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Modification of the Traditional Inkjet Printer for Printing a Three-Dimensional (3D) Tissue Engineered Human Cell-Matrix System

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Abstract: Bioprinting has grabbed attention in recent years. Bio-medical engineers have introduced Bioprinting as a cheap and affordable method to construct and fabricate tissues using cells and positioning methods along with cell culture techniques to study the behavior of living organisms. The applications such as the use of perfused three-dimensional (3D) human cardiac tissues for toxicological research, drug testing, and screening or personalized medicine. Moreover, it helps to develop in-vitro tissue test systems to explore basic cellular behaviors, disease progression, and treatment options. This study aims to modify a standard thermal inkjet printer to accomplish Bioprinting. Cell viability was determined. We made two types of printing, 3D, and 2D. The 3D printing is much closer and mimics the in vivo condition and allows more cells to be printed per unit space.

Keywords: Bioprinting, fabricated tissues, clogging nozzles, 3D printing.

1. Introduction

The fields of tissue engineering and regenerative medicine seek to construct biological substitutes to restore and maintain normal function in diseased and injured tissues. Biological structures are highly organized and heterogeneous, composed of multiple different cell types and extracellular matrix (ECM) components in precise locations. Even simple structures of the engineered Tissue composed of only one cell type, such as articular cartilage, contain highly organized ECM arranged in ways that facilitate the biological functions of that structure. Therefore, biological substitutes must mimic the normal structure as much as possible to provide the same functionality as the native Tissue. Engineering these complex tissue components requires new methods of combining cells, growth factors, and biomaterials in ways that facilitate Tissue and organ morphogenesis. In addition, tissue engineering can create in vitro test systems to evaluate pathologies for various conditions and develop novel pharmaceuticals [1].

Bioprinting or direct cell printing is an extension of tissue engineering, as it intends to create completely new organs. It uses bio-additive manufacturing technologies, including laser-based writing, inkjet-based printing, and extrusion-based deposition. Bioprinting offers great precision in cell space rather than providing scaffold support alone. Although still in its infancy, this technology appears to be more promising for advancing tissue engineering toward organ fabrication, ultimately mitigating organ shortage and saving lives.

Cui and his coworkers [1] applied inkjet printing technology to repair human articular cartilage, showing its promising potential for high-efficiency direct tissue regeneration. One of the advantages of inkjet printing is that surfaces, where the cells are printed and patterned do not have to be flat, which favors cell printing which is useful in future cases where the surface is human skin. To maintain a clean printing environment, the ink cartridge should be cleaned before and after use. This also helps avoid the crystallization of salts and other biological material in the cartridge that could cause blockages [2].

The technical printing issues are mostly related to the nozzles, resolution, and accuracy of the printheads. The main sources of resolution error are the printhead itself, the distance from the printhead to the substrate where the drop will be placed (drop distance), and mechanical vibrations [3]. Such printheads are not designed to deliver material to an exact point repeatedly. This error depends on the type of printhead used and cannot be changed — it must be included in the design of the biological structure. The error associated with drop distance is similar to the printhead error. Finally, when creating structures on a micrometer scale, tiny vibrations originating from the movement of the system or even the air conditioning in the room can significantly affect the final resolution of the printing system. These sources of error can be somewhat overcome by holding the printhead on a stationary platform and using a moveable stage beneath the printhead to create the biological structure [4].

Our objectives are to achieve low-cost Bioprinting using an inkjet printer by applying some modifications to the printer and cartridge. The printer prints tissues and tests, including angiogenesis imaging, wound healing test, and healthy/cancer tissues. The relations and characteristics which help in any future research, such as clogging inside the nozzle tip with high viscosity at the same time and misplacement due to the surface tension, will be outlined.

2. Materials and Methods

The materials used in this work include Hewlett Packard, HP ™ DeskJet 656c, Hp cartridge 20 (black), Fibrinogen & thrombin, and Human skin fibroblast (HSF). In the initial printer preparation step, the printer's plastic case should be removed, and the printer's interior must be cleaned. The paper feed detector was replaced with a wire to trick the system. The cartridge is cleaned and sterilized by removing the upper cap and then sonicating for 15 mins, finally spraying ethanol inside and outside. Further in the next step, prepare the cell suspension using HSF and PBS with a concentration of 1M cells in 1 mL, for 3D printing 2:1 fibrin gel was used. Cells were stained with g-actin to be imaged later under the fluorescence microscope. Finally, the cell suspension is pipetted into the cartridge, a software "Microsoft word" with a line pattern to print multi-levels on the same spot. The cells were imaged under fluoresce microscope to do the observation.

This work needs to convert the HP DeskJet 656c, where the implemented technique should work with many commercial inkjet printers. However, older printers tend to work better as they use ink cartridges with larger diameter nozzles, which do not clog as easily. In addition, older printers tend to use mechanical paper feed sensors that are easier to bypass. Printers with optical sensors can be tricked but using a small strip of paper on the far edge of the printer during each cycle, but it is a bit more difficult than the original mechanical system. The commercially available printers that work the best have low resolution (DPI). Higher-resolution printers tend to clog more easily. The resolution of the HP Deskjet 500 is 300 DPI. Many commercially available printers (HP Deskjet series and others)

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have a resolution of 600 DPI. This type of printer can be used with only a small increase in nozzle clogging issues that can be alleviated using careful cleaning [2]. The step to convert the HP DeskJet 656c includes removing the printer's top plastic case. Afterward, unscrew the button/display light panel from the top of the printer, clean the inside, and locate the cables supplying power to the paper feed mechanisms. Then unplug them from the motherboard, and bypass the detection mechanism by affixing a string or wire loop to serve as a manual pull handle. The HP DeskJet 656c, the paper detection mechanism, is a gray plastic lever found above and behind the printing/paper feed mechanism. A stage in front of the paper feed mechanism should be created to bring the desired printing slides to a level just below the cartridge print head. In this work, the foam shipping holder for 15 mL centrifuge tubes was used with several microscope slides taped in place in the printing region to bring the final level of the slides to the desired height. The printer can be placed inside a standard biohazard cabinet or tabletop laminar flow hood to work in the aseptic environment.

Further, to convert Stock HP Ink Cartridges (HP 20 Black ink cartridge), it is initially required to remove the cartridge from its packaging and stabilize the body of the cartridge (black portion) either with a clamp or vise to leave a green top clear of any obstacle. After that, it is required to grasp the green top of the cartridge and twist it back and forth several times until it breaks free using pliers or an adjustable wrench. Finally, flush the reservoirs with water after removing the plastic protective tape covering the printer contacts and print head and ensuring the reservoir is empty of any remaining.

Ink Cartridge cleaning is the process that needs to be conducted after converting the HP DeskJet 656c and stock HP Ink Cartridges (HP 20). This step requires fully submerging the cartridge in a beaker full of de-ionized water and sonicating it for 15 minutes before and after printing. After sonication, ensure there is no excess water on the cartridge and spray 70% ethanol into the cartridge to create a more aseptic environment. It must be ensured that the ethanol has dried before adding the bio-ink printing solution.

In the bio-ink preparation process, the cells need to be cultured until it ready to passage. For 3T3 fibroblasts, the cells seed on T-75 flasks at approximately 1.3x104 cells/cm2 in Dulbecco's Modified Eagles Medium (DMEM) with 10% Fetal Bovine Serum (FBS). Culture cells for two days in an incubator at 37 °C and 5% CO2. in this process, it also needs to make the fluorescent g-actin stock solution at a concentration of 50 μg/ml in Phosphate Buffer Solution (PBS).

The next step is passage cells. For 3T3 fibroblasts, remove media from a flask and rinse twice with PBS. Cover cells with 5 ml 0.25% Trypsin EDTA and incubate for 5 mins. After that, add 5 ml of fresh medium and pipette the cell suspension into a 15 ml conical centrifuge tube and centrifuge at 1000 rpm for 5 min. Aspirate spent medium. Resuspend the cells in PBS with the fluorescent g-actin stock solution to create bio-ink. Bio-ink should have a final concentration of g-actin of 10 μg/ml and a cell concentration of 1x105 cell/ml. This concentration has been optimized to limit the number of cells per drop and clogging of the printer head.8, noted that 250 μl of bio-ink prints three coverslips of a line pattern.

For applying the Bioprinting, the printer should be warmed up first, and prepare the printing surface on the center of the stage. After a printing pattern file is created with any drawing software, the prepared cartridge with desired cell suspension can be loaded—pipette suspension into the small circular well at the bottom of the cartridge compartment. Use approximately 100-120 μl of solution. The printed drop size is around 130 pico-liters. Print the file with the HP Deskjet 656c Printer. For best results, print smaller patterns multiple times (5) by changing the number of copies desired in the word processing program, as in figure 1.

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(**a**) (**b**)

Figure 1. Modification process of (a) modified regular printer into Bioprinting; (b) Testing of the 3D Bioprinting

3. Results

As a result of this work, it is indicated that the Bioprinting went as expected, with no unusual events except the nozzles clogging and printing errors. The printing was conducted in 2D and 3D. The main difference between 2D and 3D is using the fibrin gel in bio-ink. Also, as maintained earlier, cells were stained to be imaged under the fluorescence microscope. It may discuss the five images for each type, count the cell viability, and take the average of cells per unit space for each type.

As part of this experiment, a comparison between the images for each type was to determine the average number of cells in each printing and the printer accuracy in cell printing. The formation of fibrin gel and clogged nozzles is a limiting factor leading to minimizing the printing time and, thus, the shape, complexity, and printing area. Lastly, to determine the homogeneity of printing, five samples were taken randomly to indicate the difference and illustrate the preciseness of the printing.

In 2D printing, after preparing the cell suspension and pipetting into the cartridge with a concentration of 1×10^6 in 1 mL of PBS' phosphate buffered saline', here in the next five samples, the number of cells was counted, the green fluorescence objects are the cells after being labeled using g-actin. While counting, we ignored the small object as it is not identifiable as cells. Also, the number of cells in some samples is estimated as the high concentration of cells in the same spot.

Figure 2(a) and figure 2(b) both illustrate the same spot where figure 2(b) is 40x zoomed to show a cell which is very clear to the image and shows high contrast with the surroundings. Figures 2(c), 2(d), 2(e), and 2(f) are the rest of the samples, and it is clear that the number of cells is steeply varying for each sample, showing that it is inhomogeneous, the average distance between cells is estimated 300-400 μm. Sample No. 2 curries the lowest homogeneity distribution and cell number, while sample No. 5 has the highest. Sample No. 4 has the best homogenous distribution cells placed well in a block shape. In Table 1 are the samples and statistics of cell distribution in space. The average number of cells is 11 for an area of 4.4×10-6 m2 or about 4.4-millimeter square. The standard deviation was 4.6.

Compared to 2D, 3D printing has more cell number 'density' due to the attachment afforded by fibrin gel '2:1 used'. The cells in 3D show more formal distribution and shorter distances between cells. The same number of cells was used while the cell suspension along with fibrin gel was 1.5 mL Samples No.8 and No.10 have been very similar to 2D outputs, and this is due to the formation of fibrin gel inside the cartridge.

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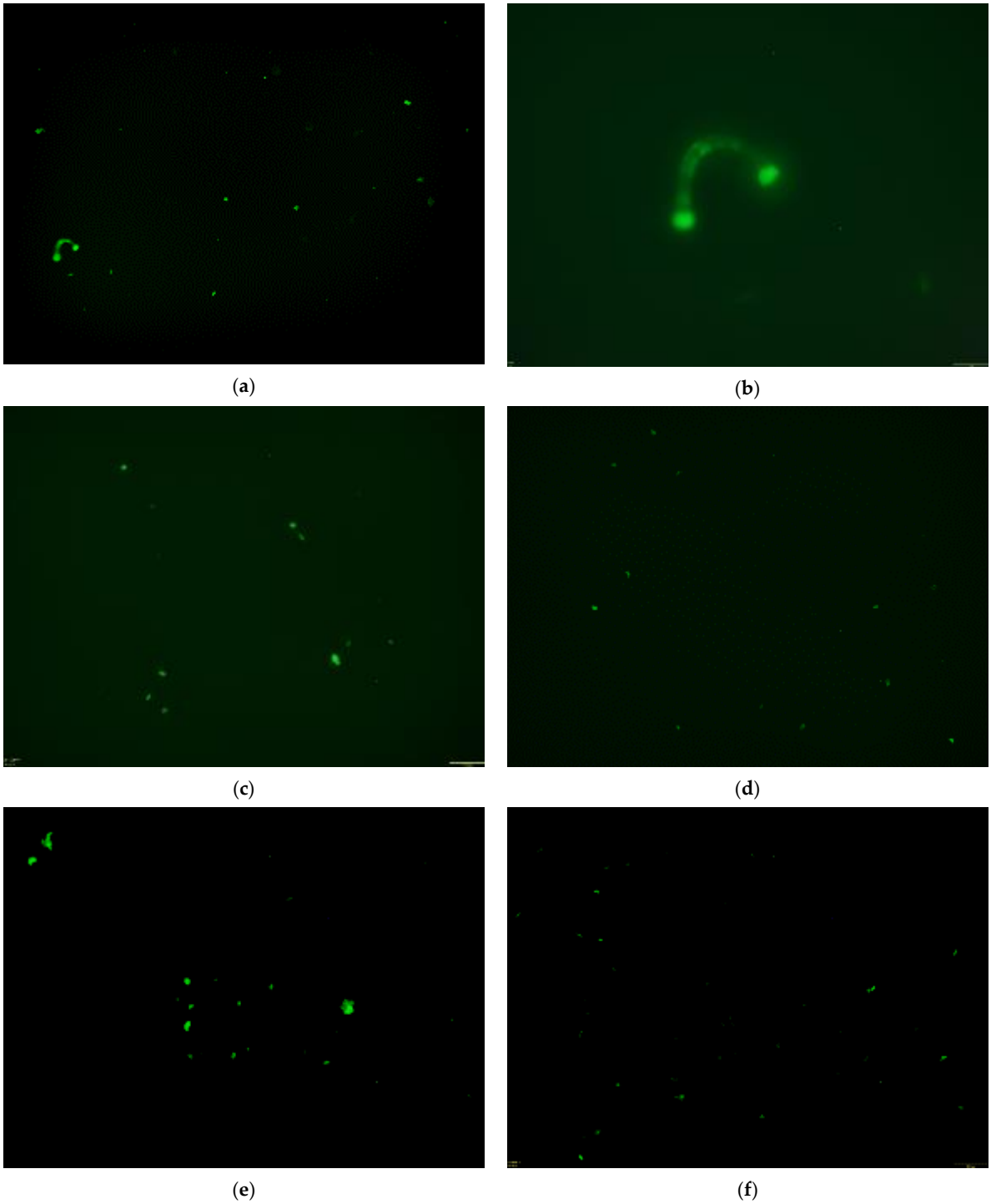
Table 1. 2D samples and their cells number per area of 4.4×10^{-6} mm^2

Sample	Number of cells
Sample 1	9
Sample 2	7
Sample 3	10
Sample 4	11
Sample 5	19



(**a**) (**b**) (**c**) (**d**) (**e**) (**f**)

Figure 2. 2D printing: (a) sample 1; (b) sample 1 (enlarged); (c) sample 2; (d) sample 3; (e) sample 4; (f) sample 5.

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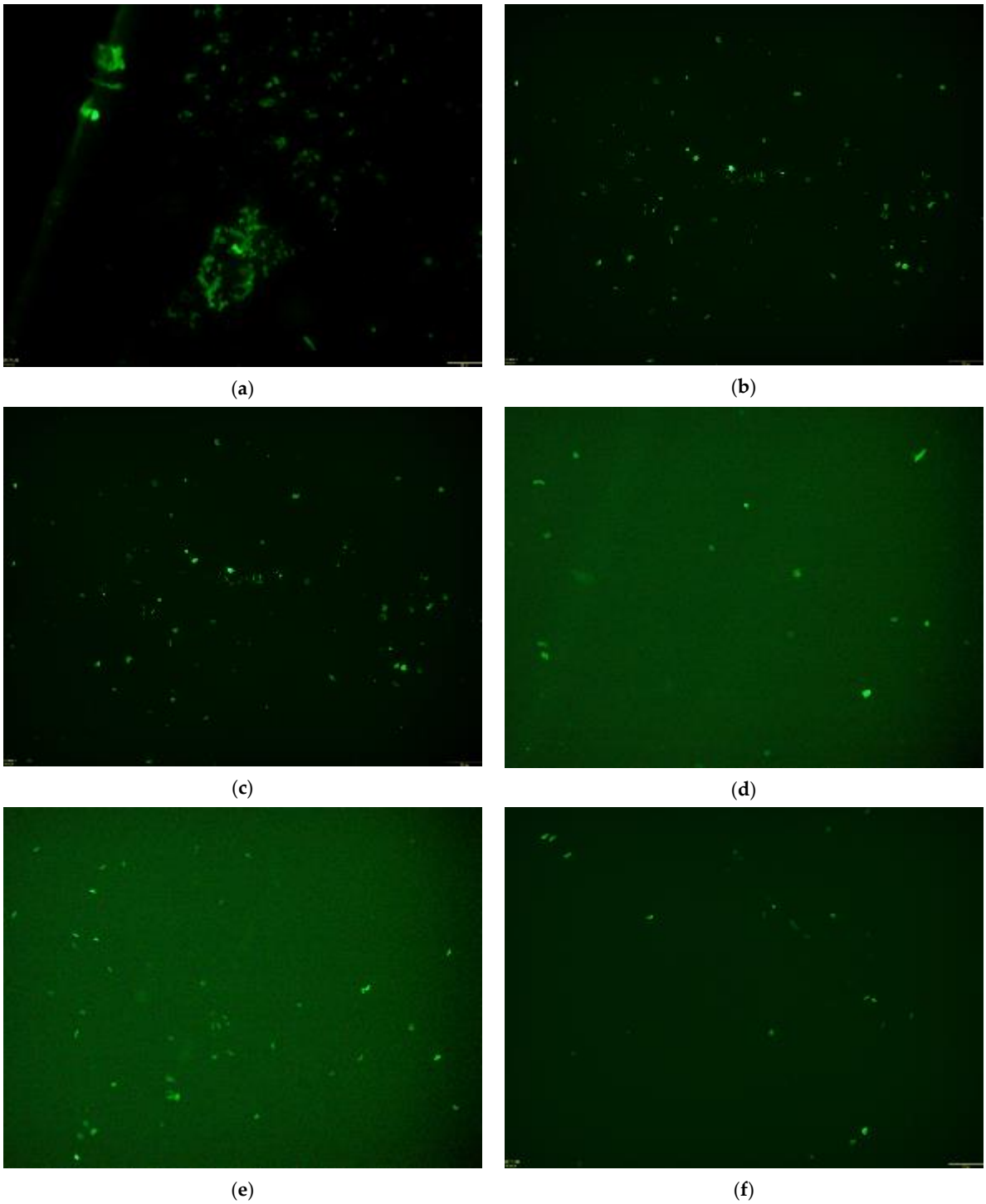
Table 2. 3D samples and their cells number per area of 4.4×10^{-6} mm²

Sample	Number of cells
Sample 6	50 – 70
Sample 7	30 – 40
Sample 8	16 – 20
Sample 9	25 – 30
Sample 10	20 – 25



(**a**) (**b**)

(**c**) (**d**)

(**e**) (**f**)

Figure 3. 3D printing: (a) sample 6; (b) sample 7; (c) sample 7; (d) sample 8; (e) sample 9; (f) sample 10.

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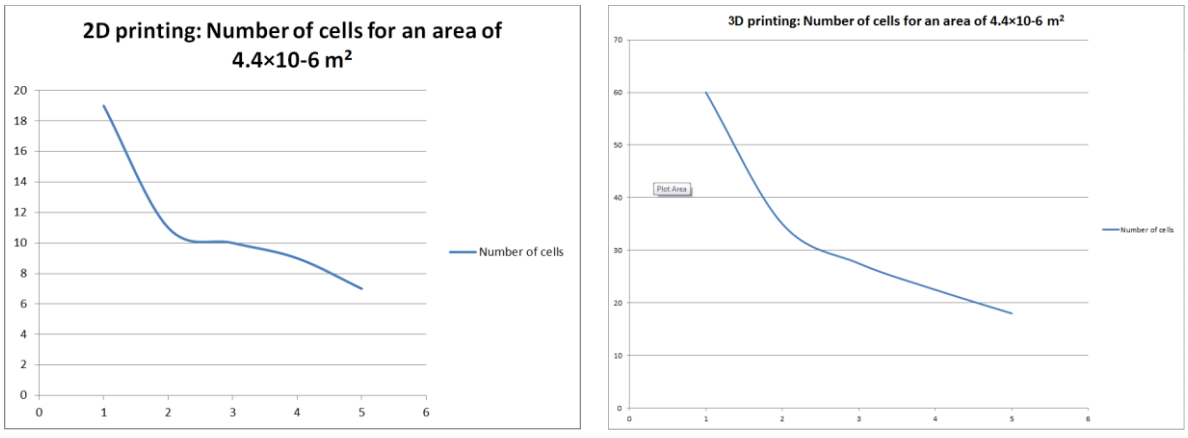
Figure 3(a) shows the highest cell density per unit space with a sharp edging 'lining.' Figures 3(b) and 3(c) are for the same specimen with a different threshold level to compare the difference in different threshold levels. However, no difference was noted, and cell absence was detected. Table 2 determines the number of cells for the five samples, the average number of cells 28-37 for an area of 4.4×10-6 m2 or said as 4.4 millimeters squared. The standard deviation was 16.5. Compared to the standard deviation in 3D with 2D, the output dispersion is higher in 3D, justified by the formation of fibrin gel formation and thus declines the output of ink-jetted cells. Sample 6 curries the highest cell number and the most formed shape. Samples 8 and 10 had the lowest cell number. As a compression, no significant indication of stability in printing quality, and the number of cells keeps declining gradually.

4. Discussion

Newer inkjet printers are not suited for cell bioprinting because the orifice size on the new printheads is significantly less than the size of a cell. Older printheads released in the late 1990s are theoretically capable of single-cell precision depending on the size of the output orifice [5]. This is the reason for selecting the HP Deskjet 656c. From the obtained result, we find that the results vary as the printing goes on. Here, we spot the light on the possible sources of error. First, about the printhead resolution, the main sources of resolution error are the printhead itself, the distance from the printhead to the substrate where the drop will be placed (drop distance), and mechanical vibrations.

Printheads are not designed to deliver material repeatedly to an exact point. This error depends on the type of printhead used and cannot be changed. As a result, it must be considered while designing the biological structure. Printheads are typically held several millimeters above their intended target. Any errors in the direction of the ink drop will be magnified as the distance from the printhead to the target increases. Finally, when creating structures on a micrometer scale, tiny vibrations originating from the movement of the system or even the air conditioning in the room can significantly affect the final resolution of the printing system. These sources of error can be somewhat overcome by holding the printhead on a stationary platform and using a moveable stage beneath the printhead to create the biological structure [5].

Inkjet cartridges suffer from low throughput. This is mainly due to the deposition of salts in the microfluidic channels during the printing process. It often occurs when water evaporation from the bio-ink drops leaves behind solid salts that block the channel orifice. Once the channel is clogged, it is virtually impossible to restore full functionality to that channel, presented in Fig. 3.



(**a**) (**b**)

Figure 3. Result from Comparison (a) the number of printed cells goes gradually down, and a gradient is about to stabilize; (b) the number of printed cells went dramatically down.

Furthermore, cellular debris and other contaminants can also clog the microfluidic chambers. As a result, inkjet cartridges typically can only print 400,000 cells per cartridge before failure [6]. When comparing the 2D printing, cells during printing on a glass slide will float because of weak attachment. Thus, the resolution is getting lost, while in 3D printing, the cells show a more shaped and edged pattern due to the existence of fibrin gel. The weakness of fibrin gel is clogging the nozzles quickly. To overcome the problem, we suggest using two cartridges or a multi-champers cartridge and preparing the cell suspension separately.

5. Conclusions

The difficulty faced through Bioprinting mostly centered on homogeneity and nuzzled clogging. Bioprinting using a modified printer can be successful in research if it can be translated as a helpful device for researchers and enterprises. The use of human skin fibroblast cells can be replaced with another type of cells with a smaller orifice to extend the printing period. The findings were challenging to be encouraging in clinical testing even though it affords cheap and affordable methods with low spatial resolution. The field is still young and needs more to be performed in cell viability and proliferation. In recommendation, it can be used more than a single type of cell for further studies. For the 3D Bioprinting, more working in fibrin gel concentration and fabrication time. As our method is based on thermal inkjet delivery, further studies for the different delivery methods and testing the viability of cells and their proliferation. It is important to develop it for in-situ printing as the recent searches go to repairing the external organs such as skin. More work must be done to overcome the clogging nozzles and extend the printing axis to have more complex and larger structures.

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