Video Article

Creating Transient Cell Membrane Pores Using a Standard Inkjet Printer

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URL: https://www.jove.com/video/3681
DOI: doi:10.3791/3681

Keywords: Bioengineering, Issue 61, bioprinting, inkjet, cell, actin, fluorescence, transfection

Date Published: 3/16/2012

Citation: Owczarczak, A.B., Shuford, S.O., Wood, S.T., Deitch, S., Dean, D. Creating Transient Cell Membrane Pores Using a Standard Inkjet Printer. *J. Vis. Exp.* (61), e3681, doi:10.3791/3681 (2012).

Abstract

Bioprinting has a wide range of applications and significance, including tissue engineering, direct cell application therapies, and biosensor microfabrication.[1-10] Recently, thermal inkjet printing has also been used for gene transfection.[8,9] The thermal inkjet printing process was shown to temporarily disrupt the cell membranes without affecting cell viability. The transient pores in the membrane can be used to introduce molecules, which would otherwise be too large to pass through the membrane, into the cell cytoplasm.[8,9,11]

The application being demonstrated here is the use of thermal inkjet printing for the incorporation of fluorescently labeled g-actin monomers into cells. The advantage of using thermal ink-jet printing to inject molecules into cells is that the technique is relatively benign to cells.[8, 12] Cell viability after printing has been shown to be similar to standard cell plating methods[1,8]. In addition, inkjet printing can process thousands of cells in minutes, which is much faster than manual microinjection. The pores created by printing have been shown to close within about two hours. However, there is a limit to the size of the pore created (~10 nm) with this printing technique, which limits the technique to injecting cells with small proteins and/or particles. [8,9,11]

A standard HP DeskJet 500 printer was modified to allow for cell printing.[3, 5, 8] The cover of the printer was removed and the paper feed mechanism was bypassed using a mechanical lever. A stage was created to allow for placement of microscope slides and coverslips directly under the print head. Ink cartridges were opened, the ink was removed and they were cleaned prior to use with cells. The printing pattern was created using standard drawing software, which then controlled the printer through a simple print command. 3T3 fibroblasts were grown to confluence, trypsinized, and then resuspended into phosphate buffered saline with soluble fluorescently labeled g-actin monomers. The cell suspension was pipetted into the ink cartridge and lines of cells were printed onto glass microscope cover slips. The live cells were imaged using fluorescence microscopy and actin was found throughout the cytoplasm. Incorporation of fluorescent actin into the cell allows for imaging of short-time cytoskeletal dynamics and is useful for a wide range of applications.[13-15]

Video Link

The video component of this article can be found at https://www.jove.com/video/3681/

Protocol

1. Converting the HP DeskJet 500

It should be noted that this technique should work with many commercially 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 is a bit more difficult than the mechanical system to "trick". The currently 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. There are many commercially available printers (HP Deskjet series and others) that 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 (section 3).

1. Remove the top plastic case of the printer by unlocking several plastic clips from the bottom base of the printer and slowly lifting the top off.
2. Unscrew button/display light panel from top of printer, leaving it connected to the printer's motherboard.
3. Clean the inside of the printer, especially the areas where the ink cartridge rests and where printing occurs.
4. Locate the cables supplying power to the paper feed mechanisms and unplug them from the motherboard.
    1. In the HP DeskJet 500, these are found just below the paper tray towards the left of the front.
5. Locate paper detection mechanism. Bypass the mechanism by affixing a string or wire loop to serve as a manual pull handle.

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1. In the HP DeskJet 500, the paper detection mechanism is a gray plastic lever found above and behind the printing/paper feed mechanism.

6. Create a stage in front of the paper feed mechanism (where the paper would be fed from and deposited) in order to bring the desired printing slides to a level just below the cartridge print head.
    1. For our experiments, 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.

7. To maintain aseptic technique, the printer can be placed inside a standard biohazard cabinet or tabletop laminar flow hood.
8. It is important to note that modifying a commercially available ink-jet printer will usually void the printer's manufacturer warranty.

2. Converting Stock HP Ink Cartridges (HP 26 Black ink cartridge)

1. Remove the cartridge from its packaging, leaving the protective tape covering the printer contacts and print head for the time being.
2. Stabilize the body of the cartridge (black portion) either with a clamp or vise (simply firmly gripping the cartridge by hand usually worked as well), leaving the green top clear of any obstacle.
3. Using pliers or an adjustable wrench, grasp the green top of the cartridge and twist back and forth several times until it breaks free.
    1. This should not take much force, but the top is no longer needed, so it is okay if it breaks when removing it.

4. Using screwdrivers, pry off the clear plastic piece now exposed.
    1. Again, this should not take much force, but it will not be needed, so it is okay if it breaks during removal.

5. Empty any remaining from the reservoir.
6. Remove the plastic protective tape covering the printer contacts and print head.
7. Thoroughly flush the reservoirs with water.
    1. Use a pipette or syringe to push water through the channels.
    2. Water will likely leak from the print head during this process; this is acceptable, and does not cause any damage to the functionality of the cartridge.

8. When the water runs clear, allow the cartridge to dry.

3. Cleaning Ink Cartridge

1. In order to maintain a clean printing environment, the ink cartridge should be cleaned before and after use.
    1. This also helps with avoiding crystallization of salts and other biological material in the cartridge that could cause blockages.

2. Fully submerge the cartridge in a beaker full of de-ionized water, and sonicate for 15 minutes before and after printing.
3. After sonication, remove the cartridge from the water, and shake out excess water.
4. Spray 70% ethanol into the cartridge to create a more aseptic environment.
    1. Ensure that the ethanol has dried before adding the bioink printing solution.

4. Making Cell Suspension - "Bioink"

1. Culture cells until ready to passage.
    1. For 3T3 fibroblasts: Seed cells on T-75 flasks at approximately $1.3x10^4$ cells/cm^2 in Dulbeccos Modified Eagles Medium (DMEM) with 10% Fetal Bovine Serum (FBS). Culture cells for two days in an incubator at 37 °C and 5% CO_2.

2. Make fluorescent g-actin stock solution at concentration 50 µg/ml in Phosphate Buffer Solution (PBS).
3. Passage cells.
    1. For 3T3 fibroblasts: Remove media from flask and rinse twice with PBS. Cover cells with 5 ml 0.25% Trypsin+EDTA and incubate for 5 mins.
    2. 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.

4. Resuspend the cells in PBS with fluorescent g-actin stock solution to create bioink.
    1. Bioink should have a final concentration of g-actin of 10 µg/ml and a cell concentration of $1x10^5$ cell/ml. This concentration has been optimized to limit amount of cells per drop and clogging of the printer head.[8]
    2. Note that 250 µl of bioink prints three cover slips with the pattern shown in **Figure 2**.

5. Bioprinting

1. Power on and let the printer warm up.
2. Place desired printing surface (here, we use 22 mm x 22 mm coverslips) on the center of the stage prepared in step 1.6.
3. Create a printing pattern file.
    1. Open Microsoft Word (or any other drawing software), and draw the desired pattern.
    2. Pick a desired print pattern for cell printing in the premade file.

4. Load the prepared cartridge with desired cell suspension.
    1. Pipette suspension into the small circular well at the bottom of cartridge compartment. Use approximately 100-120 µl of solution. The printed drop size is around 130 picoliters.[8]

5. Print the file with the HP Deskjet 500 Printer.
    1. For best results, print smaller patterns multiple times (5), by changing the amount of copies desired in the word processing program.

6. Printer will warm up again, then cartridge will move to the "ready position".
7. When cartridge moves into the ready position (slightly to the left of the ink drip well below and stays there), pull up on the paper feed mechanism wire, and printing should commence.
    1. For multiple copies of the printing, the paper feed mechanism will have to be released after every cycle or page printed. The cartridge will then return to the "ready" position, and the paper feed mechanism wire should be raised again.

8. The printer will print onto the coverslip placed on the center of the printing stage in step 5.2 (see **Figure 2**).

6. Representative Results

The representative results of the entire conversion process of a standard, off-the-shelf HP Deskjet 500, standard HP 26 series cartridges will create a printer with the capability of printing cell solutions for many types of analysis as stated before. The completed printer after conversion is shown in **Figure 3**, with a printing stage to place the microscope cover slip onto. The printer can be useful in analysis in many fields, including but not limited to: single cell mechanics, tissue engineering, gene transfection, biosensor micropatterning, and direct cell therapies.[1-10, 16-22]

In this example, an HP Deskjet 500 printer and HP 26 series ink cartridges were modified for bioprinting. Using this printer setup with a bioink consisting of a fibroblast cell suspension in a g-actin monomer solution, cells were printed onto glass microscope coverslips. **Figure 1** illustrates representative results of printed fibroblast cells, which show incorporated fluorescent actin monomers. The results were obtained in a controlled aseptic environment in which the cells were printed into customizable patterns.

The pattern used in this example was created in Microsoft Word (**Figure 2**). This pattern created a continuous line of the printing solution across the majority of the microscope slide. **Figure 4** shows the straight line in which the bioink and cells are mutually deposited. It should be noted that immediately after printing, there is an increase of background fluorescence because the bioink solution, in which the cells are suspended, contains free excess fluorescent actin monomers. This background fluorescence significantly decreases after the addition of growth media on the cells (**Figure 1**), which washes excess monomer from the substrate.

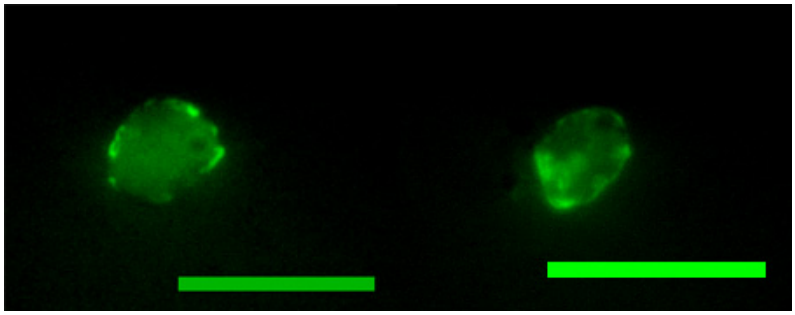

Figure 1. Representative images of 3T3 fibroblasts 3 hours after printing using the modified inkjet printer. The interior of the cells show incorporated fluorescently tagged actin monomers. Scale bars represent 50 µm.

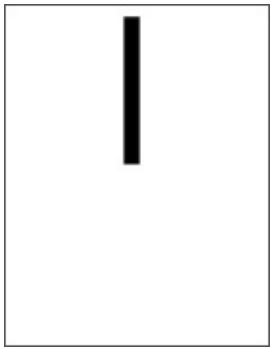

Figure 2. Design used to print bioink.

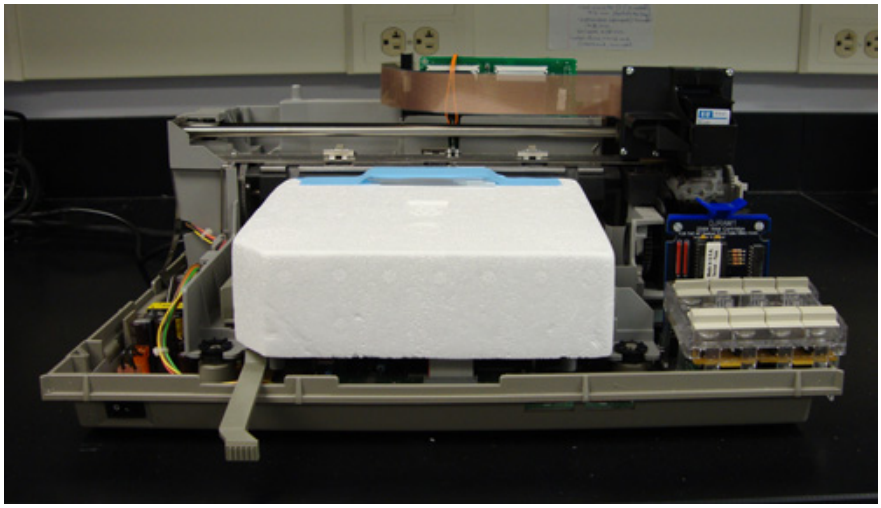

Figure 3. Printer after conversion with Styrofoam stage. The orange wire in the middle bypasses the paper feed lever sensor.

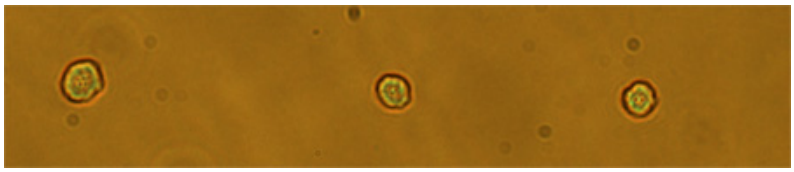

Figure 4. Representative image of 3T3 fibroblasts printed in a localized printing zone. Microscopy image taken 5 minutes after printing at 20x magnification.

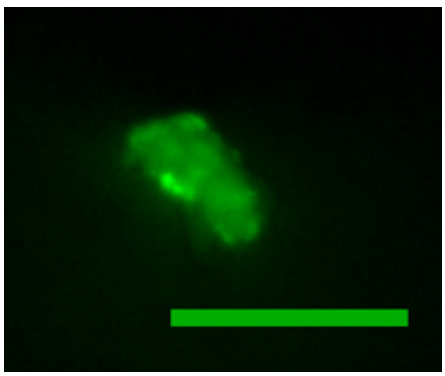

Figure 5. Fluorescence image (40x magnification) taken 3 hours after printing showing a fibroblast with internalized fluorescent actin monomers. Scale bar represents 50 µm.

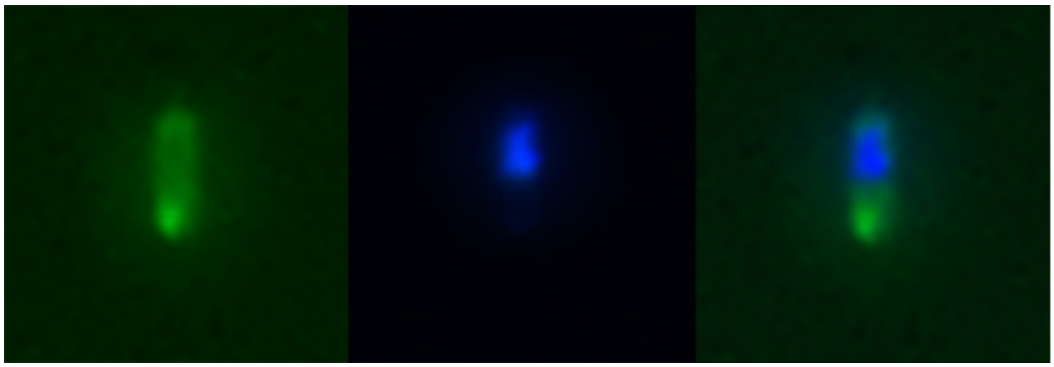

Figure 6. Fluorescent microscopy (20x magnification) of a cell taken 15 minutes after printing. The image shows both g-actin (green) and the nucleus (blue). From left to right: g-actin with Alexa Fluor 488, nucleus with DAPI, and an overlay of both.

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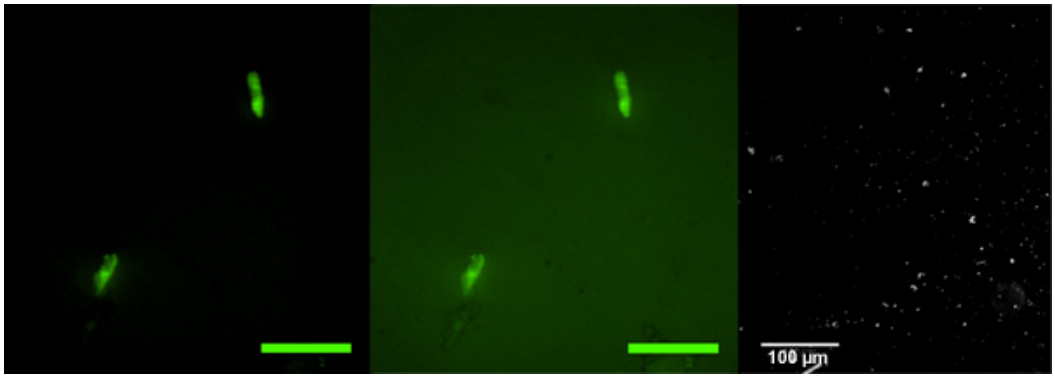

Figure 7. Fluorescent microscopy showing two fibroblasts in a line pattern 3 hours after printing. Image on left shows fluorescently labeled actin monomers inside cells. Image on right is an overlay of the fluorescent channel with the background image to show that although there may be some debris on the slide (bottom left corner), it does not fluoresce. Scale bars represent 50 µm if size not indicated the far right is a Control group of non-printed cells incubated for 3 hours with fluorescently tagged monomers. This control is to show that monomers could not penetrate the cell membrane without membrane permeabilization by cell printing.

Discussion

The process to convert a standard inkjet desktop printer for bioprinting is not particularly difficult. The most challenging step is determining how to bypass the paper feed mechanism, which is dependent on the brand and model of printer used. However, this is relatively simple when the paper feed sensor is mechanical as described here. For models with optical feed sensors, other techniques may need to be employed to trick the printer into thinking it is using paper; for instance, one can run a small piece of paper through the printer while it prints on the microscope slide. Bypassing the paper feed mechanism is likely to be the most difficult step in applying these procedures to different printer models.

When constructing a stage to hold the coverslips for printing, it is important to ensure proper alignment and height. The stage should allow for the coverslip to be placed in the middle of the printing area. In addition, it should place the coverslip at an adequate height to allow clearance for the print cartridge to pass over the slide without disrupting it. The exact height of the stage will depend on the printer model.

To ensure that printed cells do not get washed away, the slides were placed in an incubator immediately after printing for approximately 30 minutes to allow for cell attachment before additional cell growth media was added. Because the cells were printed in a solution that includes large amounts of PBS the cells did not dry out and remained viable. Cells were imaged using fluorescence microscopy to visualize the distribution of the tagged g-actin monomers inside the cell (**Figures 1, 5-7**). When printing with 3T3 fibroblasts, **Figure 5** shows a representative result, with the actin causing much of the cell to fluoresce, but showcasing lines of increased brightness. The incorporation of fluorescently tagged g-actin into the cytoskeleton is useful for the study of cytoskeletal dynamics and cellular mechanics.[12-15] A limitation of this technique is that it is only applicable for molecules and proteins that have diameters smaller than approximately 10 nm.

To ensure that cells were actually being processed by the converted printer, DAPI (1:5000 in PBS) was added to the bioink in place of half of the volume of pure PBS. The fluorescent stain DAPI binds to amino acid rich portions of DNA located in the nucleus. Therefore DAPI is a useful stain in fluorescently imaging nuclei of the printed cells. **Figure 6** shows a representative image of a cell displaying both Alexa Fluor 488 (actin) and DAPI (nucleus) fluorescence with an overlay image of both. **Figure 7** also illustrates how the cells will fluoresce once the g-actin monomers have been incorporated while non-biological material that has been deposited into the sample will not fluoresce because of the absence of g-actin monomers.

One important consideration for bioprinting is the aqueous media being used for the bioink. It was found that using standard cell growth media with serum yielded inconsistent results. This is most likely due to clogging of the print-head nozzles by serum proteins. The use of PBS increased the consistency of printed patterns and the number of cells deposited. One drawback to using PBS is that cells should not be left in the suspension for prolonged periods of time. However, fibroblasts in these examples were able to tolerate the bioink conditions for at least an hour with no change in cell viability. This is consistent with several prior findings, which report that cells printed via thermal inkjet mechanisms have been shown to have high viability rates.[2-8]

This modified printer setup can be used for applications other than cell printing. [16-22] Matrix proteins, such as collagen or fibronectin, can be easily printed onto substrates with this technique, which can be useful for cell patterning. For instance, collagen type I printed into line patterns will result in aligned collagen substrates that can be used for *in vitro* cell culture studies.[17] In addition to matrix proteins, other molecules, including growth factors, can be reliably localized on substrates for cell studies and potential therapeutic applications.[18]

A major limitation of the design described in the above steps is that this printer is not capable of printing in more than one dimension. This limits the potential for use in patterned applications such as scaffold printing. To allow for 3D printing, a specialized stage needs to be used. The stage needs to have incremental height adjustments for layer-by-layer deposition of bioink.

Bioprinting has shown promise as an efficient and cost-effective method for tissue engineering, gene transfection, micropatterning and microarray fabrication.[1-12, 16-22] The future applications of this type of device are numerous, including: creation of controlled and patterned cellular microenvironments, incorporation of macromolecules into cell cytoplasm, and deposition of cells onto scaffolds and structures that do not occur naturally or efficiently.

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Disclosures

We have nothing to disclose.

Acknowledgements

The authors would like to acknowledge Dr. Thomas Boland for the idea of using the HP DeskJet printers for cell printing. Funding for this project from NSF RII-EPS 0903795 and NIH K25 HL0922280.

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