

Devices for Biological Systems: On-Chip Horizontal Gene Transfer and 3D-Printed Microfluidic Applications

Martin D. Brennan

December 10 2015

1. SPECIFIC AIMS

Microfluidics, or the manipulation of fluids at the microscale, has unfolded over the last 20 years as a valuable technique for a wide range of applications. Unique properties emerge upon miniaturization of fluidic systems, for example, viscous flow and diffusion dominate at the microscale allowing better predictability of reactions and environmental control. A significant step forward in microfluidic development was the adoption of MEMS style fabrication techniques that allowed rapid prototyping of devices with custom geometries. As a research tool microfluidic devices are often presented as a lab-on-a-chip solution due to miniaturization and ability of high-throughput approaches, such as droplet microfluidics, which can generate large sample sizes with little resources. Applied to biological research microfluidic devices are often designed as culture platforms that can enable probing, analysis and imaging of biological systems in a novel way. We would like to present, in detail, a droplet microfluidic approach for investigating gene transfer between a single pair of cells, a 3D-printed oxygen control system for a 24 well plate, as well as 3D printed micropipette.

Aim 1: Demonstrate a microfluidic approach for co-encapsulation of two strains of strep to observe single gene transfer events. Horizontal gene transfer (HGT), the transfer of genes between organisms via transduction, conjugation or transformation, allows rapid alterations to the genome resulting adoption of antibiotic resistance and virulence, for example, directly from the genes of other cells. It has been observed that an ideal environment for HGT is in *in vivo* polyclonal biofilms of *Streptococcus pneumoniae*¹⁻⁴. This rapid gene transfer is thought to be a major factor in the persistent and long term infections as the genes adjust to both the immune response and antibiotic interventions. In the lab, genetic transformation is studied in populations of cells cultured in suspension although in *in vivo* biofilms the frequency of recombination events is far greater and the resulting number of bases that are transferred much more extensive^{5,6}. Furthermore, current studies using cell suspensions are only able to be analyzed as whole populations and do not reveal individual cell-cell interactions which may be crucial to understand this phenomena. We propose a method for co-encapsulation of pairs of bacteria in droplets for the study of single cell interactions of gene transfer. Current studies use relatively large populations and cannot discern single recombination events. Using a microfluidic based droplet encapsulation method, recombination events between pairs of cells can be isolated and analyzed individually. We aim to encapsulate a competent attacker and a non-competent victim cell in a droplet, incubate and allow transfer of DNA, then recover and perform whole genome sequencing.

Aim 2: Develop an easy to use and fabricate oxygen control insert for a 24-well plate. Oxygen control is important for mimicking conditions experienced by cells *in vivo* but is often overlooked or ignored by cell and tissue researchers in part due to the inconvenient or expensive equipment required. Many solutions for oxygen control have been met with microfluidic methods, although they usually require custom microfabrication⁷. 3D printing has emerged as a method for directly printing complete microfluidic devices. We would like to expand one of our previous oxygen control inserts, for a 6-well plate, to a 24-well plate. With 3D-printing we hope to simplify fabrication and increase functionality of the design by adding a distribution network to reduce the need for compressed gas tanks and manual setup.

Aim 3: Develop a 3D printable micropipette and compare its performance to commercial versions. The open source development model, initially applied to software, is thriving in the development of open source scientific equipment (Open Source Lab (book), OpenPCR(project)) due in part to increasing access of 3D printing. Open design 3D-printable lab equipment is an attractive idea to the scientific community because it allows development and access of new customizable technologies without a price tag⁸⁻¹⁰. While printable micropipette designs can be found on part sharing websites none allow adjustment to known volumes nor have any been rigorously validated. We are working towards an assembly of printable parts and simple hardware that will actuate a 1 mL syringe allowing the user to read and adjust the displacement to a desired volume. The pipette should be intuitive to use compared to a commercial pipette and have comparable accuracy and precision.

2. SIGNIFICANCE

Gene Transfer In a Droplet

Rapid gene transfer in pneumococcus is the main factor in the persistent and long term infection as the colony adjusts to the immune system and antibiotic treatments. It is known that a polyclonal biofilm provides an ideal environment for gene transfer but is difficult to observe what exactly is happening. Isolation and observation of single cell-to-cell interactions would provide a valuable insight for the mechanism of gene transfer. This would simulate the smallest polyclonal unit to confirm attack of a competent cell on a victim and the resulting recombination from a single event. This approach could be used to determine the amount of transfer from one recombination. With the method we hope to investigate if proximity and confinement will play a role in increasing the amount of transferred genes over macro suspensions or populations.

3D-Printed 24-Well Oxygen Control Insert

Typically cell culture studies are performed at 21% oxygen, atmospheric oxygen conditions, although levels that cells experience *in vivo* are less than 21%⁷. For example, tumors are generally hypoxic as cancer cells rapidly outgrow their vasculature creating a poorly perfused, hypoxic inner region. Studying cancer cells under controlled hypoxic conditions is important in understanding the pathophysiology because research has shown hypoxia may enhance aggressive phenotypes, tumor progression, metastasis, and resistance to therapy¹¹⁻¹³. Hypoxia is known to alter the transcription of many genes which are under the activity of the HIF (hypoxia inducible factor) family of transcriptional factors¹⁴⁻¹⁶. To better study the role of oxygen levels in cancer gene expression, a gas controlled culture system is required. Our oxygen control system will allow the addition of oxygen control to a familiar culture platform, the 24-well plate.

3D-Printed Micro-Pipette

The cost of scientific equipment is often a hurdle for researchers. Open design tools can give researchers a cost cutting option. 3D printing as a form of additive manufacturing has existed for decades although the recent availability of inexpensive desktop printers have made it feasible for researchers, designers and hobbyists to design and print prototypes, functional parts, and even consumer goods. Open design scientific tools are increasingly meeting the needs of

researchers. The development of a 3D printed pipette which is a ubiquitous tool for biomedical research can demonstrate the utility of this method.

3 INNOVATION

Gene Transfer In a Droplet

Current studies of gene transfer involve populations of cells in suspension. Co-encapsulating a pair of pneumococcus strains in 10 um droplets to observe single attack and HGT events would be a novel approach.

3D-Printed 24-Well Oxygen Control Insert

No 3D-printed microfluidic devices have been demonstrated for oxygen control. This is the first 3D-printed device incorporating gas permeable membranes to facilitate oxygen control in cell culture.

3D Printed Pipette

Open design 3D-printable pipettes are available yet none allow adjustment to a volume *a priori*, or before the pipette is characterized. The scale marks on the syringe can be used to adjust this pipette to the desired volume accurately without verifying with a scale. In addition our pipette allows transfer from 15 mL conical tubes.

4 APPROACH

Aim 1: Gene transfer In a Droplet

Preliminary Results

We have designed a flow focusing, droplet generating device that produces monodisperse water in oil (w/o) droplets of ~5-10 um in diameter. The device has two inputs for the aqueous phase, reserved for the two strains of pneumococcus, and one input for FC-40 oil w/ 2% v/v pico-surf at the continuous phase. Droplets are produced and flow downstream to a ~1 cm² holding chamber and then to an outlet. The droplets can be halted in the holding chamber by stopping the flow. They remain relatively still and are very stable due to the surfactant preventing coalescence.

We tested encapsulation with 1 um green and red fluorescent polystyrene beads serving as surrogates for pneumococcus. With a concentration of around 4×10^9 beads per mL Overall occupancy is around 20% of droplets with 5% of occupied droplets having dual occupancy of a red and green bead..

We also used E. coli in the device although the LB media interfered with imaging due to index of refraction effects.

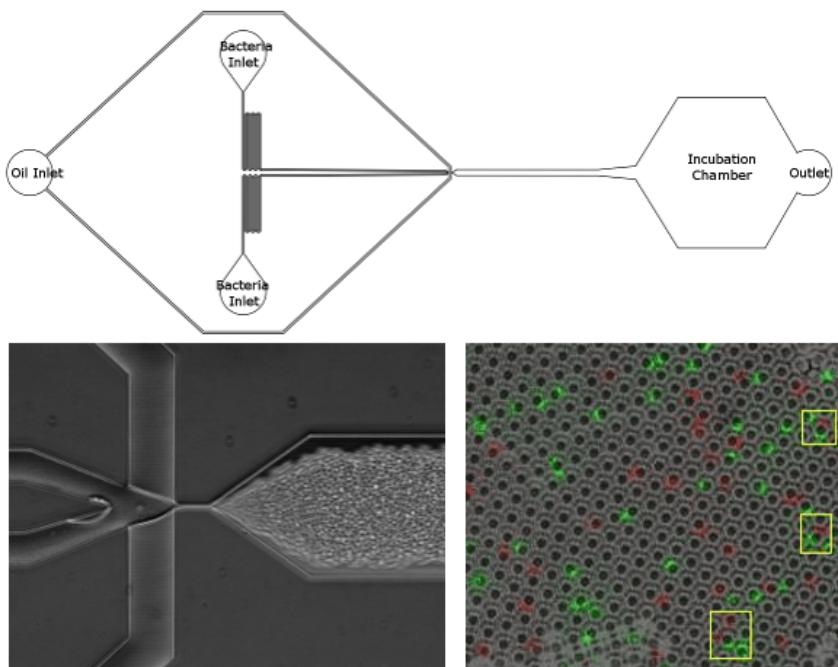


Figure 1. Design of flow-focusing device (above). Micrograph of droplet generation (lower left). Red and green beads in droplets (bottom right). Yellow boxes indicate co-encapsulation of a red and green bead.

HGT model strains

We have two strains of *Streptococcus pneumoniae* to investigate HGT. The 'attacker' strain CP2204 is a non-virulent derivative of the wild type D39 and is modified with the resulting genotype: cps comA::erm hlpA::GFP::CAT rifR. The changes make it unable to secrete competence-stimulating peptide (CPS), leaving it unable

to become competent unless CSP is added to the media. It also is modified to not form a polysaccharide capsule making it non-virulent. GFP expression was added as well as resistance to chloramphenicol (antibiotic) and Rifampin (antibiotic-inhibits RNA polymerase).

The 'victim' strain is derived from MD5037 (accession number ERR129172), after several crosses the strain is CP2215 with the genotype: cps nov-1 hlpA::RFP::CAT comE::spc. These changes make it unable to sense CSP and therefore unable become competent at all. It also expresses RFP and is resistant to chloramphenicol, novobiocin and spectinomycin (antibiotics).

Approach moving forward:

The first step is to perform encapsulation with the pneumococcus attacker and victim strain in the device. A chilled suspension of cells prepared with CSP will be introduced into the device. When droplet formation stabilizes, the droplets in the chamber will be halted by stopping the flow. Next, the device will be placed on a heated microscope stage to warm the cells, activating them, and then incubated at 37C. Fluorescence imaging of the GFP/RFP expressing cells can be used to observe occupancy and killing during the 20 min incubation. The droplets can then be collected off chip and for emulsion breaking to release the surviving bacteria. The resulting cell suspension can then be diluted such that only single cells will be plated into a multi well

plate. Whole genome sequencing can then be used to elucidate transfer events on resulting cultures. Great-granddaughter generation will have all 8 possible recombinations.

Intermediate testing of this approach may be valuable as well, for example free DNA added to the cell suspension should be taken up and incorporated by a competent cell. A selectively competent pneumococci strain can be added in one inlet and CSP and free DNA coding for antibiotic resistance can be added to the other inlet. The resulting culture can then be treated with the antibiotic. Any surviving cells can then be sequenced to confirm transfer of the gene.

Expected Results:

We expect to be able to encapsulate pairs of attacker and victim pneumococcus and use fluorescence imaging to confirm and characterize occupancy. We hope to then observe and confirm attack of a competent cell on a victim cell. Upon whole genome analysis we hope to determine the amount of gene transfer from a single event by comparing the genomes of the original attacker and victim cell.

Potential Difficulties:

Encapsulated cells may not become competent or be able to perform a successful attack. The encapsulation process may be too traumatic for the cells making them unable to become competent. Successful attack and lysis may rely on more than a single cell-cell interaction. Lysis of one cell may inadvertently trigger lysis in the second cell due to released signaling molecules or digestive agents that are compounded by confinement in the droplet. Cells may not be able to physically meet each other due to unforeseen effects of fluid flow in the droplets.

3D-Printed 24-Well Oxygen Control Insert

Preliminary results

We have designed and characterized a 3D-printed part that with the addition of a PDMS membrane controls oxygen in an off-the-shelf 24-well plate. The 24-well insert was designed to control gas in 4 rows of 6 wells each. Each of the 4 rows can be controlled independently from an input and also incorporate an integrated distribution network and hose barbs to simplify device operation. The 24 pillars extend into each well leaving a ~500 µm gap between the diffusion membrane and the culture surface. Diffusion occurs rapidly across this gap allowing control of the dissolved gas environment around the cells. A distribution network stems from the central input that equalizes the flow along each path length by varying the channel width to the proximal, intermediate, and distal wells.

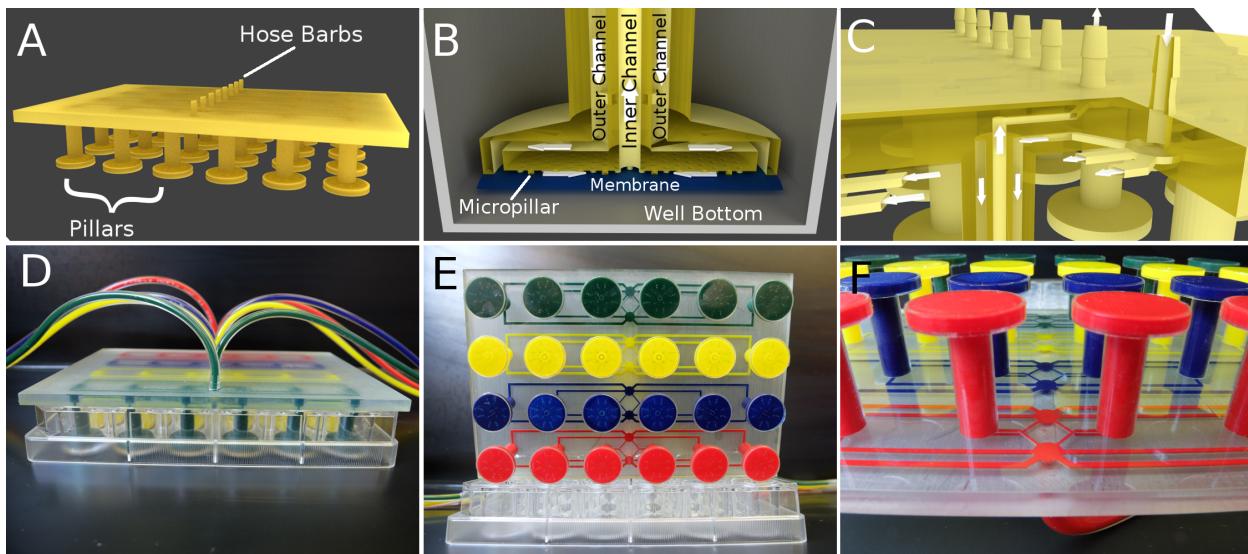


Figure 2 (A-C) CAD rendering of the device. (D-F) Photographs of the device with dye added to visualize channels.

Approach moving forward:

PDMS is a convenient material for proof-of-concept but alternative membrane materials will be explored to further simplify assembly of the device. PTFE and various thermoplastics may serve as more convenient and manufacturer friendly material. These materials need to be considered with oxygen permeability, biocompatibility, mechanical properties and methods of adhering in mind. The design can be developed further to increase functionality and convenience in cell studies. Studies of more than 24 hours usually require the media to be exchanged. When the device is removed and or the media is exchanged with fresh media this breaks the desired oxygen environment. We would like to build into the device the ability to exchange media for long term studies. The current design applies a single oxygen condition to the entire well. Additional fluidic features can be added to allow patterning of the oxygen environment within the well¹⁷.

Expected Results:

We expect to be able to assess several materials to replace the PDMS membrane of the current device. We can characterize the oxygen permeability and explore bonding methods with the device. We would also expect to be able to add a simple media exchange feature to the design. A media exchange port can be added to allow aspiration and addition of fresh media without removing the insert.

Potential Difficulties:

Exchange of fresh media even if the device remains in place may disrupt the desired oxygen environment. We can test the extent of this deviation by detecting changes in the oxygen levels during the exchange. The exchange system may also require room for a port at the bottom of the well which will leave a larger area of the culture are not covered by the oxygen environment.

Aim 3: 3D Printed Pipette

Preliminary Results

We have developed a design of printable parts and hardware at a cost of about \$5 USD that can actuate a 1 mL syringe to a variable user defined displacement with comparable accuracy to commercial pipettes costing \$100 or more. In addition, the pipette operates in a similar fashion to a commercially available micropipette. The user can adjust the volume to be dispensed by noting the displacement against the graduations printed on a 1mL syringe which is a component part of the printed pipette. We have characterized our printed pipette's performance compared to commercial micropipettes and found it is competitive (table 1). We have also tested its user friendliness by taking data from novice users' first use of the pipette.

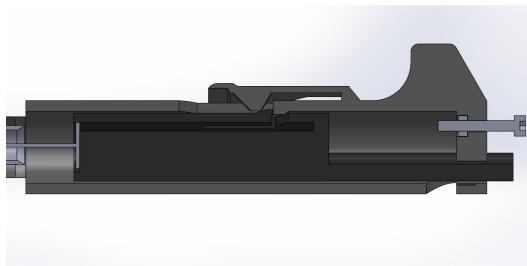


Table 1. Comparison of error between the printed and commercially produced pipette

Volume		Systematic error	Random error	
10 μL	printed	2.17 %	$\pm 0.217\mu\text{L}$	1.96% $\pm 0.196\mu\text{L}$
	commercial	3%	$\pm 0.3\mu\text{L}$	1% $\pm 0.1\mu\text{L}$
20 μL	printed	0.33%	$\pm 0.066\mu\text{L}$	1.18 % $\pm 0.236\mu\text{L}$
	commercial	2.50%	$\pm 0.5\mu\text{L}$	0.70% $\pm 0.14\mu\text{L}$
50 μL	printed	1.17%	$\pm 0.585\mu\text{L}$	0.50% $\pm 0.25\mu\text{L}$
	commercial	1%	$\pm 0.5\mu\text{L}$	0.30% $\pm 0.15\mu\text{L}$
200 μL	printed	2.52%	$\pm 5.04\mu\text{L}$	0.69% $\pm 1.38\mu\text{L}$
	commercial	0.6%	$\pm 1.2\mu\text{L}$	0.20% $\pm 0.4\mu\text{L}$

Figure 3 A cross-sectional view of the printed pipette assembly.

Approach moving forward:

As we continue to develop this printable micropipette design we will aim to add features, make it more user friendly and accurate. One critical feature missing is ejection of the pipette tip. This would make it a more of a viable option compared to a commercial pipette. As an ejector arm may not be feasible at this time we envision a disposable box to pull off the pipette tips as they are inserted. Current actuation of the pipette requires operation by pushing with thumb and releasing with the forefingers. Re-designing the pipette as push-push operation with only the thumb in the same position should improve overall ergonomics making it more user friendly.

Expected Results:

We expect to be able to simplify the operation of the pipette by developing a push-push operation which will also improve ergonomics. We also expect to develop a tip ejection method that will provide user experience more similar to commercial pipettes.

Potential Difficulties:

A push-push operation will likely require additional specialized hardware complicating the assembly and increasing the price. A built in tip ejection system may not be possible with the current state of consumer grade printers.

References:

1. Marks LR, Mashburn-Warren L, Federle MJ, Hakansson AP. Streptococcus pyogenes biofilm growth in vitro and in vivo and its role in colonization, virulence, and genetic exchange. *J Infect Dis.* 2014;210(1):25-34. doi:10.1093/infdis/jiu058.
2. Hiller NL, Ahmed A, Powell E, et al. Generation of genic diversity among *Streptococcus pneumoniae* strains via horizontal gene transfer during a chronic polyclonal pediatric infection. *PLoS Pathog.* 2010;6(9):e1001108. doi:10.1371/journal.ppat.1001108.
3. Golubchik T, Brueggemann AB, Street T, et al. Pneumococcal genome sequencing

- tracks a vaccine escape variant formed through a multi-fragment recombination event. *Nat Genet.* 2012;44(3):352-355. doi:10.1038/ng.1072.
- 4. Chewapreecha C, Harris SR, Croucher NJ, et al. Dense genomic sampling identifies highways of pneumococcal recombination. *Nat Genet.* 2014;46(3):305-309. doi:10.1038/ng.2895.
 - 5. Croucher NJ, Harris SR, Barquist L, Parkhill J, Bentley SD. A High-Resolution View of Genome-Wide Pneumococcal Transformation. *PLoS Pathog.* 2012;8(6):e1002745. doi:10.1371/journal.ppat.1002745.
 - 6. Mostowy R, Croucher NJ, Hanganese WP, Harris SR, Bentley S, Fraser C. Heterogeneity in the frequency and characteristics of homologous recombination in pneumococcal evolution. *PLoS Genet.* 2014;10(5):e1004300. doi:10.1371/journal.pgen.1004300.
 - 7. Brennan MD, Rexius-Hall ML, Elgass LJ, Eddington DT. Oxygen control with microfluidics. *Lab Chip.* 2014;14(22):4305-4318. doi:10.1039/C4LC00853G.
 - 8. Fullerton JN, Frodsham GCM, Day RM. 3D printing for the many, not the few. *Nat Biotechnol.* 2014;32(11):1086-1087. doi:10.1038/nbt.3056.
 - 9. Waldbaur A, Rapp H, Länge K, Rapp BE. Let there be chip—towards rapid prototyping of microfluidic devices: one-step manufacturing processes. *Anal Methods.* 2011;3(12):2681. doi:10.1039/c1ay05253e.
 - 10. Zhang C, Anzalone NC, Faria RP, Pearce JM. Open-Source 3D-Printable Optics Equipment. *PLoS One.* 2013;8(3). doi:10.1371/journal.pone.0059840.
 - 11. Vaupel P, Mayer A. Hypoxia in cancer: significance and impact on clinical outcome. *Cancer Metastasis Rev.* 2007;26(2):225-239. doi:10.1007/s10555-007-9055-1.
 - 12. Vaupel P, Harrison L. Tumor hypoxia: causative factors, compensatory mechanisms, and cellular response. *Oncologist.* 2004;9 Suppl 5(Supplement 5):4-9. doi:10.1634/theoncologist.9-90005-4.
 - 13. Gilkes DM, Semenza GL, Wirtz D. Hypoxia and the extracellular matrix: drivers of tumour metastasis. *Nat Rev Cancer.* 2014;14(6):430-439. doi:10.1038/nrc3726.
 - 14. Ke Q, Costa M. Hypoxia-inducible factor-1 (HIF-1). *Mol Pharmacol.* 2006;70(5):1469-1480. doi:10.1124/mol.106.027029.
 - 15. Semenza G. Signal transduction to hypoxia-inducible factor 1. *Biochem Pharmacol.* 2002;64(5-6):993-998. <http://www.ncbi.nlm.nih.gov/pubmed/12213597>. Accessed April 16, 2015.
 - 16. Semenza GL. Regulation of mammalian O₂ homeostasis by hypoxia-inducible factor 1. *Annu Rev Cell Dev Biol.* 1999;15:551-578. doi:10.1146/annurev.cellbio.15.1.551.
 - 17. Oppegard SC, Nam KH, Carr JR, Skaarup SC, Eddington DT. Modulating temporal and spatial oxygenation over adherent cellular cultures. *PLoS One.* 2009;4(9). doi:10.1371/journal.pone.0006891.