonly 0.5–1 μm × 1–4 μm, and usually occurring singly or in pairs. The organism is present in the intestinal tract of humans and other animals and is common in soil and water. The numerous strains, some of which are enteropathogenic, are commonly distinguished serologically by their O, K, and H antigens. *E. coli* takes part in bacterial conjugation and other forms of genetic transfer, and can be infected with some bacteriophages and plasmids.

DH5 alpha is a strain of *Escherichia coli* used in gene cloning using pUC and similar plasmid vectors and permitting alpha complementation. The alpha complementation, a phenomenon in which the N-terminal sequence 1–56 of b-galactosidase from *Escherichia coli*, the a-peptide, is able to restore enzyme activity to a mutant form of the enzyme such as lacZDM15 that has the sequence deleted. [6]

To prove the drug screening process of this project, *Escherichia coli* has been chosen as the living organism to be deposited and evaluated under the presence of difference antibiotics.

2.1.2. Antibiotics

According to Oxford Dictionary of Biochemistry and Molecular Biology [6], the antibiotics are any of numerous substances of relatively low M_r produced by living microorganisms (and also certain plants) that are able selectively and at low concentrations to destroy or inhibit the growth of other organisms, especially microorganisms. Also included are the many semi- or wholly synthetic organic compounds with similar antimicrobial properties. [6]

The antibiotics chosen for this project have been limited to three of the most common antibiotics in different categories and available in the market due to the advantages propose by this technology explained in section 4.6. The antibiotics were penicillin, antimycotic, and kanamycin sulfate.

Penicillin. (sometimes abbreviated PCN or pen) is a group of antibiotics derived from Penicillium fungi [7]. Penicillin antibiotics are historically significant because they are the first drugs that were effective against many previously serious diseases such as *Syphilis* and *Staphylococcus* infections. Penicillins are still widely used today, though many types of bacteria are now resistant. [6]

Antimycotic is a group of antibiotics also known as antifungal. Antifungal work is performed by exploiting differences between mammalian and fungal cells to kill the fungal organism without dangerous effects on the host. Unlike bacteria, both fungi and humans are eukaryotes. Thus fungal and human cells are similar at the molecular level. This makes it more difficult to find or design drugs that target fungi without affecting

human cells. As a consequence, many antifungal drugs cause side-effects. Some of these side-effects can be life-threatening if the drugs are not used properly.[8]

Kanamycin Sulfate is an aminoglycoside antibiotic, available in both oral and intravenous forms, and used to treat a wide variety of infections. Kanamycin is isolated from *Streptomyces-kanamyceticus* [9]. Kanamycin is used in molecular biology as a selective agent most commonly to isolate bacteria (e.g., *E. coli*) which have taken up genes (e.g., of plasmids) coupled to a gene coding for kanamycin resistance (primarily Neomycin phosphotransferase II [NPT II/Neo])

2.1.3. The Importance of Screening Volume

Applications are driven by the desire to miniaturize processes in order to achieve cost efficiencies. In High Throughput Screening (HTS), higher density lower volume assay plates are being investigated because of the combined effect of decreased reagent/consumable USC and increased throughput (more assays per plate). Genomics research is pushing toward very high density micro-array of DNA. The more cDNA 'spots' that can be arrayed per slide, the more genetic variations that can be investigated [10].

Technology stimulated in the area of assay automation and miniaturization have advanced until the point that current well plates used for screening are in the range of 1536 wells utilizing 2.5-10 µl per well (see Figure 1 for further detail).

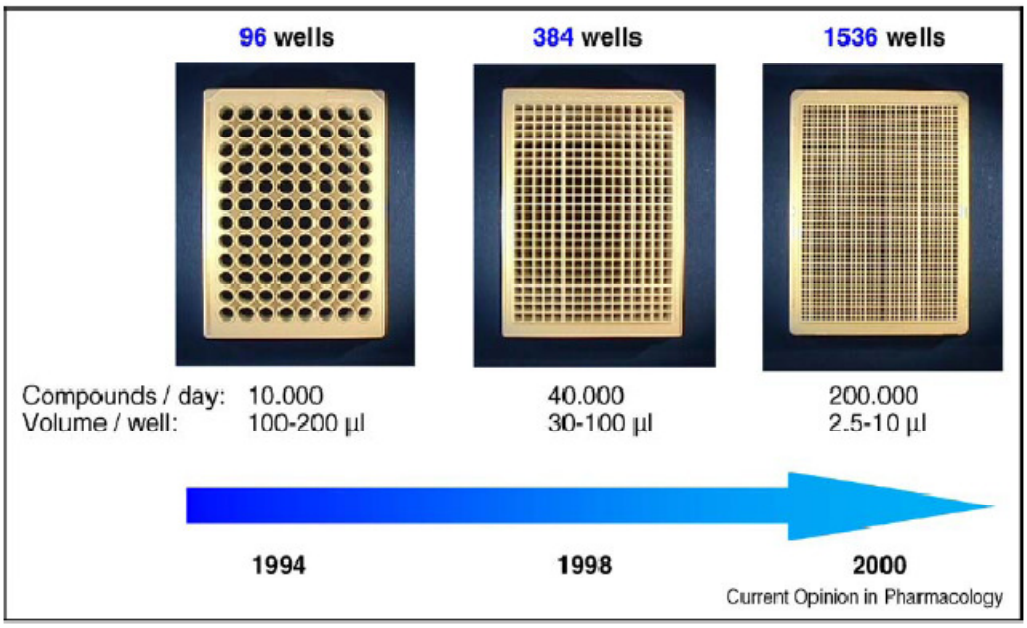

Figure 1. The trend toward miniaturization in screening. The figure shows the different types of microtiter plates used for compound storage and highthroughput screening. In the early and mid 1990s, the main screening format has been the microtiter plates with 96 wells per plate (96w-MTP). This plate type has been largely replaced in recent years with 384w-plates and in some cases even with 1536w-plates (384w-MTP, 1536w-MTP). Despite the fact that all these plate types have the same footprint, they can carry different numbers of distinct reaction wells and thereby enable miniaturization and higher throughput in the screening process.[5]

It is of vital importance for this research project to demonstrate the capabilities of the inkjet printing technology to downsizing the volume usage for delivering biochemical substances for drug screening applications.

2.2. Inkjet Bioprinting

Recently, the inkjet printing technique has attracted much attention as a useful tool for the fabrication of cellular patterns on a substrate. In this technique, precise target positions on a substrate can be assigned by computer-assisted deposition. Inexpensive commercially available printers can be used in these experiments with little modification. Several studies have shown the successful creation of cellular patterns on

substrate by using this inkjet printing technique [11-17]. There are two main strategies, printing with living cells and printing with cell adhesion molecules [18].

Boland and coworkers reported that Chinese hamster ovary cells and primary embryonic hippocampal or cortical cells can be directly printed onto a substrate with a desired pattern without loss of cell function [16-17]. The printing of cell adhesion molecules such as collagen or cell growth/differentiation factors such as FGF-2 and CNTF with a desired pattern onto a substrate has also been demonstrated using inkjet printers [12-14].

2.3. Other Engineering Tools for Small Volume Delivering

Current engineering tools offered for dispensing small volumetric rate provides a solution for drug screening under low volume; however, there are limitations associated with it. These typically involve time (limited throughput) and reagent consuming (micro-, nanoliter scale volumes) instrumental tools and facilities like clean rooms, photolithography, liquid handling robotics, complex detectors, and software for data processing [19].

2.3.1. Microdrop Technologies[20]

Microdrop Technologies is the leading provider of equipment, software and services for advanced micro-dispensing and inkjet printing applications. The technology from microdrop is a versatile tool for liquid handling and material deposition (adhesives, coatings, waxes, nanoparticles, polymers, DNA, proteins, cells, etc.). The main 3 technologies from Microdrop included:

1. Microdrop – Inkjet technology
2. Nanojet – Valve technology
3. Dropjet – Continous drop generation

Table 1. Microdrop Technologies

Characteristics	Microdrop – Inkjet Technolgy	Nanojet - Material Deposition by Valve Technology	Dropjet - Continuous Drop Generation
Single drop volumes*:	25 - 500 pl	> 200 nl	-
Droplet diameter*:	30 - 100 µm	-	60 – 200 µm
Volume variation*:	approx. 1 %	approx. 5 %	approx. 2 %
Viscosity range:	1 - 100 mPas (unheated)100 – 10,000 mPas (heated)	0 – 50 mPas	1- 50 mPas
Drop rate*:	0 - 2000/s	-	20 – 80 kHz
Max. throughput:	1 µl/s	approx. 2 ml/s	-
***depending on the liquid**			

Microdrop – Inkjet technology

The microdrop technology uses the principle of the ink jet printing technology. The core of the microdrop dispensing head consists of a glass capillary which is surrounded by a tubular piezo actuator. At one end the capillary is formed to a nozzle (diameter 30 to 100 µm). Applying a voltage pulse, the piezo actuator contracts and creates a pressure wave which propagates through the glass into the liquid. In the nozzle region the pressure wave accelerates of the liquid. A small liquid column leaves

the nozzle, breaks off and forms a droplet which flies freely through the air. Depending on the nozzle sizes (30 to 100 µm) volumes of 25 up to 500 pl (corresponding to drop diameters of 35 to 100 µm) are generated.

A limitation of the microdrop inkjet technology is the low throughput associated with the fact that only use a single nozzle for dispensing any given substance; in contrast, with the several set of nozzles utilized by the proposed inkjet technology (HP printer described in section 4.6) which allows a high throughput.

Nanojet - Material Deposition by Valve Technology

The Nanojet system is the right choice for applications where volumes from 200 nl and larger are needed to be dispensed. The Nanojet technology forms a liquid jet by means of a time/pressure-controlled dispenser system. Liquid from a pressurized reservoir is pressed into the dispenser head which consists of a micro valve and a nozzle. By opening the valve, liquid flows through the nozzle and forms a well-defined jet. The integrated micro valve switches the jet on and off. Since the micro valve switches at a very fast rate, volumes down to 200 nl can be dispensed. The special nozzle geometry forms a laminar jet, which is stable over a wide range before breaking off into droplets.

Dropjet - Continuous Drop Generation

Dropjet – a combination of Microdrop and Nanojet technology – allows the generation of a large number of drops with a well-defined size. This is of interest in applications like micro-encapsulation, aerosol research, spray and micro-sphere production.

A jet is generated by pressurized liquid flowing through a nozzle. The jet is modulated by an integrated piezo actuator causing defined fluctuations of the jet diameter at the nozzle outlet. The fluctuations grow over time, constrictions are built and after a certain distance the jet disintegrates into droplets.

The drop volume is defined by the flow rate, the frequency of modulation and the nozzle diameter of the system. The smallest possible drop size - approx. two times the size of the nozzle diameter - is defined by the smallest wave length of the periodic fluctuation. The maximum volume is limited by the stable separation of the drops. At larger distances of the constrictions the liquid tends to separate into more than one drop with different volumes.

3. HYPOTHESIS AND OBJECTIVES

3.1. Hypothesis

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.

3.2. Objectives

a) Demonstrate the bacteria viability when processed by the inkjet technology.

b) Demonstrate the bacteria functionally when processed by the inkjet technology.

c) Evaluate the speed of this technology to classify between a high throughput screening process and ultra high throughput process.

d) Explore the scope of this technology regarding the smallest amount of bacteria that can be delivered, downsizing the substances being used therefore reducing the development cost.

e) Demonstrate drug screening capability under diminutive volumetric constraint.

4. MATERIALS AND METHODS

4.1. Agar Films Preparation

Print substrates were made from soy agar. 7ml of 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 with a calculated layer thickness of about 1 ± 0.12 mm. This substrate provides the sufficient nutrients for bacterial proliferation.

4.2. 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 (described in section 4.9) [21]. This solution was diluted to different concentrations of bacterial suspensions for subsequent printing. 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.

4.3. Viability Evaluation of the Bacterial Array

The viability of the Efficiency™ DH5α cells (Invitrogen, Stockholm, Sweden) contained in the printed alginate microparticles 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). The samples were viewed using a fluorescent microscope, and the viability of the cells was evaluated by counting the number of cells stained with SYTO 9 (green) through method described in section 4.13, and this number was compared to the total number of cells. The viability results of the printed samples were compared to the controls, in which Efficiency™ DH5α cells were manually plated onto a standard tissue culture plate (BD Biosciences, San Jose, CA).

4.3.1. Staining Procedure

1) Combine equal volumes of Component A and Component B in a microfuge tube and mix thoroughly.
2) Add 3 µL of the dye mixture per mL of bacterial suspension.
3) Mix thoroughly and incubate at room temperature in the dark for 15 minutes.
4) Trap 5 µL of each stained bacterial suspension between a slide and an 18 mm square coverslip.
5) Observe in a fluorescence microscope equipped with any of the filter sets listed in Table 2. Live gram-negative organisms should fluoresce green and gram-positive bacteria should fluoresce red.
6) To optimize the staining pattern, repeat steps 1 through 5 with different mixtures of Component A and Component B. The volume of the dye mixture added to the cell

suspension in step 2 may also be varied; however, adding more than 3 µL of DMSO (from the dye mixture) per mL of bacterial suspension may adversely affect staining.

Table 2. Characteristics of common filters suitable for use with the LIVE BacLight Bacterial Gram Stain Kit.

Omega filters *	Chroma filters *	Notes
XF25, XF26, XF115	11001, 41012, 71010	Longpass and dual emission filters useful for simultaneous viewing of SYTO 9 and hexidium iodide stains
XF22, XF23	31001, 41001	Bandpass filters for viewing SYTO 9 alone
XF32, XF43 XF102, XF108	31002, 31004 41002, 41004	Bandpass filters for viewing hexidium iodide alone
* Catalog numbers for recommended bandpass filter sets for fluorescence microscopy. Omega® filters are supplied by Omega Optical Inc. (www.omegafilters.com). Chroma filters are supplied by Chroma Technology Corp. (www.chroma.com).		

4.4. 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 [22], 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.

4.4.1. Purification Procedure

1) Pick a single colony from a freshly streaked selective plate and inoculate a starter culture of 2–5 ml LB medium containing the appropriate selective antibiotic. Incubate for approximately 8 h at 37°C with vigorous shaking (approx. 300 rpm).

2) Dilute the starter culture 1/500 to 1/1000 into 3 ml selective LB medium. Grow at 37℃ for 12–16 h with vigorous shaking (approx. 300 rpm).
3) Harvest the bacterial cells by centrifugation at 6000 x g for 15 min at 4℃.
4) Resuspend the bacterial pellet in 0.3 ml of Buffer P1. Ensure that RNase A has been added to Buffer P1. The bacteria should be resuspended completely by vortexing or pipetting up and down until no cell clumps remain.
5) Add 0.3 ml of Buffer P2, mix thoroughly by vigorously inverting the sealed tube 4–6 times, and incubate at room temperature (15–25℃) for 5 min.
6) Add 0.3 ml of chilled Buffer P3, mix immediately and thoroughly by vigorously inverting 4–6 times, and incubate on ice for 5 min.
7) Centrifuge at maximum speed in a microcentrifuge for 10 min. Remove supernatant containing plasmid DNA promptly
8) Equilibrate a QIAGEN-tip 20 by applying 1 ml Buffer QBT, and allow the column to empty by gravity flow.
9) Apply the supernatant from step 7 to the QIAGEN-tip 20 and allow it to enter the resin by gravity flow. The supernatant should be loaded onto the QIAGEN-tip promptly. If it is left too long and becomes cloudy due to further precipitation of protein, it must be centrifuged again before loading to prevent clogging of the QIAGEN-tip.
10) Wash the QIAGEN-tip 20 with 2 x 2 ml Buffer QC. Allow Buffer QC to move through the QIAGEN-tip by gravity flow.
11) Elute DNA with 0.8 ml Buffer QF. Collect the eluate in a 1.5 ml or 2 ml microcentrifuge tubes (not supplied).

12) Precipitate DNA by adding 0.7 volumes of room-temperature isopropanol to the eluted DNA. Mix and centrifuge immediately at ≥10,000 rpm for 30 min in a microcentrifuge. Carefully decant the supernatant.
13) Wash DNA pellet with 1 ml of 70% ethanol and centrifuge at 10,000 rpm for 10 min.
14) Air-dry the pellet for 5–10 min, and redissolve the DNA in a suitable volume of ddH2O.
15) To determine the yield, DNA concentration should be determined by both UV spectrophotometry at 260 nm and quantitative analysis on an agarose gel. For reliable spectrophotometric DNA quantification, A260 readings should lie between 0.1 and 1.0.

4.4.2. Transfection Method

1) Allow FuGENE® HD Transfection Reagent, DNA, and diluent to adjust to +15 to +25℃. Vortex for one second or invert the FuGENE® HD Transfection Reagent vial to mix.

2) Dilute 2 ug DNA with 96 ul serum-free DMEM medium (without antibiotics or fungicides).

3) Add 6 uL FuGENE® HD transfect ion Reagent to tubes containing diluted DNA: Pipet the FuGENE® HD Transfection Reagent directly into the medium containing the diluted DNA without allowing contact with the walls of the plastic tubes.

4) Mix and incubate the transfection complex at room temperature for 15 min: Vigorously tap the tube or vortex for one to two seconds to mix the contents.

5) Add the transfection complex to cells: Remove culture vessel from the incubator. Removal of growth medium is not necessary. Add the transfection complex to the cells in a drop-wise manner or add below the surface of the medium. Swirl the wells or flasks to ensure distribution over the entire plate surface.

6) Incubate cells and assay the results: Following transfection, incubate the cells for 18–72 hours prior to measuring protein expression.

4.5. Design Software

The printer patterns for the *E. coli* solution and antibiotics were designed using PowerPoint software (Microsoft Inc., Redmond, WA). The color scheme was adjusted to print the desired compounds from their respective cartridges.

4.6. Printer and Cartridge Modification

The modifications to the HP photosmart printer model D5360 (Hewlett-Packard, Palo Alto, CA) were limited to removing the rubber and the metallic rolls of the center area of the compact disc tray feeder; moreover, the rubber cleaners, which are used to clean the cartridges nozzles, were removed along with their respective springs use to maintain contact with the cartridge when needed.

A black and color ink-jet cartridges (HP 74 and 75 respectively) 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 a bacterial printing suspension. This procedure proved to be an effective method for the cleaning and sterilization of the

cartridges. This was determined by imprinting cover slips with sterilized water filled cartridges and subsequent absence of colonies on agar-coated cover slips 2 days after incubation at 37ºC.

Color cartridges (i.e. HP 74) contain three different compartments which deliver its content as illustrated in Figure 2. These three compartments along with the black cartridge compartment allow us to be able to print four different substances at the same printing session.

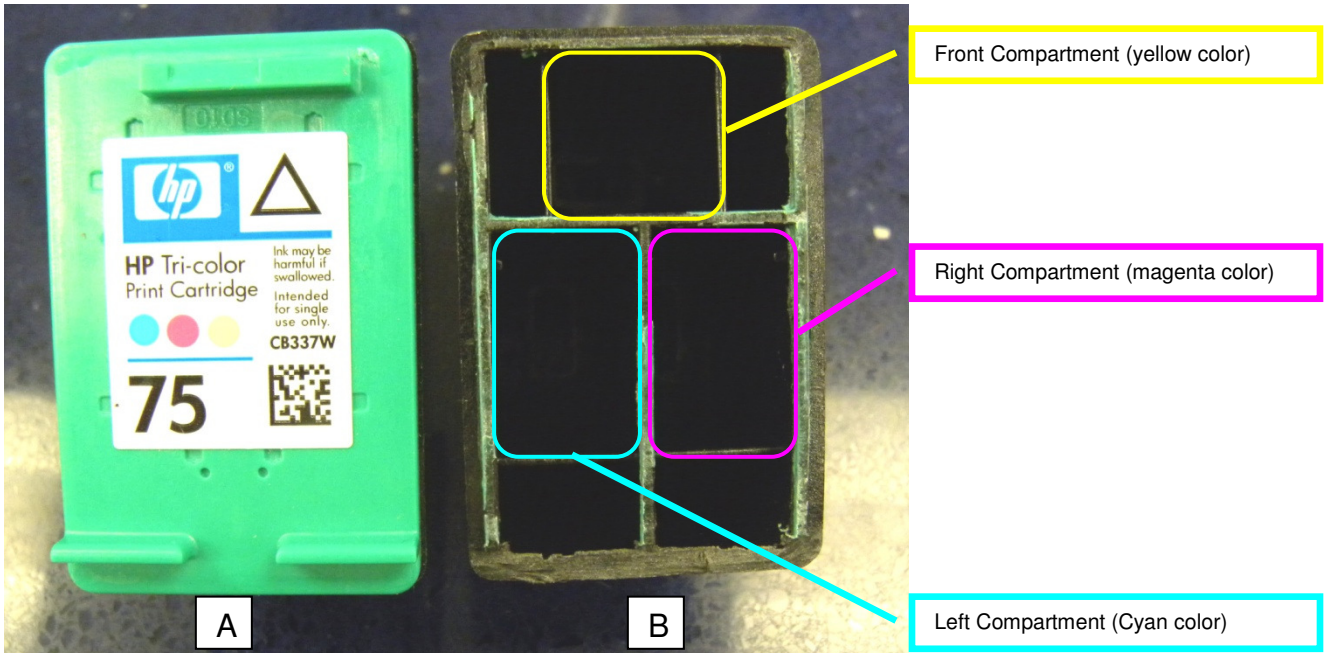

Figure 2. Color cartridge. A) Closed, B) Opened

4.7. 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.

4.8. X, Y Capability

It is shown for the first time the usage of a modified compact disc inkjet printer HP D5360 photosmart. Previous work has been done modifying a regular paper printer [12, 14, 16-18, 23-31]; moreover, the advantage gained by this model consists in allowing printing in a two dimension platform, given more versatility to the living organisms printing deposition process.

4.9. Dilution Biological Assay for Cell Concentration Determination

In determining the number of organisms present in water, milk, and food, the standard plate count (SPC) is universally used. It is relatively easy to perform and gives excellent results. [21]

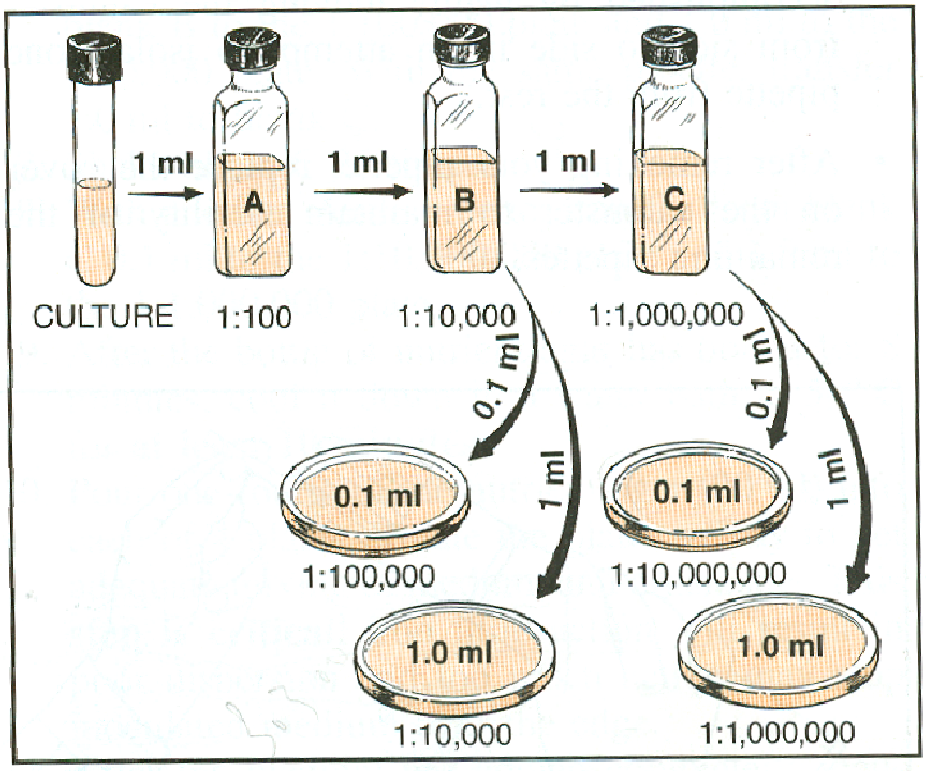

Figure 3. Quantitative plating procedure

The original Benson method [21] was slightly modified to match available laboratory materials as following description. The procedure consists of diluting the organisms with a series of sterile water blanks as illustrated in Figure 3. By using the dilution procedure indicated here, a final dilution of 1:1,000,000 occurs in blank C. From blanks B and C, measured amounts of the diluted organisms are transferred into empty Petri plates. Nutrient agar,, cooled at 50℃, is then poured into each plate. After the

nutrient agar has solidified, the plates are incubated for 24 to 48 hours and examined. A plate that has between 30 and 300 colonies is selecting for counting. It should be pointed out that greater accuracy can be achieved by pouring two plates for each dilution and averaging the counts.

Proceed as follows to dilute out four cultures of *E. coli* at different concentrations and pour four plates for each culture, as illustrated in Figure 3.

4.9.1. Materials

- 1 bottle (40ml) broth culture of *E. coli* at four different concentrations
- 1 bottle (400ml) Trypticase™ Soy Agar solution (Becton Dickinson & Co, Cockeysville, MD)
- 4 Petri plates
- 1 ml pipettes
- Sterile 49.5 ml water blanks
- Canister for discarded pipettes

1) A bottle of nutrient agar was prepared. While it was being heated, 3 49.5 ml sterile water blanks were labeled A, B, and C. Also, 4 Petri plates were labeled as 1:10,000, 1:100,000, 1:1,000,000, and 1:10,000,000. In addition, indication of the amount to be pipette into each plate was labeled (0.05 or 0.5 ml).
2) Absorbance value was measured for each of the four cultures under wavelength of 600nm.
3) Shake the cultures of *E. coli* and transfer 0.5 ml of the organism to blanks A, using sterile 1ml pipette. After using the pipette, it was discarded into canister.

4) Blanks A were shaken 25 times in an arc of 1 foot for 7 seconds with the elbow on the table. Forceful shaking not only brings about good distribution, but it also breaks up clumps of bacteria.
5) With a different 1 ml pipette, 0.5 ml were transferred from blanks A to blanks B.
6) Blanks B were shaken about 25 times in the same manner.
7) With another sterile pipette, 0.05 ml were transferred from blanks B to the 1:100,000 plate and 0.5 ml to the 1:10,000 plate. With the same pipette, 0.5 ml were transferred into blanks C.
8) Blacks C were shaken 25 times.
9) With another sterile pipette, 0.05 ml were transferred from blanks C to the 1:10,000,000 plate and 0.5 ml to the 1:1,000,000 plate.
10) The nutrient agar was boiled for 8 minutes, and then it was left to cool down in a water bath at 50℃ for at least 10 minutes.
11) 20 ml of the nutrient agar were poured into each plate. Plates were rotated gently to get adequate mixing of medium and organisms. This step is critical! Too little action will result in inoculated medium over the edge.
12) After the agar was cooled completely, they were incubated at 37℃ for a minimum of 48 hours, inverted.

4.9.2. Counting

The Petri plates were place upside down and using a felt pen each colony was marked as being counted. Plot of the absorbance value against cell numbers were obtained after this counting process for the four culture concentrations.

4.10. 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. 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 cartridges and results were obtained.

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 (see Figure 4) were printed utilizing both solutions onto glass slides to correlate the dot size with the volume dispensed
3) Glass slides were exposed to 100 ± 15 ℃ 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 model (Fisher Scientific, Pittsburgh, PA).
5) Knowing the solution concentration and dot size being print volume was determined.

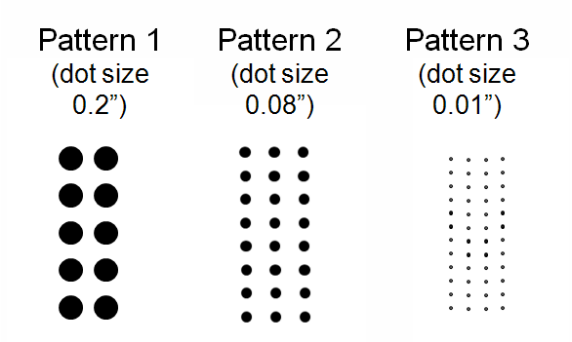

Figure 4. Patterns printed for volume determination

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 utilized.

4.11. 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 prepared black cartridge (as described in section 4.6) was 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 as illustrated in Figure 5 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%.

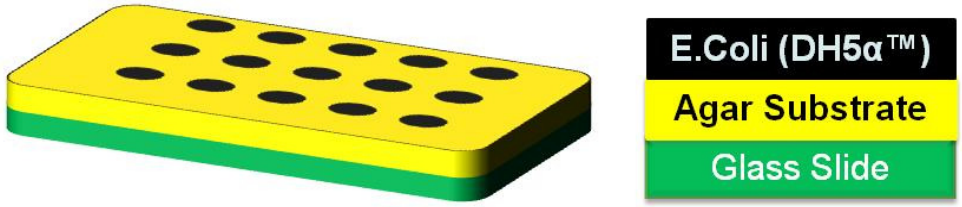

Figure 5. Diagram of *E. coli* deposition onto agar coated glass slide.

4.12. Chip Design

The compact disc tray was modified to support different size glass slides and cover slides. To verify the dispensing of the substances being printed the disc tray support was modified to allocate PH paper at both ends of the printing area, printing pattern was design in such a way that if substances was being dispensed over the PH paper, it will change color due to the dispensed substance, indicating the good condition of the cartridge nozzles.

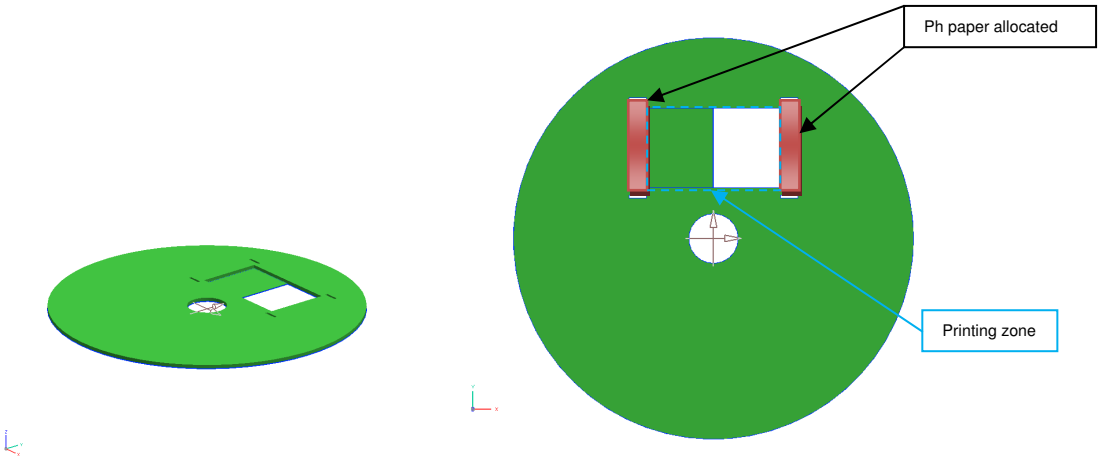

Figure 6. Rendered image of a CD tray modified to allocate diferent sizes glass slides. Pictures show the one for 24x40 mm glass slides.

4.13. Image Processing – Photoshop

The technique of selection of similar features on digitized images has previously been utilized on previous bioengineering research projects [32-34]. The selection of a specific color on a digitized image is a “procedural” selection, i.e., using the information of color saturation, hue, and luminosity intrinsic to every pixel in the image. To make a selection of a specific color, the cursor of the Magic Wand tool is clicked on any object on the image displaying the desired color/chromogen. The selected area is automatically highlighted. To specify how broad a range of color the Magic Wand tool should include in the selection, the Tolerance value in the Magic Wand Options palette can be set to a number between 0 and 255, with lower numbers indicating a small range of colors. Using the Select Similar command, all pixels on the image are highlighted that fall within the selected color range and are not touching the initial selection. Because the selected area is automatically highlighted on the screen, the selection process can be controlled at every step and necessary corrective measures can be taken. For example, the Select Grow command expands the color range to expand the selection to neighboring pixels. The selection continues to grow as often as this command is repeated. Often colors/chromogens are too similar to allow a control of separation using the above-described method. In this case, tone adjustments must be made to the original image. Using the Hue /Saturation tool in the Image Adjust menu, the saturation and/or hue of a selected color (red, yellow, green, cyan, blue, magenta, or any color selected using the Eyedropper tools) can be modified (e.g., rendering fluorescent green into a more bright green color, which now separates well from the background green chromogen as seen in Figure 9).

Once the different chromogens are selected, quantification is accomplished using the Histogram command in the Image menu. This display is rarely if ever used by graphic designers but rather serves as an internal measurement of total distribution as the basis for automated image manipulation (map commands). When Histogram is selected, a display appears on the screen depicting the gray levels (black /white) or the luminosity (color) of all pixels within the selected area, including median and standard deviation. Furthermore, this display shows the number of pixels that are covered by the selected area. Because the number of pixels reflects a surface area on the image, important spatial information can be obtained for the specific chromogen (and hence the bacterias expressing fluorescent green protein) and can be quantify expressed as number of pixels representing the bacteria numbers. Determining a number of pixels equivalent to a single bacteria.

4.14. 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 [35], 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.

In a single slide, six dots were dispensed in sequential lines, a line was used for evaluating each of the three different antibiotics (Antimycotic, Penicillin, Kanamycin Sulfate), letting as control a line printed with a modified layer 3 with no antibiotic. Cell count was done as described in section 4.13 and results were plotted.

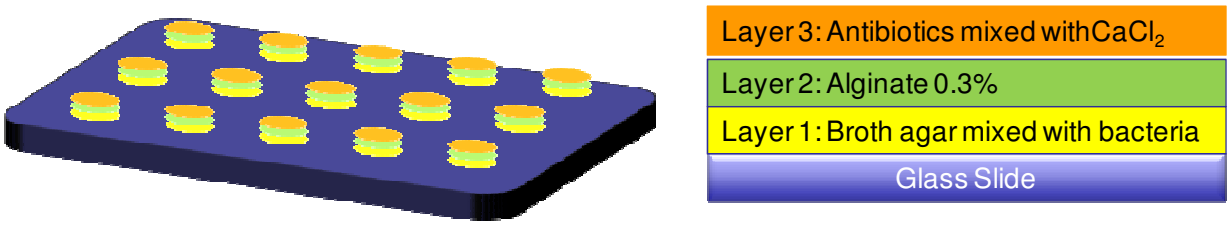

Figure 7. Schematic illustration of capsules dispensed on glass slides

4.15. Statistics

All results were presented as mean ± standard deviation. The grouped data were statistically compared to analysis of variance (ANOVA) and a two-sample Student's t-test.

5. RESULTS AND DISCUSSION

5.1. 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.

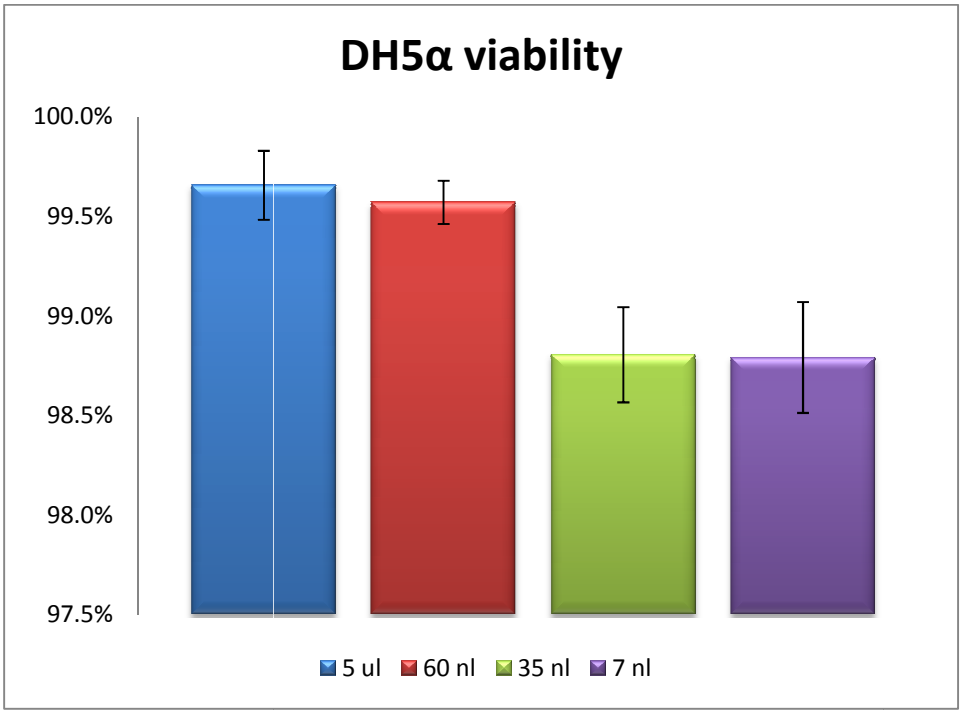

Figure 8. Viability of the bacteria-loaded microparticles at different volume rates (n=3) read immediately after printing. The samples rated in nanoliters were printed, and the sample rated in microliters was manually dispensed onto standard agar culture plates. All samples were well above 98% viability.

The viability test proves that the inkjet printing 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.

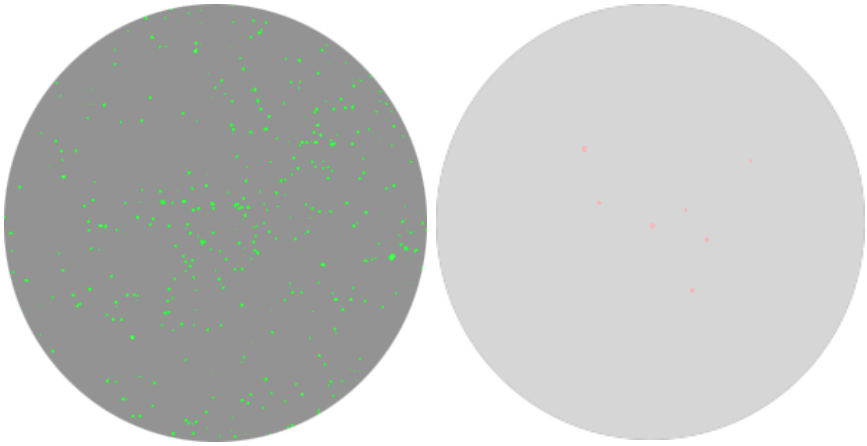

Figure 9. Examples of image adjusted to better visualize the live (green) and dead (red) cells. Pictures show control sample # 1.

5.2. 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 method described in section 4.13 founding that both samples groups were significantly equal ($p>0.05$).

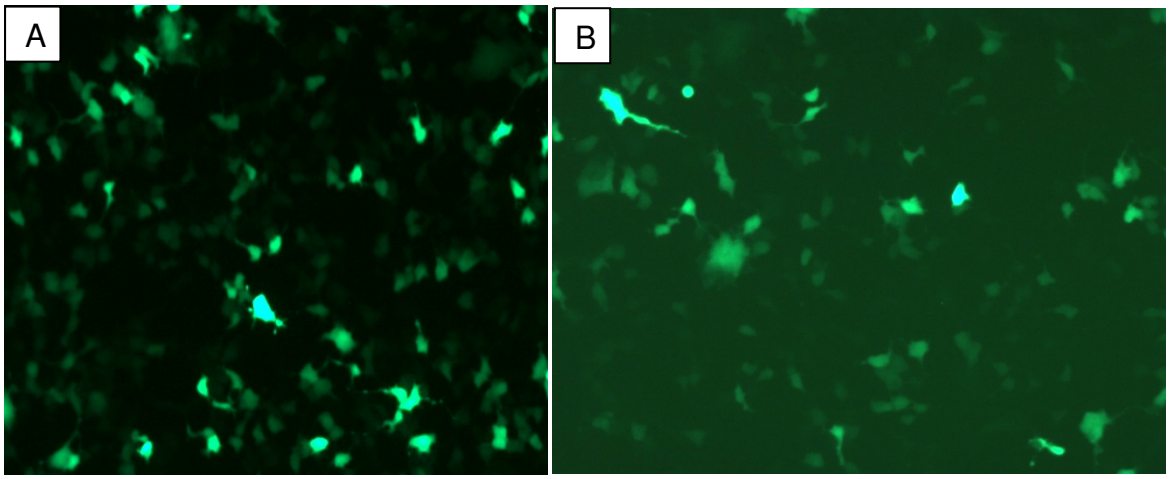

Figure 10. (A) Animal cells expressed GFP plasmids collected from non printed *E. coli, (B) Animal cells expressed GFP plasmids collected from non printed E. coli*

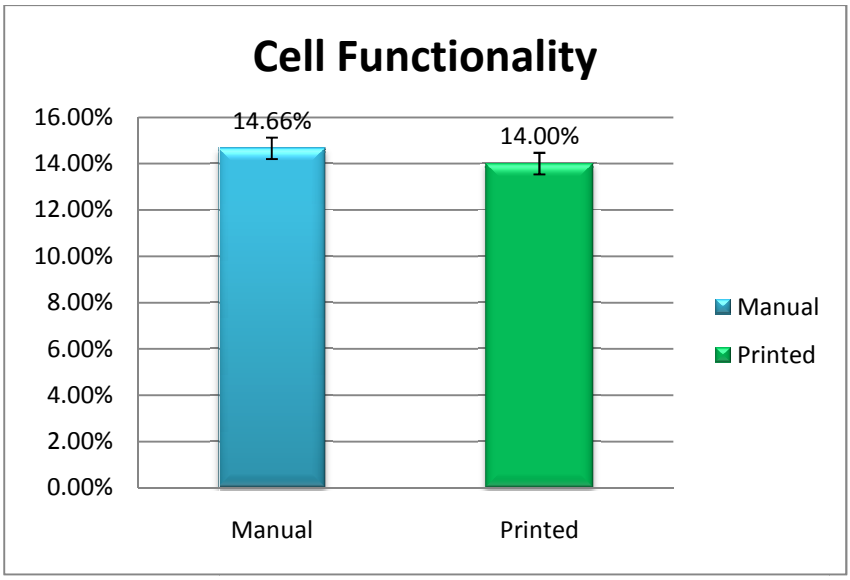

Figure 11.Percentage of GFP expressed on cells collected from manual and print dispensed samples. Samples are significantly equal (p>0.05) (n=3)

Genetic material (GFP plasmid) in the bacteria was not affected significantly by the printing process.

5.3. High Throughput

The method described in section 4.7 was able to generate capsules of 150–240 µm in diameter at a rate as high as 213 particles per second. This allows us, in theory, to run more than 18 million targets per day. This result locates the inkjet technology above the ultra-high throughput which corroborates its candidacy to solve the bottleneck associated to the target evaluation within the drug discovery process.

5.4. Cell Concentration

Four different *E. coli* solutions were prepared and analyzed under standard plate counting method for cell concentration determination. The absorbance values obtained at 600 nm wavelength for the four solutions were 0.339, 0.775, 1.066, and 1.891. After being plated as described in section 4.9 cell colonies were count after 48 hours incubation process, resulting in a cell concentration of 2 x 10^7, 4.1 x10^7, 7 x 10^7, and 1.2 x 10^8, respectively. Data was plotted adding the natural value of 0,0 (absorbance value, cell concentration) and different trend lines were draw. A positive correlation was found within the two set of data (correlation = 0.9953), which confirms the direct relation between the absorbance value and the cell concentration. A lineal trend line was found to be the best line fit (regression line) (R-squared = 0.9883), which resulted in a lineal equation that describes the relation of the cell concentration with the absorbance value. The resulted equation was:

$$Cell\ Concentration\ (y) = 7x\ 10^{-7}\mathrm{OD}_{600}\ (\mathrm{x})$$

[Equation 1]

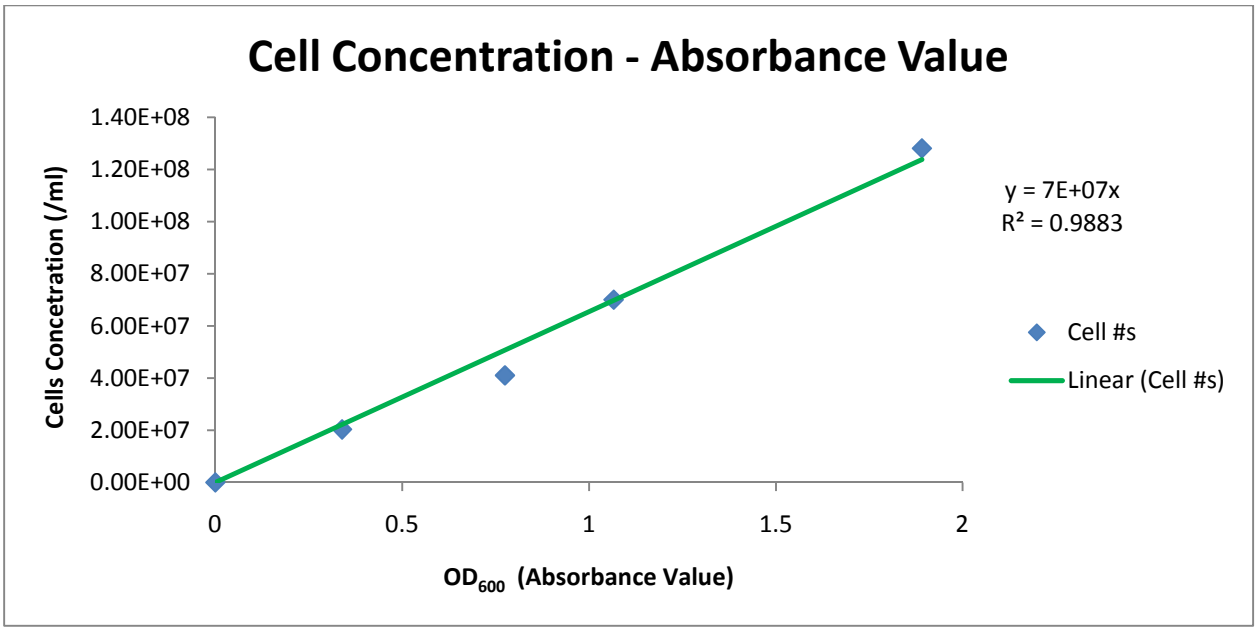

Figure 12. Graphical representation of the dilution biological assay for cell concentration. A linear trend line is fitted under R-squared value of 0.9883.

5.5. Volume Determination

To determine the volume, of such small fluid dispensing, a control test was used to dispense 100 µl of a calcium chloride ($CaCl_2$) and sodium chloride (NaCl) solutions onto glass slides. The practical volume obtained for the $CaCl_2$ solution was 89.37 µl and for the NaCl solution was 96.7 µl which represent a percentile error of 10.63 and 4.30, respectively. Sodium Cloride (NaCl) solution was found to be the most reliable substance to be use as control for the volume determination test; therefore, the rest of the tests were made using only this solution.

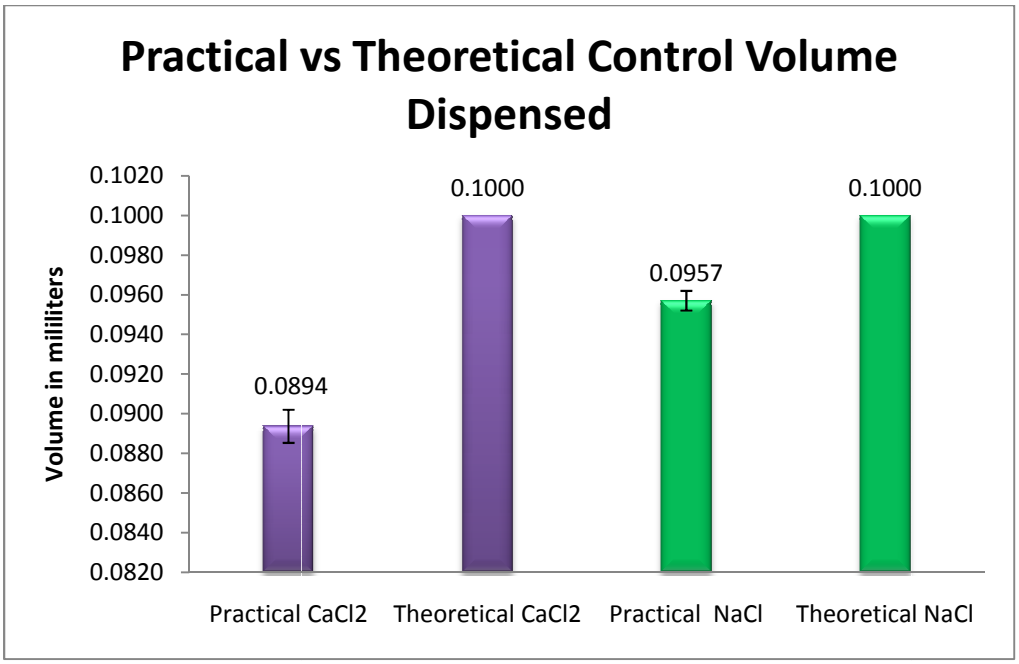

Figure 13. Practical and theoretical volume delivered (n=3). Note that practical volume for NaCl solution is closest to the theoretical volume and have a small standard deviation.

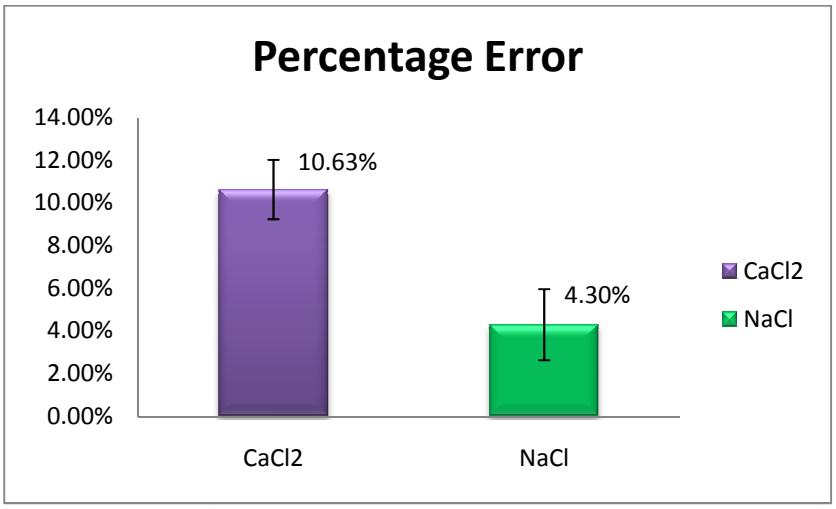

Figure 14. Percentage error of $CaCl_2$ and NaCl solutions. From this it was decided to plot the volume-area curve using NaCl solution.

Three different patterns undergoing different diameter size dots were printed onto a glass slides and volume was determine by prior method described. The patterns, illustrated in Figure 4, were of 0.2, 0.08, and 0.01 inches according to the software

which ultimately corresponded to an actual printed area of 26.86, 6.54, and 0.92 mm^2, respectively. Volume resulted in 250, 50, and 7 nanoliters in the same order. Data was plotted adding the natural value of 0,0 (area, volume) and different trend lines were draw. A positive correlation was found within the two set of data (correlation = 0.99915), which confirms the direct relation between the area of the dot and the volume dispensed. A polynomial trend line was found to be the best line fit (regression line) (R-squared = 1.0), which resulted in a polynomial equation that describes the behavior of the volume with respect to the printed area. The resulted equation was:

$$volume\ (y) = 3.0789\ area(x)^3 - 20.746\ area(x)^2 + 7643.5\ area(x) + 3\ x\ 10^{-7}$$

[Equation 2]

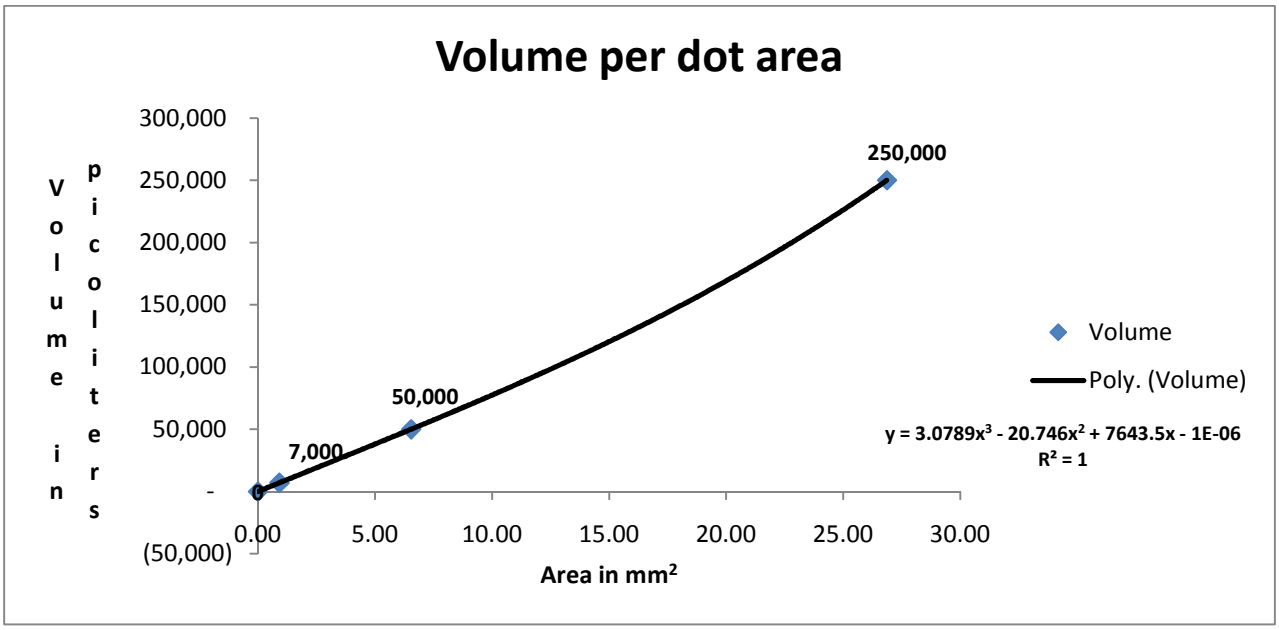

Figure 15. Area vs. volume per dot scatter diagram. Polynomial fit trend line (black). The equation describing the trend line allows us to predict volume knowing the printed area.

Upcoming volumes described within this research project were determinate using [Equation 2].

5.6. Smallest Reliable Printing Dot Font Size

It is desirable that every assay the amount of bacteria cells delivered in each dot remain as constant as possible. Different font sizes dots were printed and the numbers of cell were count through method described in section 4.13. It was found that the smallest reliable dot printed was under font size number 3, which delivered an average number of 22.33 *E. coli* cells per dot with a standard deviation variance of ± 34.28%. Dots sized 1 and 2 were reported under a bigger ± standard deviation than their respective means (1.4, 3.33, respectively).

Using [Equation 2] volume was determined to be 180 picoliters per dot for the font size 3. This represent the main advantage of this project research 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.

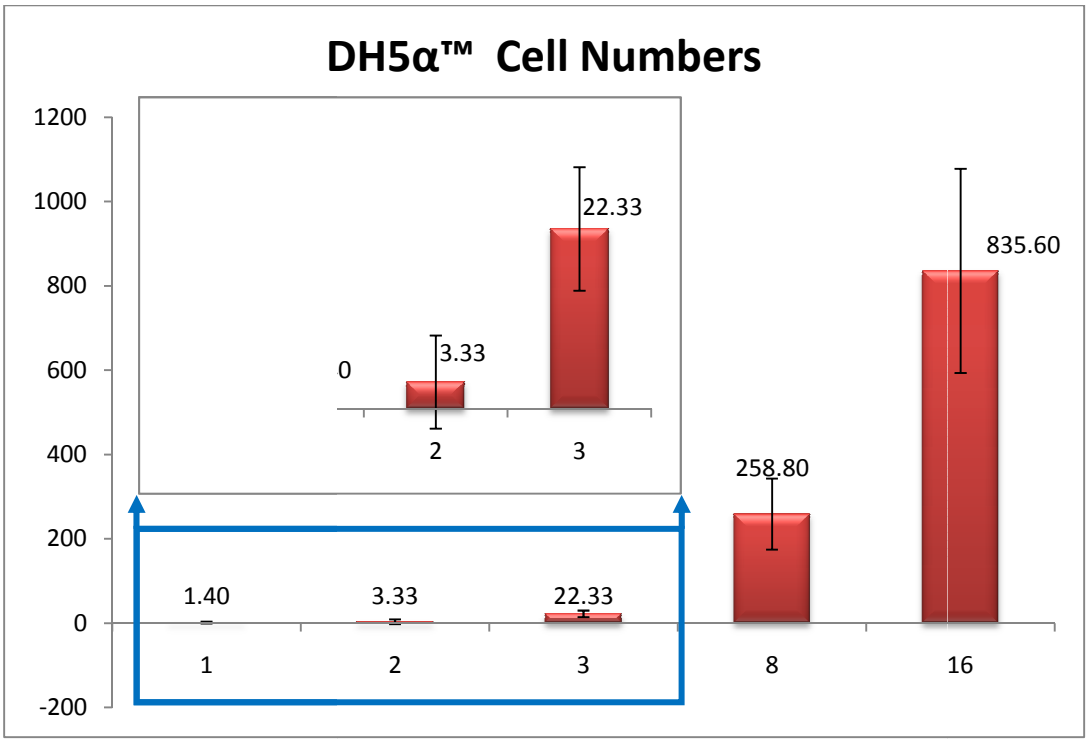

Figure 16. Number of DH5α™ cell at different font sizes (n=3)

5.7. Drug Screening (*E. coli* vs. Antibiotics)

Once proven that the dot font size 3 was the smallest reliable dot to print, it was ejected onto a glass slide the proposed methodology (described in section 4.14) for evaluation of the *E. coli* behavior under different antibiotics presence. At the mean value of 180 picoliters and 23.74 bacteria cells per dot at a cell concentration of 7.623×10^7, it was found that the three antibiotics restricted the viability of the cell below the 50% of the control sample test with no antibiotic presence. Moreover, to correlates methodologies a control test was done manually dispensing 5 µl of *E. coli* and each antibiotic in a spot, cell counting was done. Results were normalized with the no antibiotic sample to allow us to compare both methods. It can be appreciated in Figure 18 that both method 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

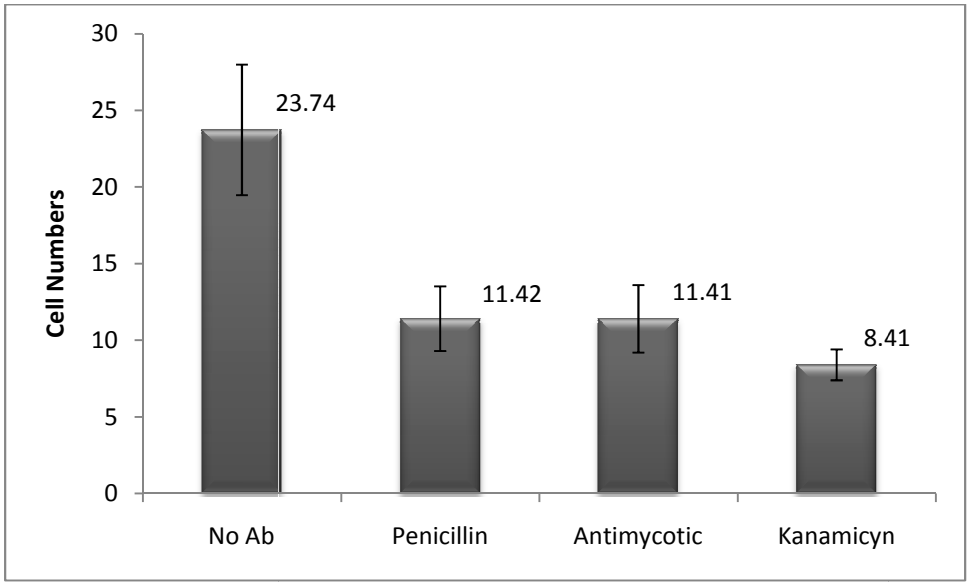

Figure 17. Printed microparticles (n=6) comparing *E. coli* behavior under different antibiotics and under no antibiotics presence

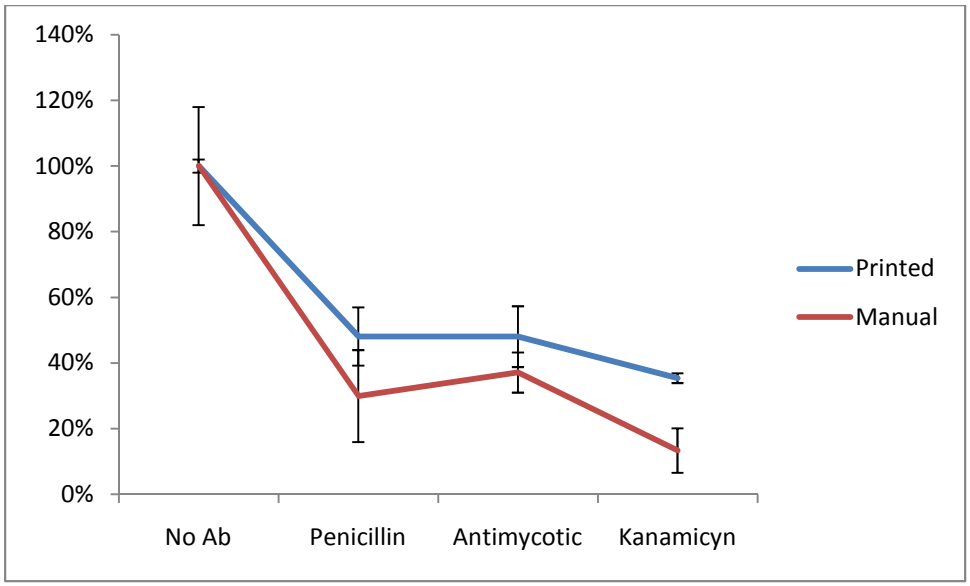

Figure 18. Normalized graph comparing behavior of *E. coli* under different antibiotics presence by printing and manual samples dispensed (n=3)

6. CONCLUSIONS

6.1. 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 high throughput. Inkjet printing technology is a promising tool for future drug screening at low cost. The present deposition method represents a low cost possible solution to stimulate the drug discovering process for developing countries (more info in 6.3).

6.2. Limitations – Further Place to Improvement

The antibiotics used within this research project were utilized under 50% concentration mixed with calcium chloride ($CaCl_2$); however, different antibiotics concentrations can be tested to evaluate the behavior of the bacteria under those parameters.

Work has to be done to design a chamber like base plate that can be adapted within the compact disc tray that provides a more viscous and humid environment for cell deposition. Avoiding the need of entrapping cells through the ejection of layers such as alginate, which due to its particular viscosity it is difficult to printed due to clogging nozzles issue.

Furthermore, an area of investigation that might improve the scientific value of such technology includes the approach to better mimic the body environment during the printing process.

6.3. Challenges and Perspectives (Potential Applications)

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 [36]. 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.

Even though, it has been demonstrated that the inkjet technology is a promising tool for future drug screening at low cost, future evaluation test can be done using different biomaterials; such is the case of the emerging project of screening breast cancer cells with different drugs for potential clinical treatments in collaboration with Texas Tech University Health of Sciences (TTUHS). More targets can be identified for future development of this methodology under the drug screening field.

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CURRICULUM VITA

Born in Ciudad Juárez, Chihuahua, México in a bright Sunday, May 15th, 1983, Jorge Iván was the last of three sons of Benjamin Rodríguez Peña and Marina Dévora Miranda. In the fall of 2005, he obtained his Bachelor of Science under the major of Electrical-Mechanical Engineering with a specialty in Advanced Manufacturing in his Hometown University, Technological Institute of Ciudad Juarez (Instituto Tecnológico de Ciudad Juárez). While pursuing his bachelor's degree, he starts working in March 2004 with Delphi Automotive Systems, a worldwide automotive company, in the area of product engineering; it stayed in the company remained after bachelor's graduation until December 2009, reaching the position of Product Engineer. While full-time employed, in the spring of 2008, he enrolled into the adventure of pursuing the Master of Science degree in Mechanical Engineering at The University of Texas at El Paso. After serving for more than five years for the company he decided to fully devote his self into research in the emerging bioengineering area lead by Dr. Tao Xu at The University of Texas at El Paso. In 2010, he participate as a contest speaker during the 4th Annual Research Colloquium organized by Texas Tech University Health Sciences Center, and was recognized as the best oral presentation within the student category presenting this thesis work.

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