Utilising inkjet printed paraffin wax for cell patterning applications

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Abstract: We describe a method to prepare patterned environments for eukaryotic cells by inkjet printing paraffin wax onto a substrate. This technique bypasses the requirement to create a master mould, typically required with the use of polydimethylsiloxane techniques and the printed structure could be immediately used to guide cell proliferation. In a space of 2–3 hours, the desired pattern could be created with computer assisted design, printed and have cells seeded onto the scaffold, which could reduce the cycle time of prototyping micropattern designs. Human dermal fibroblasts and RN22 Schwann cells were seen to proliferate within the fabricated patterns and survive for more than 7 days. Additionally, the wax constructs could be readily removed from the substrate at any stage after cell seeding with the cells continuing to proliferate. Thus, we report on a simple but novel approach for the controlled physical positioning of live cells by wax inkjet printing.

Keywords: cell patterning, bioprinting, paraffin wax

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1. Introduction

Research into cell patterning and spatial coordination are growing fields, as new technologies enable researchers to accurately position populations of cells and promote the design of better biological systems[1,2]. Geometry and topology are important factors that affect anchorage-dependent cells[3–5], as living cells actively investigate their surroundings, which can influence function and morphology[6]. Cell behaviour can be better elucidated if there was a technique that allowed the rapid creation of appropriate environments to better understand the dynamic mechanism that affects cell architecture, polarity, morphology, survival and division within their surrounding environments[3,7,8]. With much interdisciplinary use of mechanical techniques being applied in the further study of tissue engineering, much has been learnt recently about cell behaviour in a microenvironment and the creation of microstructures,
that are essential in the understanding of fabricating microdevices to control cell-substrate interactions\cite{9–12}. The importance of such research was highlighted in a special themed issue of Soft Matter in 2014 on cells in patterned environments\cite{11}. Being able to control the deposition and location of cells onto a surface allows the creation of scaffolds suitable for tissue engineering, biosensors, the formation of neuronal networks, cell-based assays and for the study of cell-cell interactions.

The current “gold standard” for preparing patterned environments for cells uses PDMS (polydimethylsiloxane) during the construction of lab-on-a-chip devices and micropatterning\cite{14}. With the ability to modify mechanical, optical and chemical properties, patterning on glass\cite{15} or silicon\cite{16}, PDMS is a versatile material. An essential requirement when using PDMS is the creation of a master mould, into which PDMS can be poured, cured and removed to create the desired construct. There has been research in reducing and making the process easier, as the pattern design can be created on the master mould through soft-lithography, or through etching into silicon. The photomask can be created using high resolution printers\cite{15} or photo-plotters\cite{17}, but a clean room environment is required for fabrication. Solid object printers have been used to create the initial patterns for the master mould\cite{18}, with resolutions of >250 μm. Other techniques have also been investigated by researchers, which deposit a cell attractive or repelling agent onto a substrate, with cells thereafter physically restrained to grow within the confines\cite{19,20}. Such approaches, however, do not allow the user to remove such a physical confine at a later time.

The use of PDMS is advantageous if several copies of the master pattern are required. However, when the user requires a large range of variances in their design during prototyping, a typical need exists to create tens, or even hundreds of master moulds. In contrast, the direct patterning of a design using wax printed on a substrate bypasses the conventional process of using PDMS. Potential applications of our proposed method are found in the field of simulation studies and optimising microdevice prototypes\cite{6,21}.

Using inkjet printing to produce patterned environments offers fewer limitations compared to previously mentioned techniques that use UV exposure, photomasks and organic and toxic solvents, and require long processing times, complicated machinery, etching and multiple steps. Avoiding these drawbacks would aid in the fabrication of microfluidic devices in research and industry settings, as the turnaround times inherent in these techniques can be reduced, from around 24 hours (with the majority of this time being used for mould preparation), to hours.

With respect to inkjet printing for cell guidance, this typically involves depositing a biologically active molecule such as fibronectin, collagen and/or polymers, to selectively adhere cells at specific places on a substrate\cite{21,22}. The use of wax for the creation of microfluidic devices has been used to create paper and glass-based devices as a simple and inexpensive method using commercially available materials\cite{23–25}. This ability to create biosensors has been investigated to an extent with inkjet printing technology\cite{26–29}.

In this paper we have described for first time inkjet printing paraffin wax on tissue culture plastic and on a plain glass substrate and these formed structures were then used to guide cell attachment, spreading and proliferation, without further processing steps. Previous research using paraffin wax for the creation of microfluidic devices described wax deposition integrated with paper, film or combined with PDMS stamping\cite{30–33}. The current approach for cell patterning involves creating the desired pattern by CAD software (Figure 1A), bypassing the need to create a master mould or use of PDMS, and thereafter the immediate seeding of cells after fabrication (Figure 1B). The inkjet printing system was able to move through three

![Figure 1. (A) Wax was printed in the desired micrometre scale shape on a substrate; (B) cells were seeded onto the substrate and left to attach, spread and proliferate; (C) when required, the wax could be physically removed, to leave the cells in situ.](image-url)
axes (x,y,z) and was therefore not limited to 2D design structures. 3D and topologically irregular surfaces could be printed. At any point during the experiment, the wax could be removed to allow cells to freely migrate on the substrate, permitting the further study of cell behaviour (Figure 1C).

2. Materials and Methods

2.1 Inkjet Printing System

A single nozzle piezoelectric inkjet device (MicroFab, Texas, USA) was used to print paraffin wax. Specifically, a Jetlab4 xl-A table top printing platform with position accuracy and repeatability of 25 µm and 5 µm respectively, with a 50 µm orifice diameter (PH-04a Polymer Jet™) high-temperature, drop-on-demand printhead, was used. This was a drop-on-demand printhead that was connected to the cartridge reservoir through an integrated filter. It allows print-on-the-fly and point-to-point printing, through vector and raster printing modes.

Such a system had a 30 mL stainless steel reservoir and the system could heat up to 240°C. A CT-PT4 four-channel pressure controller was used, made by Microfab to maintain a slight negative pressure within the system to control the creation of the correct nozzle meniscus level for optimal jetting. The print head was made of a glass capillary tapered to the stated orifice size and encased in a metal body surrounded by a piezoelectric actuator. JetDrive III software was used to drive the electronics to control the generation of a waveform to tailor the jetting parameters of the print heads. Prior to jetting, all the tubings, reservoirs and the print head were flushed with 1% Micro-90 cleaning solution and distilled de-ionised water.

2.2 RN22 Schwann Cells and Dermal Fibroblasts

Rat RN22 Schwann cells were purchased from the European Collection of Cell Cultures (ECACC) (Public Health England, Porton Down, Salisbury, UK). Human dermal fibroblasts were obtained from abdominoplasty or breast reduction operations according to local ethically approved guidelines (under an HTA Research Tissue Bank license number 12179). All cell types were cultured independently and grown in Dulbecco’s Modified Eagle Medium (DMEM) containing 10% (v/v) fetal calf serum (FCS), 1% (v/v) glutamine, 1% (v/v) penicillin/streptomycin, and 0.5% amphotericin B (under serum-free conditions) in a humidified atmosphere with 5% CO₂ at 37°C. Prior to seeding, cells were grown to near confluence, and detached with 0.05% trypsin/EDTA (GIBCO, Invitrogen, Karlruhe, Germany). A Neubauer chamber was used to count the cells. Passages 20 to 22 were used for RN22 Schwann cells and dermal fibroblasts respectively.

2.3 Paraffin Wax

Paraffin wax was placed inside the cartridge reservoir and the printing system was heated up to 75°C for printing, while the printing platform was heated to 30°C to improve the topography of the printed structures. Wax viscosity was measured with a rheometer (AR 2000, TA Instruments) at different temperatures (Figure 2). When the temperature reached above 60°C, the viscosity was less than 10 mPa·s (10 centipoise). Specification guidelines from Microfab stated that inks should have a viscosity below 20 mPa·s (20 centipoise) for successful droplet formation during inkjet printing. At the temperature of printing (75°C) the viscosity was 6.02 mPa·s (6 centipoise), which was within optimal printing limits.

2.4 Wax Patterning

Using a combination of Microsoft Paint and MS Windows-based computer aided design software environment (Jetlab4, Microfab), varying shapes and designs of wax structures were created with varying channel widths and complexity onto tissue culture plastic and glass substrates. Inkjet printing parameters were optimised to create a single wax droplet per ejection, with droplet spacing of 40 µm between each droplet to create an impermeable scaffold block of wax. The volume of wax that was ejected from the piezoelectric print head could be manipulated through the fine tuning of its printing parameters, such as the
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voltage, rise and echo time, which alters the size and velocity of the wax droplet to create optimum conditions to print on varying surfaces and resolutions. When required during the study, the wax was removed by physical lifting with a scalpel.

2.5 Cell Seeding

After the wax template had been printed onto the substrate (tissue culture plastic or glass substrates), the sample was placed in a petri dish and cells were seeded at $2 \times 10^4$ cells per sample in 1 mL, and left in the incubator for 60 minutes. After this time, the sample was supplemented with 10 mL cell medium to cover the entire substrate, and left to proliferate for up to 7 days and wax removal when necessary, during which images were captured to record cell growth along the substrate with and without the patterned wax.

2.6 Analysis of Samples

Images were obtained using an inverted Olympus CK40 phase contrast microscope. Images were captured of samples prior to cell seeding, after cell seeding and after wax removal through physical lift off with a sharp scalpel.

2.7 Image Processing

All image processing was performed with ImageJ (U.S. National Institutes of Health). The orientation field was obtained using the ImageJ plugin, OrientationJ. The colour survey was set with the following settings — Hue: Orientation, Saturation: Coherency, Brightness: Original-Image. With this, it was possible to better visualise the orientation of cells along the patterned substrate, with and without the wax template over time.

2.8 Confocal Fluorescence Microscopy

For confocal fluorescence imaging, RN22 Schwann cells and dermal fibroblasts were seeded on the scaffolds at $2 \times 10^4$ cells per sample, stained with phalloidin-fluorescein isothiocyanate (FITC) for F-actin filaments and 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) for nuclear staining.

Samples were fixed with 3.7% formaldehyde in PBS for 30 minutes at room temperature and permeabilised with 0.1% (v/v) Triton X-100 in PBS for 30 minutes. Phalloidin:FITC was added at 1:1000 in PBS in combination with DAPI at 1:1000 (300 nM) for 30 minutes, washed and stored in PBS at 4°C until imaging. Cells were washed with PBS (x3) for 5 minutes between each step.

A confocal scanning microscope (Carl Zeiss LSM-510-META, Germany) with magnification ×10 and ×40 long-range water-dipping lenses were used. FITC channel ($\lambda_{ex} = 485$ nm; $\lambda_{em} = 520$ nm). DAPI ($\lambda_{ex} = 400$ nm; $\lambda_{em} = 460$ nm). Image acquisition and analysis were carried out with Carl Zeiss Laser Scanning Systems LSM 510 software.

3. Results and Discussion

3.1 Inkjet Printing of Wax Guides

A variety of designs were created that were suitable for isolating and connecting islands of cells on substrates (Figure 3A–C). Under high magnification, as exemplified in Figure 3A single wax rows of droplets appeared to have a slightly uneven topography due to scalloping behaviour. The final shape and surface texture of the resultant printed structure was dependent on conditions that include the wettability of the substrate and its temperature, print head temperature, gap distance from the print head to the substrate and droplet material[34–36]. The scalloping behaviour was due to the droplets being cooled quicker than optimal during jetted flight, after landing on the substrate, merging with the previous deposited droplet and partially retained their individual rounded contact lines[37]. Printing wax allowed the creation of a range of different complexities and channel widths that allowed the creation of thick impermeable blocks, to channels as small as 30 µm. The smallest dimensions that can be created with the wax struts is a single line of inkjet printed paraffin wax. Using a 50 µm diameter print-head nozzle, wax lines with a minimum width of 50 µm could be created to act as a barrier between each compartment.

3.2 Cell Seeding

Human dermal fibroblasts and RN22 rat Schwann cells were seeded and imaged to show cell compartmentalisation and connection within the wax structures. Figure 4A and B show images taken after 24 hours of cell culture with fibroblasts and Schwann cells on a glass substrate, respectively. Figure 4C and D show cells that have proliferated after 5 days, where the cells were able to grow in a wax-containing environment on tissue culture plastic. No cells were observed growing across and over the wax structures, showing how this technique was effective at impeding the cell interactions between individual compartments and creating separate environments for collections of
cells to grow.

With isolated arrays, cells proliferated in random shapes with no obvious direction (Figure 4A and B), but when a linear patterned structure was created, cells aligned in the direction of the structure (Figure 4C and D). Figure 5 show cells aligning to the same orientation as the channel, and through the use of ImageJ software, the orientation of cells could be clearly observed following the direction of the channel. Cell alignment could be investigated with this methodology as in the research reported by Duclos et al,[8], who described how NIH-3T3 mouse embryo fibroblasts aligned on confined strips from 30 µm to 1.5 mm.

After fibroblasts or Schwann cells were seeded, they adhered, had spread and thereafter proliferated into the desired positions and orientation. The wax structures were easily removed from the samples with a sharp scalpel, which was used to physically peel the wax off, to leave the cells to grow without any space limitations. Figure 6 shows images of the fabricated wax structures, patterned cells before and after wax removal. Cells maintained their position and orientation for at least 7 days after seeding as shown.

3.3 Cell Proliferation After Removal of Wax

A wax scaffold with channel widths of 40 µm and 30 µm connected together at one end were created and fibroblasts were seeded into the structure and cultured. After 2 days of culture and prior to wax removal, fibroblasts had aligned within the shape of the open channels (Figure 7A). Upon wax removal, cells were not limited to the channel space (Figure 7B) and had spread to cover the substrate, which was observed 24 to 48 hours after wax removal (Figure 7C and D). The wax printing technique could therefore be used initially to deposit cells in the desired areas spatially. The ability of the wax to be removed from the substrate, without the addition of new substances into the environment, allowed for a new method to analyse cell migration and proliferation on open substrates to be studied over time. We observed a high level of alignment to the direction of channels with diameters of less than 160 µm, and consequently the authors of this paper suggest that this work would have immediate applications in cell migration and proliferation studies, commonly used in wound healing and cancer cell migration research.

Cells were able to remain attached onto the substrate even after wax removal. An exception to this occurred when cell concentration exceeded confluence. From this study, when cell density began to reach confluence (typically more than 300 cells/mm$^2$) an increased proportion of cells detached. All cells within the scaffold were removed when cell density reached above 700 cells/mm$^2$. It was postulated that this was
Figure 4. Micrographs of (A) fibroblasts after 24 hours on glass substrate, (B) RN22 Schwann cells after 24 hours on glass, (C) fibroblasts after 5 days cultured on tissue culture plastic, (D) RN22 Schwann cells after 5 days cultured on tissue culture plastic, with inkjet printed wax scaffolds. Bars = 50 µm, 40 µm, 100 µm and 100 µm respectively.

Figure 5. Micrographs showing (A) a wax pattern on glass where fibroblasts are proliferating and orientating along the channel after 2 days in culture and (B) the same picture after processing with OrientationJ to highlight the alignment of cells within the channel. Channel widths are 40 µm and 60 µm and bar = 100 µm.

cau sed by adhered cells depositing a proportionate amount of extracellular matrix along their local environment, which had spread over time and was able to bind onto the substrate, wax and cells. When there was a high concentration of cells, the volume of extracellular matrix that was produced was enough to create a sheet on which the cells grew on, and allowed the cells to be peeled off along with the wax when the
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Figure 6. Light microscopy and confocal micrograph images that showed Schwann cells proliferated and aligned between two compartments through an open channel; (A) printed paraffin wax on glass; (B) confocal image immediately after wax removal, after 2 days of Schwann cells cultured on wax structures, stained for actin (green; phalloidin-FITC) and nuclei (blue; DAPI); (C) Schwann cells proliferated and remained within the confines of the wax structures, with the channel width that spanned several cell widths; (D) confocal image of Schwann cells immediately after wax removal, after 7 days of culture, cells were seen firmly adhered and aligned with the orientation of the channel stained for actin (green; phalloidin-FITC) and nuclei (blue; DAPI). Channel widths averaged 30 µm (i.e., the confines of the wax). Bars = 100 µm, 100 µm, 200 µm, 200 µm respectively.

wax was lifted off. This could be considered a limitation of this study; as the extracellular matrix deposited caused the cells to adhere on the wax.

4. Conclusion

A new method of creating cell guided structures through the use of paraffin wax deposited by inkjet printing has been presented. Channel widths of 30 µm and wax widths of 50 µm could be produced. RN22 Schwann and dermal fibroblast cells were seen to proliferate for over 7 days as exemplars, with and without wax present, on tissue culture plastic and glass substrates. The design of the wax pattern could be easily changed and directly patterned onto a substrate through CAD, without multiple processes or use of lithography techniques to create a master mould, which is advantageous when experimental procedures require slight variances in each microdevice design to be fabricated. Such examples include optimising the channel width, length, height and complexity of a prototype lab-on-a-chip for cell study. Compared to other cell guidance techniques, this technique does not require the use of harsh chemical treatments, long procedures to create the desired structures, is low cost and easy to fabricate. During cell seeding, the wax scaffold can be removed at any point, so that users can study the behaviour of cells that are allowed to proliferate freely on the substrate. Limitations can arise with this technique when studies involve lifting the wax off when growing cultures to confluence; due to the deposition of extracellular matrix from adhered cells that glue the constituents of the surrounding environment together. More cells are lifted off with increasing cell density and narrower wax channels. A smooth substrate was used in our studies; however the inkjet printing platform was able to move through three axes (x, y, z) and therefore is not limited to 2D design structures. Non-flat surfaces can also be printed on, along with topologically
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Figure 7. Micrographs showing (A) fibroblasts aligning within wax structures on glass, after 2 days of cell culture on wax structures, (B) immediately after wax removal, (C) 24 hours and (D) 48 hours after wax removal taken in the same sample region of interest. Each image was processed with OrientationJ to highlight the alignment of cells within the channel. Bar = 50 µm.

irregular surfaces as a substrate.
Future research will involve the application of patterned environments with co-culture of cells. Organised formations of cell constructs can be created which would be of benefit to fields such as neuronal research. Nerve cells could be patterned onto a sample within an organised wax structure, and once the nerve cells have adhered and the wax has been removed, support cells can be seeded on the sample to create a patterned co-culture environment.

Conflict of Interest and Funding
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