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Design and Implementation of a Two-Dimensional Inkjet Bioprinter

Matthew E. Pepper, *Member*, IEEE, Cheryl A. Parzel, Timothy Burg, *Member*, IEEE, Thomas Boland, Karen JL Burg, *Member,* IEEE, and Richard E. Groff, *Member*, IEEE

Abstract—**Tissue engineering has the potential to improve the current methods for replacing organs and tissues and for investigating cellular process within the scope of a tissue test system. Bioprinting technology can aid in the difficult task of arranging live mammalian cells and biomaterials in viable structures for tissue engineering purposes. This paper describes a system, based on HP26 series print cartridge technology, capable of precisely depositing multiple cell types in precise patterns. The paper discusses the research, design, and implementation of the printing system, which permits control of droplet firing parameters, including firing energy, speed, and spacing. The results demonstrate the system's fine patterning ability of viable cells, including two-dimensional patterned co-cultures of two cell types. The system has been specifically designed with the flexibility to be extended to print more than two cell types and/or materials simultaneously and to layer printed patterns to form three-dimensional constructs. With these features, the printing system will serve as the foundation for a biofabrication system capable of three-dimensional cell co-cultures, i.e. tissue test systems.**

I. Introduction

For almost two decades, tissue engineering has been defined as "the application of engineering methodology to the branches of life science with the goal of developing biological substitutes that restore, maintain, or improve tissue function" [1]. The goal of tissue engineering is to reduce the need to implant foreign, permanent materials that may cause chronic inflammatory or immunologic responses. The domain of tissue engineering has recently been expanded to include the development of *in vitro* tissue test systems, with which one can explore basic cellular behaviors, disease progression, and treatment options [2]. A common need in almost all tissue engineering applications is the ability to position, in a reasonable timeframe, large quantities of cells and biomaterials within a three-dimensional (3D) volume.

Many approaches have been suggested to build cellular systems. Conventional cell seeding methods use static, e.g. in a well-plate, or dynamic, e.g. in a stir flask or bioreactor, loading of a volume of cells onto a biomaterial scaffold. Generally, the goal is to achieve uniform cell deposition on a surface or within an open-cell volume [3]. This approach lacks spatial control of the seeded cells and results in random placement of cells on the construct [4]. Better control of cell locations is likely to benefit the construct, with designed placement of every cell being the ultimate limit. An approach that can address controlled cell and/or biomaterial placement is generally referred to as free-form fabrication or micro fabrication. Many of the approaches to rapid-prototyping of mechanical components are analogous to the free-form fabrication of biological tissues. Our goal is to develop a means to precisely deposit small numbers of cells or small volumes of biomaterial.

Drop-on-demand printers have been investigated for cell deposition over the past decade. The technology used in desktop printing systems has been exploited by modifying the ink cartridges to replace the existing ink with a cell solution, "bio-ink". To date, bioprinting work has been largely accomplished using proprietary drivers and embedded software for either Hewlett-Packard [2, 5, 6, 7, 8] or Canon inkjet printers [9, 10]. The main disadvantage of this approach is the limited customizability of the printed patterns and droplet firing parameters. These limits make it difficult to collect data pertinent to the printing process, such as statistics of how many cells are ejected per drop, how much energy is being delivered to each drop and how droplet firing parameters affect the bio-ink.

There has been some effort to access and optimize printing parameters to match the specifics of cell printing; most notably, Boland and coworkers describe modification of the software driver for an HP500 series printer that exposes the printing parameters in software [7]. Moreover, the printer's paper feed mechanism has been modified to permit two-dimensional cell printing. This seminal work with the HP500 printer demonstrates the HP26 as a capable technology for placing cells, but use of the printer itself limits the types of substrates on which bio-ink can be printed and the types of experiments that can be run [5]. In pursuit of a core technology upon which a three-dimensional biofabrication system will be constructed, we return to a two-dimensional bioprinter. This system is designed such that the droplet firing parameters may be controlled in order to print a wide variety of materials, print patterns that may be changed on the fly via software, and incorporate additional workstations and tools, such as computer vision and thermal processing, into the system.

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Matthew E. Pepper, Richard E. Groff, and Timothy Burg are affiliated with the Department of Electrical and Computer Engineering and the Institute for Biological Interfaces of Engineering at Clemson University, Clemson, SC 29634-0915 USA (corresponding author Timothy Burg: 303 e-mail: tburg@clemson.edu).

Cheryl A. Parzel, Thomas Boland, and Karen J.L. Burg are affiliated with the Department of Bioengineering and the Institute for Biological Interfaces of Engineering at Clemson University, Clemson, SC 29634 USA (e-mails: parzel@clemson.edu, tboland@clemson.edu,kburg@clemson.edu)

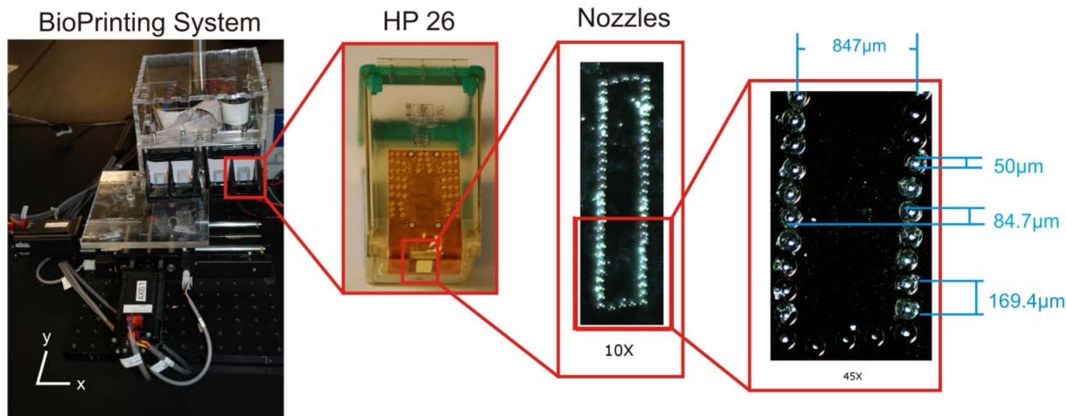

Fig. 1 Our current BioPrinting system with print head and 2D stage, inset shows where the HP26A engages. The HP26A has a flex circuit on the back which attaches to the fifty nozzles located at the bottom of the cartridge. These fifty nozzles are arranged in two columns of twenty-five, with a vertical offset of 84.7μm and horizontal offset of 847μm. Each nozzle has a diameter of 50μm.

This paper presents a custom bioprinting system, designed specifically to be flexible and extensible. This new bioprinting system allows expandable functionality such as printing multiple cell types per sample and integrating custom vision systems. Most importantly, the prototype system will serve as the basis for a 3D printing system currently under development.

II. Bioprinter System Architecture

The bioprinting system consists of three major components: (i) the cell delivery system, including ink jet cartridges and custom drive electronics, (ii) the motion system, consisting of a two-axis commercially available positioning stage, and (iii) control hardware and software.

A. Cell Delivery System

The HP26 inkjet cartridge was selected as the means of cell delivery for the bioprinting system because of its proven ability to print cells. A variety of cell types have been printed with this cartridge and shown to be viable in culture after printing [2, 5, 6, 7, 8, 11]. The HP26A cartridge is easy to disassemble and clean, is capable of a high droplet firing rate, has appropriately sized nozzles for the bioprinting application, and is widely available. In the original HP520C inkjet printer, this cartridge delivers 180,000 drops per second or 3,600 drops per nozzle per second. Thus if one cell was deposited per drop, a clinically relevant sample of ten million cells could be deposited in less than one minute.

The HP26 cartridge consists of an ink storage chamber, a print head containing the nozzles, and an electrical interconnect on the back of the cartridge (Fig 1) [12]. The nozzles in the print head are 50μm in diameter and are arranged in two vertical columns of 25 nozzles each. Within each column, the nozzles are separated vertically by 169.4μm. The cartridge's native vertical printing resolution, 300dpi, is defined by the 84.7μm spacing between a nozzle and its closest vertical neighbor in the other column. Later generations of HP inkjet cartridges provide higher vertical resolution by using smaller diameter nozzles packed closer together. Unfortunately smaller nozzles cause damage to cells during printing, presumably due to high shear forces during drop ejection.

The HP26 is a thermal inkjet cartridge. Each nozzle contains a 30 Ω resistor, which is heated by application of a constant voltage pulse of specified duration. The rise in temperature causes a vapor bubble to form, expelling a drop of liquid from the nozzle. The electrical interconnect on the HP26 cartridge permits direct electrical connection to each of the 50 nozzle resistors. This simple interface permitted the design of custom drive electronics to address and fire individual nozzles as directed by a real-time control system. The energy delivered to the drop (which is proportional to the pulse duration) and the timing of the drops may be directly controlled through software. Each drive electronics board supports one cartridge, but multiple boards and cartridges may be used simultaneously.

The electrical interconnect on the HP26 cartridge is arranged in a pattern defined by the manufacturer. The cartridge carriage assembly for 500 series printers, still available through distribution channels, provides a mechanical fixture so that the cartridge may be repeatably snapped into position as well as a flex cable that interfaces with the cartridge and terminates in two industry-standard 30-pin connectors. The carriage assembly is incorporated into the custom bioprinting system in order to ensure reliable electrical and mechanical connections to the cartridge. The carriage assembly supports two cartridges, and the current printing system (Fig 1) has two of these carriages, for a maximum of four cartridges.

B. Motion System

The cell delivery system is held in a fixed position above the sample holder, which moves in the horizontal plane. The moving sample holder simplifies the electrical connections to the stationary cell delivery mechanism and eases the extension of the bioprinting system into a mini-factory in which the sample from one workstation to another. The sample holder accepts 3" x 1" slides.

The sample holder is attached to the platform of the motion system, currently a two axis stage from Anaheim Automation. Each axis consists of a lead screw with 6" travel and 1.5875 mm/rev thread pitch (LS100-6) attached to a DC stepper motor (23MD206D), controlled by a motor controller (PCL601). The motor controller is capable of 1/8 microstepping, which corresponds to individual linear steps of 0.992μm. The motor controller communicates over a serial connection. The motor controller provides a position-dependent output trigger signal that is used to synchronize the stage with the cell delivery system. The present stage has a maximum speed for each axis of approximately 8 mm/s. While this speed has proven sufficient for the present prototype, the system software and hardware have been designed so that the positioning system is modular and can be replaced with a faster system at a later time.

C. Control Hardware and Software

The cell delivery and motion systems are coordinated by a "host" PC running MATLAB 7/Simulink (Mathworks Inc., Natick, MA). The host PC interacts with the "target" PC, a real-time control system implemented using xPC Target (Mathworks Inc., Natick, MA) and a Quanser Q8 Hardware-In-Loop card (Quanser, Ontario, Canada). The real-time system and drive electronics are capable of sampling rates well over 20 kHz, but in the experiments reported in the following section, the system operates at 1 kHz, corresponding to a maximum of 3846 drops per second.

The host PC acts as the user interface and issues commands to the motor controller and to the target PC, while the target PC is responsible for low-level activities such as interacting with the drive electronics to fire the appropriate nozzles. Both the width of the nozzle firing pulse and the time between firing pulses is software controllable by the target PC.

The bioprinting system operates in two basic modes. In the first mode, stage motion and cell deposition are uncoupled and are directly controlled by the user, either through the MATLAB command line or automated scripts. This mode is useful for software and hardware debugging purposes to test the integrity of cartridges, characterize the printability of materials, and optimize print parameters.

In the second mode, the host PC coordinates stage motion and cell deposition in order to generate a pattern, specified as a bitmap image. The software accepts either a binary image, specifying the presence or absence of a drop at each location, or a 16 level gray-scale image, which deposits the specified number of drops at each location. The host PC divides the image into 50-pixel-high swaths, with each swath printed in one pass of the stage. The host PC transfers the swath data to the target PC and commands the motor controller to start moving the x-axis at a constant velocity over the sample area. The target PC translates the swath image data into the appropriate sequence of fired nozzles, taking into account the offset between the columns of nozzles. A position trigger from the motor controller indicates to the target PC that the stage has reached the start of the print pattern and that nozzle firing should begin. The constant velocity of the stage and constant timing between nozzle firing creates precisely positioned patterns. After printing the current swath, the host PC moves the second axis, the y-direction, by 4.235mm, the height of the swath, and then repeats the process in the reverse direction. This cycle continues until the entire image is printed.

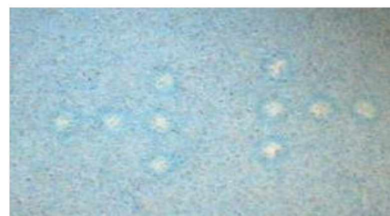

Fig. 2 Calibration patterns printed on CoCl paper. The patterns on the left and right were printed using the first and second cartridges, respectively. The relative offset between cartridges is determined from the relative offset in these patterns.

The position of the cartridge in the carriage has some variability. This does not affect patterns printed using a single cartridge, but when printing patterns involving two or more cartridges, the printing process must account for the relative variation in cartridge positions. Thus, a calibration procedure is required to account for the relative offset between the cartridges. To calibrate the system, a T-shaped pattern of dots (Fig 2) is printed by each cartridge. A rough calibration is used so that the T's are in the vicinity of one another but not overlapping. A microscope with camera is used to capture an image of the patterns, which is then processed using computer vision techniques to give the horizontal and vertical offset between the cartridges. The image to be printed by the second cartridge is shifted in order to compensate for the offset. Lighting conditions on the stage make it difficult to identify clear drops of water-based bioink on a slide. To aid identification, the calibration images are printed onto Cobalt Chloride paper, a moisture indicator. The calibration process is required for any additional cartridges and any time a cartridge is replaced in the carriage.

In this paper, the experiments involving multiple cartridges were created by printing sequentially from one cartridge and then another. The system is also capable of printing with two cartridges simultaneously.

D. Benefits of Architecture

The core benefits of this system are that it can be customized to meet specific project requirements and extended to add additional workstations and features in the future. The ability to carefully control and monitor the performance of individual nozzles led us to better understand nozzle clogging, a recurring problem in previous bioprinting experiments. These experiments led to the development of a protocol for preventing clogs using EDTA [13].

The ability to tune pulse width and time between firing has allowed us to print a wider variety of materials than previously possible. The ability to print more than two

bioinks simultaneously will be necessary to create three-dimensional cell co-cultures, and the present system is configured to make the extension easily. Moreover, the choice of moving the sample rather than the cell-deposition tool permits multiple workstations to be incorporated into the biofabrication system.

III. Demonstration of the 2-D Bioprinting System

A. Single Cell Printing

1) Cell Culture

D1 murine mesenchymal stem cells (American Type Culture Collection (ATCC), Manassas, VA) were cultured according to the distributors suggested protocol. Briefly, cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 4mM L-glutamine, 1.5g/L sodium bicarbonate, and 4.5g/L glucose (ATCC), and every 500mL was supplemented with 50mL fetal bovine serum (FBS), 5mL antibiotic/antimycotic, and 1mL fungizone. The culture medium was replaced every 48-72 hours as required, and cells were stored in an incubator at 37ºC and 5% CO_2. Cells from a non-metastatic murine mammary cancer cell line, 4T07, were maintained in the culture conditions described above for D1 cells.

To prepare cell-based bio-inks for printing, D1 and 4T07 cells were suspended in serum-free DMEM (SF-DMEM) at a density two times the desired final concentration. All cell suspensions were filtered using a 40μm sterile cell strainer. Just prior to printing, 75μL of the cell suspension was combined with 75μL of HBSS containing 1.06mM EDTA, and was subsequently deposited into the HP26 cartridge well [13]. Thus, the resulting 150μL of bio-ink consisted of D1 or 4T07 cells suspended in 50% SF-DMEM and 50% HBSS, with a final EDTA concentration of 0.53mM.

2) Preparation of Collagen Substrates

Tissue culture polystyrene microscope slides were coated with collagen using a modified method and aseptic techniques [14]. These substrates were used as surfaces for all cell patterning studies. A 2.0mg/mL collagen solution was prepared by combining 1.5mL collagen stock solution (3.0mg/mL - PureCol™) with 167μL 10x Dulbecco's phosphate buffered saline (DPBS), 225μL fetal bovine serum (FBS), and 358μL DMEM; a small volume (approximately 20μL) of 1N NaOH was added to neutralize the solution. The solution was pipetted into the center of a silicone ring attached to the slide (1/2" inner diameter) at 200μL per slide, and collagen gels were polymerized in an incubator at 37ºC and 5% CO_2 for at least 4 hours. Gel coatings were rinsed in sterile distilled water until clear and then allowed to dry in a laminar flow hood. Following drying, the collagen coatings were soaked overnight in a 1:1 solution of DMEM to FBS. Prior to printing, excess media was aspirated, and collagen coatings were allowed to partially dry in a laminar flow hood for 30 seconds.

3) Single Cell Patterning: Monoculture

D1 cells were suspended in SF-DMEM at an initial concentration of 1.5×10^7 cells/mL and subsequently combined with HBSS containing EDTA, as described above. The final bio-ink solution was comprised of 50% SF-DMEM and 50% HBSS, containing 7.7×10^6 cells/mL and 0.53mM EDTA. A volume of 150μL of the D1 cell suspension was pipetted into the HP26 cartridge well, which was sterilized using 70% ethanol. The patterns shown in Figure 3 and 4 were created using GNU Image Manipulation Program (GIMP) version 2.4 and then each pattern was printed onto a separate collagen coating. Following printing, D1 cells were allowed to attach in an incubator for 25 minutes, after which they were covered in 10% serum-inclusive DMEM. An image of each cell pattern was captured at time points 0, 24, 96, and 120 hours to show stages of cell attachment and spreading on the collagen substrates (Fig 3).

B. Dual Cell Printing

1) Fluorescent Labeling

In order to differentiate between cell types in a printed pattern, D1 and 4T07 cells were labeled prior to printing using green (Ex. 450nm, Em. 517nm) and red (Ex. 550nm, Em. 602nm) CellTracker™ probes, respectively. The CellTracker™ green stock solution was prepared by adding 10.76μL dimethyl sulfoxide (DMSO) to the lyophilized product diluted in 4.3mL of SF-DMEM. The CellTracker™ red working solution was prepared by adding 7.29μL DMSO to the lyophilized product and then subsequently diluting with 2.9mL SF-DMEM. Cells grown to confluence in a T-75 tissue culture flask were washed with 1x DPBS and incubated for 45 minutes in their respective fluorescent tag solutions.

2) Dual Cell Patterning: Co-Culture

D1 and 4T07 cells were suspended in SF-DMEM at an initial concentration of 1.5×10^7 cells/mL and subsequently combined with HBSS containing EDTA, as described above. The final bio-ink solutions were comprised of 50% SF-DMEM and 50% HBSS, containing 7.7×10^6 cells/mL and 0.53mM EDTA. To print cells in co-culture, D1 cells were first pipetted into an HP26 cartridge well, after which a designated portion of the pattern was printed onto a collagen coating. Next, a different HP26 cartridge was used to print the remainder of the pattern using 4T07 cells. The calibration system described above was used in order to ensure pattern alignment following insertion of the second cartridge. After printing, cells were allowed to attach in an incubator for 25 minutes, after which they were covered in 10% serum-inclusive DMEM. The samples were photographed using a Zeiss Axiovert 40 CFL microscope (Carl Zeiss AG Oberkochen, Germany) equipped with a 50W Xenon lamp. The images were captured using an AxioCam MRC 5, processed with Zeiss AxioVision LE 4.6, and combined using GIMP.

IV. Results and Discussion

The pictures produced by the mono-culture cell experiment (Fig. 3) demonstrate that a high resolution pattern can be successfully printed and maintained in culture for at least 120 hours. One key factor in enabling both the quickness of attachment and viability of cells during the

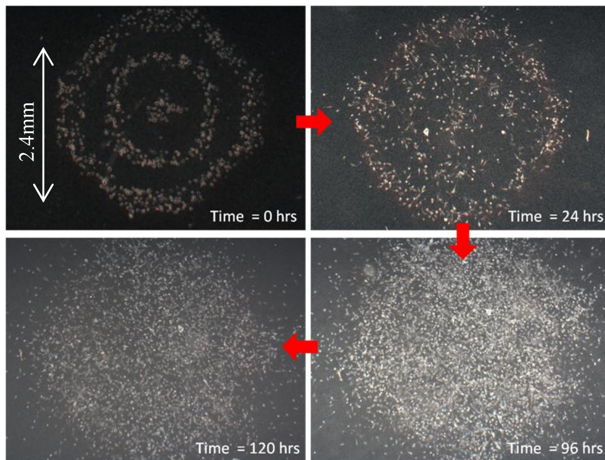

Fig. 3 Time point observation of D1 cells printed in bullseye pattern starting at the upper left and proceeding clockwise. Images of cells were captured from 0-120 hours to show stages of cell attachment, spreading, and proliferation on a collagen substrate.

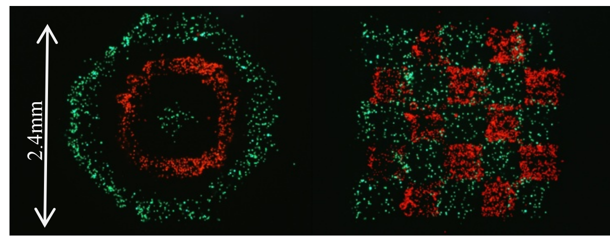

Fig. 4 Co-culture of D1 murine mesenchymal stem cells (green) and 4T07 murine mammary tumor cells (red) printed onto collagen substrates. Images were captured using a 2.5x objective at a zero time point, immediately after printing, and are 2.4mm wide.

initial incubation period was the collagen substrate on which cells were printed. In earlier trials, cells printed directly onto polystyrene slides were found to float away when culture media was applied for long-term incubation, likely because cells were printed in a serum-free medium, which is not conducive to attachment. Even the cells that did attach would not elongate or proliferate over time, as it was found that cells must be printed onto a wet substrate. The addition of a wet collagen substrate gave the cells a readily available attachment point and allowed enough moisture retention to prevent cell stress.

The D1 cells in Fig. 3 appear to enter a proliferative phase following 24 hours in culture. Because of cell proliferation, the pattern is no longer discernible after 96 hours. This point can be viewed as a positive result since cells responded to cues from their neighbors to fill in the gaps within the pattern.

Fig. 4 demonstrates the ability to create complex multiple-cell-type patterns. Of the two, the checker pattern shows that our system has the capacity to fabricate tissue test systems that mirror the non-homogeneity of real tissue. In such tissue test systems, cells will be accurately patterned into a biologically meaningful architecture, after which cellular, biochemical and physical cues will provide a microenvironment to aid in the understanding of cellular behavior.

V. Conclusion

The capabilities of the new bioprinting system will allow further understanding and refinement of the bioprinting process, as has been demonstrated by the work addressing nozzle clogging [13]. The ability to precisely pattern two or more cell types in two dimensions could serve as an enabling technology for investigations regarding cell migration, differentiation, and communication. Such experiments with planar systems will provide useful data for addressing our overarching goal of building a three-dimensional tissue fabrication system. The entire platform is designed to be incorporated as a subsystem of a biofabrication system capable of fabricating three dimensional tissue cultures that can be used to model biological systems.

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