University of Illinois at Chicago

Doctoral Thesis

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

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Declaration of Authorship

I, Martin D. BRENNAN, declare that this thesis titled, 'Devices for Biological Systems: On-Chip Horizontal Gene Transfer and 3D-Printed Microfluidic Applications' and the work presented in it are my own. I confirm that:

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"Thanks to my solid academic training, today I can write hundreds of words on virtually any topic without possessing a shred of information, which is how I got a good job in journalism."

Dave Barry

UNIVERSITY OF ILLINOIS AT CHICAGO

Abstract

Faculty Name

Department of Bioengineering

Doctor of Philosophy in Bioengineering

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

by Martin D. Brennan

The Thesis Abstract is written here. The page is kept centered vertically so can expand into the blank space above the title too. . .

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Acknowledgements

The acknowledgements and the people to thank go here, don't forget to include your project advisor. . .

Contents

List of Figures

List of Tables

Abbreviations

LAH **L**ist **A**bbreviations **H**ere

Physical Constants

Speed of Light $\quad c \quad = \quad 2.997\ 924\ 58 \times 10^8 \text{ ms}^{-\text{S}}$ (exact)

Symbols

a	distance	m
P	power	W (Js^{-1})
ω	angular frequency	rads^{-1}

For/Dedicated to/To my. . .

Chapter 1

Introduction and Background

1.1   Normoxia and Hypoxia in Cells and tissues

Oxygen makes up approximately 21% of the atmospheric air that enters the lung, yet most tissues of the body experience significantly less. Normoxia is the range of oxygen levels that tissues experience in a healthy system. Within the body oxygen levels vary depending on the specific tissue. Oxygen levels in lung alveoli are 13%, yet different regions of the brain can range from 7% to less than 1% (A list of reported oxygen levels can be found in Table oxygen in tissues). Despite the fact that normoxia for most tissues is far below 21%, culture studies are typically done at atmospheric levels. This is in effect hyperoxic, which is above physiological oxygen conditions.

1.1.1   Hypoxia inducible factor

Tissue oxygenation is a constant balancing act between oxygen availability and oxygen consumption. Small changes in oxygen levels trigger homeostasis responses in cells and tissues. Research has found that hypoxia induces extensive alterations in gene expression. The hypoxia inducible factor (HIF) family of transcription factors have been shown to activate as oxygen levels fall and the work to regulate gene expression that promotes glycolysis, angiogenesis.

1.2   Measurement of oxygen

To investigate the effects of oxygen on biological systems one first needs to find a suitable method for measuring it. Measuring oxygen in the cell environment is even more

challenging if temporal and spatial requirements are strict. Stoichiometric methods such as the Winkler method [1] are too slow and require fixing of samples.

1.2.1 Clark-style electrodes

Clark-style electrodes [2] typically use a platinum working electrode and silver chloride reference electrode with potassium chloride for the electrolyte. A voltage of about 800 mV, which is sufficient to reduce oxygen, is applied across the electrodes. Oxygen is reduced at the working electrode, thus producing electrons or current proportional to the amount of oxygen present. The electrodes and electrolyte are protected behind a gas-permeable layer of polytetrafluoroethylene (PTFE) to prevent adsorption of proteins or interfering ions from fouling the electrodes. Because Clark-style electrodes consume oxygen in order to detect it, stirring of the sample is usually required for fast response measurements. The electrode is also very sensitive to changes in sample temperature. Clark electrodes are unreliable for long-term measurements for a number of reasons which contribute to unstable readings: depletion of the electrolyte, the production of OH^- ions affecting the pH causing zero drift, and the anode becoming coated in AgCl. If used with biological samples, the protective, PTFE membrane will also lose permeability over time due to the adsorption of protein and other residues. Clark-style electrodes also suffer from low temporal and spatial resolution due to the time it takes for oxygen to diffuse across the PTFE membrane and to the electrodes. In addition, the relative size of these probes (3+ mm diameter probe) makes interfacing with microfluidic channels problematic.

1.2.2 Optical Oxygen Sensors

Formicrofluidic systems, optical oxygen sensors are the tool of choice. They have several advantages overClark-style electrodes. They do not consume oxygen so they can be used in low or no flow environments and do not suffer from fouling, making them stable for long-term studies. Where Clark electrodes require an electrical connection to each position to bemeasured and only provide a single, low spatial resolutionmeasurement, optical sensors allowmeasurement over the entire area of the sensor and at any number of discrete points. These sensors take advantage of oxygen-indicating fluoro- phores that are quenched in the presence of oxygen. The degree of quenching is determined by the oxygen partial pressure. The relationship between intensity and oxygen partial pressure is described by the Stern–Volmer equation:

equation

1.3 Methods for Global Oxygen Control and Measurement

1.3.1 Subsection 1

1.4 Microfluidic Methods for Oxygen Control

1.5 Horizontal Gene Transfer

1.5.1 History and discoveries

In 1928 Griffith reported on the discovery of the 'transforming principal' where mice that were injected with a non-virulent strain resulted in a lethal infection when injected along with a heat killed virulent strain[**?** , Griffith1928] Both virulent and non-virlent strains could then be isolated from the blood of the dead mouse. Somehow the presence of the virulent strain, although dead, passed information that allowed the non-virulent strain to transform and become virulent. A subsequent study by Avery, McLeod and McCarty demonstrated that the presence of an overlooked substance, DNA, was responsible for re-programing strains[**?** , Avery1944] At the time it was believed that some yet to be discovered protein complex was the carrier of genetic information, but after this result scientists began looking more closely at DNA. In 1951 Freeman demonstrated that a phage could re-program *Corynebacterium diphtheriae* from non-virulent to virulent[3]. Hershey and Chase found that the DNA is incorporated into the cell rather than the protein from the phage[4]. They confirmed this by producing two groups of phages, one with a radio labeled protein and one with radio labeled DNA. Watson, one of the scientist that discovered the structure of DNA was a phage scientist as well.

1.5.2 Mechanisms

Horizontal gene transfer (HGT) or (sometimes lateral gene transfer) describes the introduction of genes from an outside source which is distinct from vertical gene transfer where genes are passed from mother to daughter cells. The mechanisms of HGT are transformation, transduction and conjugation.

Transduction involves a phage which carries and infects the host cell by injecting the DNA or RNA into the cytoplasm.

In **conjugation** genetic material is passed directly during cell-to-cell contact.

1.5.3 Transformation

Transformation is the uptake of exogenous DNA from the environment by a cell[5]. Many species naturally transformable species of bacteria have been reported such as *Streptococcus pneumoniae*, Species such as *Streptococcus pneumoniae* undergo transformation naturally. Transformation was first described in *Streptococcus pneumoniae* [**?**]. In transformation the recipient cell controls the process and expresses all the proteins required for DNA up-take and homologous recombination.

1.5.4 Inducible Competence

Streptococcus pneumoniae relies on quorum sensing to activates to a state call competence. Upon reaching a density of xxxx cells release a peptide that induces competence. This peptide has been aptly named competence stimulating peptide.

1.5.5 Competence Induced Fratricide

1.5.6 Transformation in Vitro Vs in Vivo

1.5.7 Global Heath Considerations

1.6 Droplet Microfluidics

1.6.1 cell encapsulation

1.6.2 Poisson distribution

Chapter 2

Materials and Methods

2.1 3D Printed Microfluidic Devices

2.1.1 Fabrication of the 3D-printed Part

The fluidic distribution networks were designed in Autocad in 2D and then extruded to 3D with Blender. Because the channel path length to each well varied the flow resistance in each channel was balanced by adjusting the width. Each distribution network services six pillars that reach into the well of the plate leaving a 500 μm gap to the culture surface. A pipe within a pipe design was used to allow a forward and return path in each pillar that also created uniform flow across the diffusion membrane. The design was repeated to create four sets that service six wells. Hose barbs were added to each inlet and outlet port. The resulting STL file was printed by fineline prototyping with a Viper SLA system in WaterShed XC, an ABS or PBT-like proprietary material.

2.1.2 Attachment of Membranes

Gas permeable membranes were made by compressing 10:1 mixed and degassed PDMS between two glass plates that were spaced 100 μm apart with scotch tape. Molds were baked at 50°C on a hot plate to avoid bubble formation. Membranes were transfered to a transparency and placed over a cutting template and cut to size for the pillar bottoms. To attach the membranes to the 3D printed part, a small amount of PDMS was applied to the membranes and spread thin to act as a mortar. The membranes were left in place to cure overnight.

2.1.3   Flow set-up

Flow though the device was driven by vacuum with a ventri sink aspirator. This vacuum line was split evenly into four and connected to each outlet. The inlet gas was from pressurized tanks of either 0% O_2, 5% O_2, 7.5% O_2, or 21% O_2. Each gas tank was also 5% CO_2 for CO_2 buffered medium. Each pressurized tank had a multistage cylinder regulator as well as an in-line IV flow regulator. An open connection was made in the lines leading to the inlet by placing tubing from the IV regulator into the wide end of a 1000 μL pipette tip. The narrow end of the pipette tip was connected to tubing from the inlet barbs of the insert. Flow from the pressurized tank was set to exceed the flow of the vacuum so that gas was drawn from the tank condition. Flow allowed by the IV flowmeter in each of the four inlet lines was measured by temporarily diverting the flow with a three-way stopcock to a floating ball rotameter(Figure (IV flow meters)).

2.1.4   Oxygen Characterization

PtOEPK (Pt(II) Octaethylporphine ketone) sensors were created by spinning thin films from a PtOEPK in polystyrene-toluene solution. Thin film sensors were cut and fixed to the bottom of each well of a 24-well with PDMS. An oxygen scavenger solution was made by dissolving Na_2SO_3 in DI water at 2 g/L. The wells were filled with the oxygen scavenger solution and the insert device was placed in the plate. 5% CO_2, balanced N_2 was flowed through the device to flush out any oxygen. The intensity of the PtOEPK sensor was measured via epi-fluorescence imaging (Olympus XI-51). A filter cube of with ex/em was used. When the intensity stabilized images of all the sensors were taken. A motorized stage and imaging control software (metamorph) was used to memorize positions of all the wells. Each well was imaged sequentially in a 1-6, 12-7, 13-18, 24-19, order to minimize the distance and time the stage had to travel. These intensity values were considered 0% oxygen and taken as the calibration point. The plate was removed from the microscope and the insert was taken out and both the plate and insert were rinsed in DI water to wash away all the scavenger solution. DI water that was equilibrated to room temperature and atmospheric air (by placing in an open container overnight in the microscope room) was used to fill the wells. With the insert placed in the plate and 5% CO_2, balanced N_2 was flowed through the device until the intensity stabilized (45 minutes). The 5% CO_2, 5% O_2, balanced N_2 was flowed through the device. When the intensity stabilized a calibration point was taken that was considered 5% O_2. Next 5% CO_2, 10% O_2, balanced N_2 was flowed through the device and the 10% calibration point was taken. Finally the water in the plate was replaced again and 5% CO_2, balanced air was flowed through the device.

Timecourse Oxygen Characterization Data Collection: Each well was imaged sequentially at intervals of 5 minutes for a total time of 6 hours. After 15 minutes of 5% CO_2, balanced air (Three time points), the inlets were changed to: row 1: 5% CO_2 balanced N_2; row 2: 5% CO_2, 5% O_2 balanced N_2; row 3: 5% O_2, 10% O_2, balance N_2; row 4: 5% CO_2 balanced air. Summarized in table (Oxygen characterization rows)

2.1.5 Analyzing Intensities

ImageJ was used to organize images according to meta data into folders of wells including specifying calibration points. Then imageJ was used to measure intensity of each image from the time course data which was recorded into a text file for each well (Imagej macros can be found in appendix (code)). A two-site Stern-Volmer model was used to interpret the intensity data and generate oxygen values (Matlab code can be found in Appendix (code)).

2.2 3D printed adjustable micropipette

2.2.1 Design and printing

Parts were designed in CAD software and

2.2.2 Additional Parts and assembly

scale

2.2.3 Testing Procedure

2.3 Microbiology

2.3.1 *Streptococcus pneumoniae* strains

Two strains of *Streptococcus pneumoniae* were produced by Dr. Morrison of the Biological Sciences Department at UIC: CP2204 and CP2215. The strains were made to be complementary in antibiotic resistances to aid in assaying transformation events. The CP2204 strain has inducible competence so is the designated recipient where CP2215 can not become competent so it is the natural donor of DNA. Traits of these strains are listed in table 2.1

TABLE 2.1: Summary of phenotypes of strains used for transformation studies

Strain	Competence	Rifampin	Spectinomycin	Novobiocin	Label
CP2204	CSP inducible	Resistant	Sensitive	Sensitive	RFP
CP2215	non-competent	Sensitive	Resistant	Resistant	GFP

2.3.2 Culturing

Streptococcus pneumoniae strains CP2204 and CP2215 were grown separately in 12 mL of CDM with 1% CAT medium at 37°C to the desired OD (media formulas can be found in Appendix A). When the desired OD was reached suspended cultures were spun down at 8000 RCF for 8 minutes in a 4°C centrifuge. The supernatant was poured off and the cultures were re-suspended in M9 or a ratio of up to 20% CDM in M9 medium depending on the application and held at 4°C.

2.3.3 Inducing Transformation

Strain CP2204 was made competent with and inducer cocktail containing CSP, BSA, and CaCl$_2$ (The specific formula can be found in Apendeix A Table A.1). The inducer cocktail is introduced to the chilled mixture of CP2204 and CP2215. The cells can be held in at 4°C in the presence of the inducer cocktail and will remain inactivated. This suspension is then brought up to 37°C to initiate cell-cell attack and transformation. Typically these reaction suspensions are transfered to a cryogenic vials and placed in a heater block at 37°C for 30 minutes. After 30 minutes suspension are diluted ten-fold into CAT media and incubated at 37°C for an hour. This hour incubation step dilutes the inducer inactivating it and allows the generation of recombinants to emerge.

2.3.4 Plating and assaying for Drug Resistance

Suspensions were diluted in more CAT media for anticipated survivor counts. Typically suspensions were diluted by 100,000 for single drug, 100 for two drug agar, and 10 for triple drug agar. Agar was made from either CAT medium or THY medium by adding 4.5 g of agar per 300 mL of medium and autoclaving. 50 mm plates are filled with the following layers in order:

1. 3 mL Agar

2. 1.5 mL Agar + 75 μm to 1.5 mL cell suspension quickly mixed

3. 3 mL Agar

4. 3 mL drug agar

Layers are added at least a few minutes apart to allow them to set before the next layer is added. Plates are incubated at in a 37°C room for 48 hours before counting. Plates are incubated upside down to slow the diffusion of drug to the cell layer. Concentrations in the drug layer are listed in Appendix A Table A.2.

2.4 Droplet Generation and Cell Encapsulation

A microfluidic droplet generating device was used to encapsulate cells during transformation. A flow focusing device with two aqueous inlets was used to keep the cells and inducer separate until right before droplet generation.

2.4.1 Fabrication of Droplet Generating Device

Master mold fabrication. A 2D design was made in AutoCad and exported as a DFX format. Small features such as as the filter arrays were saved in a separate file and then subtracted via boolean operation in LinkCAD. SU-8 2015 (MicroChem Corp.) was spun at 4250 RPM on a 100 mm silicon wafer to a thickness of 15 μ. The wafer was baked at 95°C for 10 minutes shielded from light. The wafer was loaded into the μPG 101 (Heilderberg Instruments). The converted file from LinkCAD was imported into the μPG 101 software. The wafer was exposed at 18 mW, 100% intensity and 4x exposure time. After exposure the wafer was baked at 95°C for 10 minutes and then at 120°C for 30 minutes. I found that this additional bake at 120°C prevented de-lamination that is typical for SU-8 heights less than 50 μm. The uncured SU-8 was then washed away with SU-8 developer (MicroChem Corp.) on a shaker for 30 minutes. The wafer was then rinsed with acetone followed by IPA and blown dry with nitrogen.

Casting, assembly and treatment of device. About 30 grams of 10:1 monomer : curing-agent, PDMS (Slygard 184, Dow Corning) was mixed with a Thinky mixer for 2 minutes. About 10 grams of mixed PDMS was poured onto the master mold and spun at 2000 RPM. A 22 x 50 mm glass cover slip was layed on the uncured PDMS. The mold was placed in a vacuum desiccator to pull air bubbles from under the cover slip. The cover slip was then adjusted to be over the incubation chamber of the design and baked at 65°C to set in place. The remaining PDMS, 20 grams, is poured on the mold and degassed. The mold is then baked at 65°C for 2 hours. The PDMS is then carefully pealed from the mold containing the embedded cover slip. Holes are punched with a 1 mm biopsy punch and the PDMS is placed in contact with a glass slide after 45 seconds

of oxygen plasma exposure (Plasma Etch, Inc.) to permanently bond. The completed chip is then baked at 135°C for at least 1 hour to strengthen plasma bonding. The chip is then removed and allowed to cool before treating with hydrophobic coating. Novec 1720 (3M) is flowed through the device and then left to stand for 10 minutes. The Novec is then flushed from the chip with air and returned to a 135°C hot plate to bake in the coating and sterilize the chip for at least 30 minutes.

2.4.2 Droplet generation and Cell Encapsulation

The droplet generation chip uses a flow focusing design to make water-in-oil droplets (w/o). The Oil and aqueous channels converge at a narrow neck where the aqueous-oil interface is focused causing droplets to pinch off in a size determined by the neck width(Figure schematic of device layout). FC-40 oil with 2% (v/v) surfactant (Pico-Surf 1, Dolomite) is the continuous phase. FC-40 is a fluorinated oil that is preferred for biological applications. It has a low viscosity and is denser that water. For testing water with 1 μm fluorescent beads is used for the dispersed phase. For transformation studies cells suspended in M9 media and inducer is used. The droplet encapsulation procedure is preformed in a cold room to keep the cells inactivated until the desired amount of emulsion is produced(Figure (cold room set-up)). Depending on the experiment and chip design emulsion may be stored in off-chip tubing or can be incubated on-chip. Flow is driven with a two syringe pumps (model, brand), one for the oil syringe and one that drives both aqueous syringes. 1 m syringes (BD Biosciences) with blunted 21 gauge hypodermic needles and PTFE tubing was used. With the syringe and tubing primed the tubing is inserted into the inlet ports. The aqueous pump is started first at a flow rate of 50 μL/h. The droplet generating chip is observed through a bench-top microscope. When the aqueous channels begin making progress towards the droplet generating neck the pump with the oil syringe is started at 90 μL/h. As the flows equilibrate droplets should begin forming. The aqueous flow rate can then be reduced to 15 μL/h for the remainder of droplet formation. Droplet formation can continue until the hydrophobic surface becomes fouled or debris block the neck.

2.4.3 Emulsion Breaking

After transformation cells are released from the droplets into a bulk suspension for plating.

1. After transferring the emulsion to a micro centrifuge tube 200 μL CAT medium was added

2. The tube was Spun down at 100 RCF for 30 s

3. The separated oil was pipetted out from the bottom.

4. 400 μL of de-emulsifying agent (Pico-Break, Dolomite) was added.

5. The tube was spun down again for at 1000 RCF for 1 minute.

6. Finally the cells suspended in media are pipetted off the top of the de-emulsifying agent.

The amount of media volume in emulsion is estimated based on the packing efficiency of spheres in a sheet. The highest volume ratio of of droplets is 70%. The volume of holding chamber is 8 μL, so fully packed the volume of aqueous phase is 5.6 μL. The volume of suspension that is recovered is assumed to be 4 μL. The recovered suspension is brought up to 400 μL an considered to be diluted to by 100.

Chapter 3

Results

3.1 Main Section 1

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3.1.1 Subsection 1

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Chapter 4

Discussion

4.1 Main Section 1

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4.1.1 Subsection 1

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4.2   Main Section 2

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Appendix A

Media Recipes

	Component	Common Name	Concentration (mg/L)
1. Mineral salts	FeSO4.7H20	Iron(II) sulfate	5
	Fe(NO3)2.9H2O	Iron(III) nitrate	1
	K2HPO4	Dipotassium phosphate	200
	KH2PO4	Monopotassium phosphate	1000
	MgSO4.7H2O	Magnesium sulfate heptahydrate	700
	MnSO4	Magnesium sulfate	5
2. Amino Acids	DL-Alanine	Alanine (racemic)	100
	L-Arginine	Arginine	100
	L-Aspartic acid	Aspartic acid	100
	L-Cystine	Cystine	50
	L-Glutamic acid	Glutamic acid	100
	L-Glutamine	Glutamine	200
	Glycine	Glycine	100
	L-Histidine	Histidine	100
	L-Isoleucine	Isoleucine	100
	L-Leucine	Leucine	100
	L-Lysine	Lysine	100
	L-methionine	Methionine	100
	L-phenylalanine	Phenylalanine	100
	L-Proline	Proline	100
	Hydroxy-L-proline	Hydroxyproline	100
	L-serine	Serine	100
	L-Threonine	Threonine	200
	L-Tryptophan	Tryptophan	100
	L-Valine	Valine	100
3. Vitamins	p-Aminobenzoic acid	para-aminobenzoic	0.2
	Biotin	Biotin	0.2
	Folic acid	Folic acid	0.8
	Niacinamide	Niacinamide	1
	β-Nicotinamide adenine dinucleotide	NAD+/NADH	2.5
	Pantothenate calcium salt	Calcium Pantothenate	2
	Pyridoxal	Pyridoxal	1
	Pyridoxamine dihydrochloride	Pyridoxamine (source of B6)	1
	Riboflavin	Riboflavin (B2)	2
	Thiamine hydrochloride	Thiamine (B1)	1
	Vitamin B12	Vitamin B12	0.1
4. Sugar	Glucose	Glucose	10000
5. Nucleobases	Adenine	Adenine	20
	Guanine hydrochloride		20
	Uracil		20
6. Salts	CaCl2.6H2O	Calcium Chloride Hexahydrate	10

TABLE A.1: Formula for inducer cocktail. *typically 0.04% BSA is used to prevent non-specific binding but was reduced in these experiments to prevent fouling of they hydrophobic droplet generator

Component	Stock	unit	Inducer
CSP	250	μg/mL	0.1
BSA	4	%	0.004*
CaCl2	1	M	0.005

TABLE A.2: Concentration for drug agar overlay. This concentration assumes a 3 mL overlay layer with a 12 mL total volume per plate.

Drug	Overlay	Stock
Rifampin	40 μg/mL	20 mg/mL
Novobiocin	10 μg/mL	10 mg/mL
Spectinomycin	160 μg/mL	100 mg/mL

CAT

M9

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