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Development of a valve-based cell printer for the formation of human embryonic stem cell spheroid aggregates

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Abstract

In recent years, the use of a simple inkjet technology for cell printing has triggered tremendous interest and established the field of biofabrication. A key challenge has been the development of printing processes which are both controllable and less harmful, in order to preserve cell and tissue viability and functions. Here, we report on the development of a valve-based cell printer that has been validated to print highly viable cells in programmable patterns from two different bio-inks with independent control of the volume of each droplet (with a lower limit of 2 nL or fewer than five cells per droplet). Human ESCs were used to make spheroids by overprinting two opposing gradients of bio-ink; one of hESCs in medium and the other of medium alone. The resulting array of uniform sized droplets with a gradient of cell concentrations was inverted to allow cells to aggregate and form spheroids via gravity. The resulting aggregates have controllable and repeatable sizes, and consequently they can be made to order for specific applications. Spheroids with between 5 and 140 dissociated cells resulted in spheroids of 0.25–0.6 mm diameter. This work demonstrates that the valve-based printing process is gentle enough to maintain stem cell viability, accurate enough to produce spheroids of uniform size, and that printed cells maintain their pluripotency. This study includes the first analysis of the response of human embryonic stem cells to the printing process using this valve-based printing setup.

(Some figures may appear in colour only in the online journal)

1. Introduction

The rapidly developing field of regenerative medicine promises to repair, replace and regenerate damaged cells, tissues or organs through stem cell therapy. Human embryonic stem cells (hESCs) and induced pluripotent stem cells have the ability to self-renew indefinitely and the potential to differentiate into any cell type \cite{1–5}. Totipotent stem cells can differentiate into all cell types found in an organism, whereas pluripotent stem cells can only differentiate into those cells which are found in an adult \cite{5}. These unique potency characteristics make hESCs ideal for use in a number of applications such as modelling early embryonic development. The potentially limitless numbers of differentiated hESC progeny can also be used for clinical tissue engineering/replacement applications such as novel drug discovery and testing for the pharmaceutical industry \cite{1, 2, 6, 7}. \textit{In vitro}, hESCs typically cluster together to form three-dimensional spheroid aggregates when cultured in medium that has the growth factors removed, which maintains them in a non-adherent and undifferentiated state. After the spheroids have formed, the medium is replaced by one that doesn’t prevent the hESCs from differentiating, and the spheroids are now commonly known as embryoid
bodies (EBs). The efficiency with which specific cell types are generated within the EB is partly determined by the size of the spheroid used to create the EB. A lack of uniformity in EB size can lead to asynchronous and heterogeneous differentiation [8]. Consequently the ability to reliably create uniform EBs of specific sizes is required to generate the correct cell–cell signals needed to produce particular cell types such as cardiomyocytes [9].

Various techniques can be used to create spheroid aggregates including static suspension, rotary mass suspension, non-adhesive microwell arrays, adhesive stencils and the hanging-drop method; however, it is still difficult to obtain uniform specific sized spheroids in a controllable manner. Static suspension has limited control over the size of cell aggregates that are formed, thus the subsequent differentiation results in heterogeneous populations [10]. Rotary mass suspension successfully creates homogeneous size distribution of spheroids but the process may damage the hESCs and disrupt cell signalling which could affect subsequent cell differentiation [9, 11, 12]. Non-adhesive microwell plates have been developed in various dimensions and shapes (i.e. U and V shaped wells) in order to control the size and shape of the resulting spheroids; however the resulting EBs were mechanically forced into a disk shape and were found to be unstable and formed different cell lineages when re-suspended [8, 9]. Adhesive stencils and other surface modification techniques are only able to control the initial size of EBs [13]. The hanging-drop method is a common method to form EBs using hESCs despite the fact that the resulting EBs can vary in size, mostly due to variations in droplet volume and cell concentrations in each droplet during pipetting, and the fact that the manual method is time consuming and prone to human error [4, 14].

In the field of biofabrication, great advances are being made towards fabricating 3D tissue and whole organs with very fine spatial control of cell deposition. Rapid progress has been made in recent years in developing and testing printing techniques including those based on laser pulses, inkjets and other more novel approaches. Laser based printing techniques are accurate and reliable but they are more suited to single cell deposition and could be prone to heating problems that can cause cell damage [15–22]. Inkjet printing techniques are inexpensive, easy to set up and potentially expandable but can suffer from clogging, shear forces which can lead to cell damage, and the bio-inks must be within a specific range of viscosity and surface tension [23–34]. Electrohydrodynamic jet printing has the unique ability to create continuous streams as well as discrete droplets but the droplet sizes and dispense location are currently uncontrollable [35–38]. Acoustic based printing techniques are not reliant on nozzles to deliver the biological materials preventing any clogging problem but may have issues with heating over longer printing sessions [39–42]. Valve-based printing techniques are one of the newest additions to this list and have the advantage of being one of the gentlest techniques for printing any number of cells but, as with all other nozzle-based techniques, clogging is potentially an issue [43–46].

Although human mesenchymal stem cells (hMSCs) [47] and mouse embryonic stem cells (mESCs) [4] have been printed in the past, until now, there have been no reports of attempts to print hESCs. hESCs are known to be more sensitive to physical manipulation, more demanding in terms of their requirement for extracellular matrix coatings for routine cell culture and are more difficult to transfet with plasmid DNA. However, they do have a greater potential to generate a wider variety of differentiated cell types than hMSCs and tissues generated would be expected to yield better models of human biology than those using mESCs as precursors. Here, we report the first investigation into the response of hESCs to the printing process and associated spheroid creation procedure. A new cell printing platform has been developed, capable of depositing hESCs with precise quantity and high cellular viability, while maintaining the pluripotency of the stem cells. The combined methods of hanging-drop spheroid formation with valve-based cell deposition systems were used for the controllable and repeatable creation of uniform hESC spheroids of specific sizes. The combination of a single valve-based deposition system and the hanging-drop technique has recently been shown to be effective in producing spheroids from mESCs using a single nozzle system [4]. However, in this paper we present a dual nozzle system which enables combinatorial printing of hESCs and results in a system with increased throughput as multiple bio-inks can be printed simultaneously. In addition we used microwell plates for aggregate creation by printing cells directly into the wells. This allowed the spheroids to form in situ without the need to transfer them to a well plate, after they have formed, therefore lowering the amount of stress applied to the cells during the aggregation procedure. The response of hESCs to the aggregation procedure was investigated to determine whether it differed in any way to that of other stem cells.

2. Materials and methods

2.1. HEK cell culture

HEK293 (human embryonic kidney, line 293) cells were used for preliminary setup experiments. The popularity of this particular cell line is due to its ease of transfection using various techniques, including the calcium phosphate method [48]. The HEK293 cells were cultured in high glucose Dulbecco’s Modified Eagle Medium: Nutrient Mixture F-12 (DMEM F-12) (Life Technologies). Cells were stored in an incubator at 36.0–37.5 °C, 5.0 ± 0.5% CO₂.

2.2. hESC culture

hESC line RC-10 was supplied by Roslin Cellab Ltd for this research. The hESCs were cultured in complete StemPro® hESC SFM (supplement, DMEM/F-12, BSA, PFG basic, and 2-mercaptoethanol) (Life Technologies) supplemented with 8 ng mL⁻¹ of human basic fibroblast growth factor. Cells were stored in an incubator at 36.0–37.5 °C, 5.0 ± 0.5% CO₂. Under these conditions the cells are kept in an undifferentiated state.
2.3. Multi-well plates

60 well Terasaki plates (653102, Greiner bio-one) were used in this study. These particular plates were chosen due to their flat bottomed conical well profile, with an angle of 56°, which both lowers the surface area of the hanging droplet and removes the unwanted corners at the bottom of most standard well plates. This increases the droplets resistance to drying and potentially induces the cells to aggregate quicker and lowers the likelihood of multiple aggregates.

2.4. Cell printing platform

Two nanolitre dispensing systems, each comprising a solenoid valve (VHS 25+ Nanolitre Dispense Valve, Lee Products Ltd) with a Teflon coated 101.6 μm internal diameter nozzle (Minstac Nozzle, Lee Products Ltd) controlled by a microcontroller (Arduino UNO, Arduino). Each nanolitre dispensing system was attached to a static pressure reservoir for the bio-ink solution to be dispensed via flexible tubing. The nanolitre dispensing system and bio-ink reservoirs were mounted in a custom built enclosure on the tool head of a micrometer-resolution 3-axis CNC machine (High-Z S-400, CNC Step) controlled by a customized CNC controller (based on G540, Geckodrives) (figure 1). A relatively large nozzle diameter (compared to the size of the cells that will be printed) was selected in order to reduce the amount of shear stress that could be experienced by the cells during the dispensing process. The bio-ink reservoirs were kept as close to the valves as possible in order to minimize the amount of time it would take to charge the system with bio-ink and to purge it at the end of the experiment. It was decided to use two dispensing systems in order to speed up experiments that required multiple bio-inks to be dispensed, as described in the experiments noted in this paper.

A USB microscope was also included to enable visual inspection of the target substrate during the printing process. Due to the type of deposition system used, a direct line-of-sight view through the nozzle is not possible therefore the USB microscope was mounted at an offset angle from the cell deposition system assemblies.

2.5. Control software

The cell printer was controlled via customized control software with a graphical user interface (GUI), written in MATLAB, that allows the user to automatically populate the wells of a specific plate or manually create a pattern of droplets, with specific droplet properties (size and choice of bio-ink). A g-code file was automatically created from this information which positions the nozzles of the cell printer above each target point in turn and sends a dispense trigger signal for the valves at the preset target locations. The algorithm has two modes of operation: for a sparsely populated array it calculates the shortest possible path that passes through each target point only once in order to speed up the deposition process; for a fully populated array it raster scans through each target point. The visual system verifies the location of the nozzle to prevent miss-printing. The short impulse signal (0.1 ms or longer) that triggers the dispensing of a droplet is controlled by an Arduino UNO microcontroller to ensure accurate signal timing.

2.5.1 Optical guiding system. In addition to verifying the location of the nozzles during the deposition process, the USB microscope was also used to ensure that the cell printer is at the correct ‘home’ position before starting each deposition run.

2.6. Drop-on-demand testing

Some applications, such as transfection, require positional repeatability and very fine spatial resolution down to the micron level. In order to test the spatial resolution of our cell printer an experiment was conducted to test each axis separately and together in order to ensure that it was accurate enough to return to a previous location specified by the same coordinates without drifting either in the X-direction, Y-direction or both. The CNC machine is largely responsible for the positional repeatability but it could be affected by the lower spatial accuracy of the dispensing systems. A repeating square grid was programmed in g-code with dispensing points at the corners of the square to check that the cell printer could return to a previous position accurately.
2.6.1. Array printing. The next logical step was to increase the complexity of the grid pattern to be printed. A low resolution version of the Heriot-Watt University logo was created in MS Paint with black pixels representing the dispense points and blue food dye was added to DMEM (to improve clarity). A G-code file was created from the image using the dedicated MATLAB algorithm and fed into the cell printer.

2.7. Characterization of droplet size

The volume of bio-ink dispensed in each droplet can be altered by modifying one or more of the following factors: the properties of the bio-ink (i.e. viscosity, surface tension, etc); the diameter of the nozzle orifice; the valve on-time; and the inlet pressure. Since in the majority of future experiments the same bio-ink will be used, the properties of the bio-ink will be constant. The smallest available nozzle orifice was chosen (0.002 inch or 50.8 μm). This left the valve on-time and the inlet pressure as the only remaining factors that can be used to alter the droplet size between, or during, deposition runs. An experiment was devised to characterize the droplet size variation over a range of inlet pressures and valve on-times. All other parameters remained constant. At each inlet pressure and valve on-time combination, a set number of droplets were dispensed onto a set of weighing scales and the displayed weight recorded. Droplet volumes were obtained through a simple calculation:

\[
\text{Droplet Volume (nL)} = \frac{\text{total mass of dispensed droplets (g)} \times 10^6}{\text{number of dispensed droplets}} \quad (1)
\]

2.8. Cell viability

The viability of the dispensed HEK293 cells was assessed via Trypan Blue exclusion (T8154, Sigma-Aldrich). A control was created by staining samples taken from the bio-ink before printing. HEK293 viability was measured by viewing bright-field images under the microscope (ix0015000m Trinocular Fluorescence Microscopes) both immediately and at 24 h after printing. Between these times the control and printed cells were kept in a CO₂ incubator (Galaxy S+, RS Biotech) at 36.0–37.5 °C, 5.0 ± 0.5% CO₂.

The viability of the dispensed hESCs was assessed by examining the recorded bright-field images taken using the microscope and counting the numbers of live and dead cells using ImageJ. A control was created from the unprinted bio-ink. Images were recorded at 24 and 72 h post printing. Between these times the control and printed cells were kept in a CO₂ incubator (Galaxy S+, RS Biotech) at 36.0–37.5 °C, 5.0 ± 0.5% CO₂.
2.9. Characterization of droplet cell concentration

The number of cells encapsulated within a single droplet can be controlled by changing the droplet size (by altering the parameters discussed previously) or the initial concentration of cells in the bio-ink. The cell size also plays a part in determining cell concentrations; the larger the cell, the smaller the number of cells that can be encapsulated within a droplet of a fixed size. Therefore an experiment was devised to characterize the droplet cell concentration variation over a range of droplet sizes using a constant cell concentration. Cell numbers were obtained by viewing the droplets under the microscope (fl0015000m Trinocular Fluorescence Microscopes) immediately after printing.

2.10. Oct-4 immunofluorescence

Human ES cells were printed out and kept in a CO2 incubator (Galaxy S+, RS Biotech) at 36.0–37.5 °C, 5.0 ± 0.5% CO2 for 48 h. The now adherent hESCs were washed once with PBS and fixed using 4% paraformaldehyde for 20 min at room temperature (RT). Cells were permeabilized by washing once with PBS and then incubating with 100% ethanol for 5 min at RT. Cells were then washed three times with PBS and blocked using 1% normal goat serum (Sigma G9023) in PBST (PBS plus 0.1% Tween 20) for 1 h at RT. Blocking buffer was then replaced with fresh blocking buffer containing the primary antibody (goat-anti-Oct-4, Santa Cruz SC-5279) at a dilution of 1:200. This was incubated on a rocker table at RT for 2 h or overnight at +4 °C. Primary antibody was removed by washing three times with PBST for 5 min each at RT. An Alexa-fluor conjugated secondary antibody (rabbit anti-goat, Life Technologies, A-11078) was diluted 1:200 in 1% normal rat serum (Sigma R9133)/PBST and used for 30 min at RT in the dark on a rocker table. Unbound antibody was removed during three 5 min PBST washes on a rocker table at RT in the dark. Salt was removed by washing twice with double distilled water. Excess water was removed and the sample embedded with Vectashield containing DAPI according to manufacturer’s instructions. Samples were allowed to set at RT in the dark overnight before being analysed on a fluorescence microscope.

2.11. Combinatorial printing for aggregate creation

Due to the fact that the size of an EB will influence the differentiation path of the hESC within it, by altering one or more of these characteristics we may be able to discover how much influence they have over hESC differentiation and perhaps discover the perfect conditions required to produce different lineages. As the droplet volume and hence (approximate) initial cell numbers in droplets is easily altered by overprinting droplets encapsulating cells in medium with either of different materials or by the same material, in order to increase or decrease the concentration. By overprinting droplets encapsulating cells in medium with additional medium, the cell concentration of that droplet will decrease.

It takes less than 150 µs (with the smallest valve on-time of 100 µs) to dispense a single droplet. With the dual cell deposition systems on the cell printer, we were able to generate around 40 distinct droplets per second or around 6500 droplets per second at the same location. With minor adjustments of the dwell time it is possible to obtain more droplets, but with the use of a range of droplets sizes and therefore varying valve on-times, care must be taken not to lower it too far as the printing resolution could be affected.

A low resolution version of the Heriot-Watt University logo was successfully printed out with DMEM, proving that the cell printer is capable of printing a large and complex pattern without drifting in either direction. Unfortunately, printing such large arrays takes a relatively long time and consequently by the time the last droplet has been printed, the first few have evaporated. This would be easily solved by printing larger droplets, or by printing onto a wet substrate coated with a cell culture medium such as fibrin, soy agar gel or collagen.
3.2. Characterization of droplet size

In order to characterize the dispensed droplet sizes, the method described in section 2.7 was carried out. 10 000 droplets were dispensed onto a weighing scale (Adventurer Pro AV812, Ohaus Co) for each combination of pressure and valve on-time. The results of this study are shown in figure 3.

Using equation (1), the smallest possible droplet produced during this investigation was calculated to be $\sim 2 \text{ nL}$. Due to the relationship between the inlet pressure, valve on-time and droplet volume, setting the inlet pressure at a specific value would fix the minimum droplet size. By varying the valve on-time, different droplet sizes could be dispensed in a single print run, meaning that it is possible to regulate and vary the maximum number of cells that are deposited in a single droplet.

3.3. Cell viability

3.3.1. HEK293 viability. A single cell suspension of HEK293 cells at a concentration of approximately $1 \times 10^5 \text{ cells/mL}$ in DMEM was loaded into the cell printer and primed. Droplets were dispensed into a Petri dish and a control was created in a second Petri dish; Trypan Blue was applied to both dishes before being examined under the microscope. Ten random sample images were taken for both the printed and control groups and the number of live cells and dead cells were counted in each image. The results of this study are shown in figure 4.

The normalized cell viability was calculated to be $> 99\%$ for our valve-based cell printer. This confirms that this printing process did not appear to damage the cells or affect the viability of the vast majority of dispensed cells.

3.3.2. hESC viability. hES cells were suspended in StemPro® hESC SFM to a concentration of approximately $2 \times 10^6 \text{ cells/mL}$ and loaded into the reservoir of one of the cell deposition systems. Due to the fact that different pressures are often used in different experiments, cellular viability was investigated at a number of pressures to give a more accurate indication of viability for different circumstances.
Several droplets were dispensed at each pressure onto microscope slides, the unprinted cells were used as a control. The slides were examined under the microscope at 24 and 72 h post printing and sample images were recorded. The number of live and dead cells was counted using the ImageJ image processing program and the results are shown in figure 5.

The cell viability was calculated to be >95% after 24 h and >89% after 72 h for all pressures. This confirms that this technique does not affect the short and longer term viability of cells even those as sensitive as hESCs, which is consistent with the observations of other groups [4, 43–46].

3.4. Characterization of droplet cell concentration

10 mL of StemPro® hESC SFM with RC-10 hES cells suspended at a concentration of approximately $5 \times 10^6$ cells/mL was loaded into the reservoir of one of the cell deposition systems. 2 μL of DAPI (4′, 6-Diamidino-2-Phenylindole, Dihydrochloride) (Life Technologies) was added in order to fluorescently label and image cell nuclei. The printing process was then performed, the results of which are shown in figures 6(a) and (b).

Each droplet was ~60 nL and it appears that around 28 RC-10 cells were transferred per printed droplet of solution. However, as the number of cells transferred per droplet is mainly determined by the concentration of cells in the bio-ink and the ratio of droplet size to cell size, these results can be altered by adjusting the droplet sizes or cell concentration.

3.5. Oct-4 immunofluorescence

After performing the procedure detailed in section 2.11, the cells were examined under the microscope 72 h after printing. The results are shown in figure 7.

Three days after printing, hESCs remained positive for the Oct-4 pluripotency marker, which confirms that the printing process does not affect the pluripotency of hES cells.

3.6. Combinatorial printing for aggregate creation

Prior to using cells to create aggregates, DMEM was used to check that the cell printer could print a gradient of droplet volumes. The results are shown in figure 8.
Figure 7. Detailed views of hES cells 72 h after printing: (a) optical image; (b) fluorescence image. Scale bar: 10 μm.

Figure 8. (a) An array of DMEM (with red dye for clarity) with a gradient of droplet sizes (1.5–0 μL). (b) The same array with an opposing gradient of water overprinted to create uniform volume droplets with a gradient of DMEM.

Figure 9. Detailed views of selected wells of the printed array immediately after printing: (a) second column; (b) fifth column; (c) ninth column. Scale bar: 250 μm.

Human ESCs were suspended in complete StemPro® hESC SFM to a concentration of approximately 1 × 10⁶ cells/mL and loaded into the left reservoir. The other reservoir was loaded with complete StemPro® hESC SFM. The gradient of medium was printed into the wells of a 60-well plate first in order to reduce potential impact on the cells. Next, the opposing gradient of cells in medium was printed over the medium droplets resulting in droplets of uniform size in every well (~4 μL). This plate was then placed into an incubator and the procedure was repeated on a new plate which was examined under the microscope before joining the other plate in the incubator. The results are shown in figures 9 and 10.

The experiment was repeated with a few modifications. Complete StemPro® hESC SFM with hES cells suspended at a concentration of 3 × 10⁶ cells/mL were loaded into the left reservoir. The other reservoir was loaded with complete StemPro® hESC SFM. As before, the two opposing gradients of medium and cells in medium were printed onto a 60-well plate, resulting in droplets of uniform size in every well.
Figure 10. A graph to show the average cell numbers in each column after printing.

24 hours after printing:

![Image](a)
![Image](b)
![Image](c)

48 hours after printing:

![Image](d)
![Image](e)
![Image](f)

Figure 11. Detailed views of selected aggregates 24 and 48 h after printing: (a) and (d) first column; (b) and (e) fourth column; (c) and (f) ninth column. Scale bar: 250 μm.

(~10 μL instead of 4 μL). This plate was then placed into an incubator and the procedure was repeated on a new plate which was also placed into the same incubator. As the immediate results had been verified previously, the plates were examined under the microscope 24 and 48 h after printing. The results are shown in figures 11 and 12.

In order to quantitatively evaluate the aggregate sizes a best fit ellipse was used to calculate diameters. It was observed that a gradient in the initial concentration of cells yields aggregates with a similar gradient of sizes. In addition, higher concentrations of cells tended to yield larger aggregates and these larger aggregates exhibited more growth over the 48 h than smaller aggregates.

An unexpected result was that multiple aggregates had formed in some of the wells. Although this did not occur very often, the data seems to suggest that it will occur more often in the wells with lower initial concentrations of cells. Another interesting result was that the multi-aggregates that formed
were similar sizes to those in the same column where only single aggregates had formed. Two possible explanations exist for this phenomenon: either there is a relationship between cell density and cell mobility, and there comes a point where the cells are too widely spread to form a single aggregate; or this may be a quirk of human ESCs. An example of multi-aggregate formation is shown in figure 13.

The results presented here clearly show that aggregates can be created, with a reasonable degree of control of their size, by varying the initial cell concentration and culture time. The standardization of aggregate sizes is much greater than those achievable by manual pipetting due to the inherent heterogeneous nature of manual pipetting, especially with regards to droplet sizes and therefore initial cell concentrations. Another advantage of the cell printing approach is the increased speed of the technique; the cell printer can deposit ∼40 distinct droplets per second therefore it could populate an entire 96-well plate in less than 10 s, whereas a manual approach would take several minutes. As a consequence of this decreased time and the GUI, the cell printer is a high throughput and user-friendly operation. Further work is required to fully characterize the spheroids formed using this technique.

4. Conclusions

We developed a valve-based bioprinter and reported the first study on the response of hESCs to the printing process. This work demonstrates that the valve-based printing process is gentle enough to maintain hESC viability, accurate enough to produce spheroids of uniform size, and that printed cells maintain their pluripotency. Due to the dual nozzle setup, the system is also able to create gradients of cells and other bio-inks which, when used in conjunction with the hanging droplet technique, yields gradients of cellular aggregates. The resulting aggregates are uniform and have repeatable size or size ranges which means that they can be made for specific applications (e.g. production of macrophages or other blood cells) that require going through an EB phase en-route to the eventual terminally differentiated cell type. Differing from previous studies, we printed directly onto the microwell plate allowing us to create and culture spheroid aggregates without the need to transfer them after formation therefore lowering the amount of stress applied to the cells during the aggregation procedure. The ability to print hESCs for the generation of 3D structures will allow us to create more accurate human tissue model, which is essential to the in vitro drug development and toxicity-testing. Additionally, this may also pave the way for human stem cells to be incorporated into clinical protocols either for patient implantation of in-vitro regenerated organ or direct in-vivo cell printing for tissue regeneration.

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