Design, Analysis, and Translation of 3D Hydrodynamic Tweezer Microeddies

Valerie H. Lieu

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Reading Committee:
Daniel T. Schwartz, Chair
François Baneyx
Mary E. Lidstrom
Danilo Pozzo

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Valerie H. Lieu

Chair of the Supervisory Committee:
Chair and Boeing-Sutter Professor Daniel T. Schwartz
Department of Chemical Engineering

The drive to understand the behavior and dynamics of single cells and their connection to population-based properties has prompted the microfluidic community to develop diverse micro-trapping array systems. We present work on the use of microeddy arrays for trapping, counting, and characterizing microscale objects (particles and cells). We explore the effects of nine distinct device geometries on microeddy flow traits, and show how the eddy number, shape, symmetry, and strength are controlled by the device geometry. Microparticle trapping stability and trapping site depends on the frequency of flow oscillations as well as the device geometry. When placed in arrays, microeddies provide a high throughput platform for microparticle and cell trapping. Proper statistical design is critical for quantitatively linking single-cell measurements to population behavior. We describe a general procedure for evaluating data quality, detection, and determination limits when using arrayed trapping devices that load a homogenized mixture of micro-objects, and then trap those objects. A series of successively stringent statistical tests are used to evaluate the operational domain where micro-object trapping is described by a Poisson distribution. When particle titer (particles/ml) is well above the trap
titer (traps/ml), finite size effects and particle masking cause deviations from Poisson behavior. We use the statistical properties of the array to determine the detection limit for rare objects and uncertainty in quantitative measurements. In the dilute trapping limit, we also show that counting the empty traps is an effective method for determining titer. Statistical design is a powerful tool for creating combinations of different micro-objects, for example to study cell-cell interactions. With known trapping statistics, we show that the assembly of a predictable distribution of singlet, doublet, triplet and higher order particle clusters is possible, along with predictable sub-population ratios in each doublet or larger cluster. Because each microeddy is hydrodynamically-isolated from the others, this platform seems well suited for studying cell-cell interactions via paracrine signaling. The statistical tools and experimental design we present here can be applied to different kinds of microtrapping arrays, and should broadly help guide device system designers to support biological research.
TABLE OF CONTENTS

Chapter 1. Introduction ........................................................................................................1
   Single-cell analysis ........................................................................................................... 1
   Microparticle trapping ................................................................................................. 3
   Steady Streaming Flow and Hydrodynamic Tweezer ..................................................... 4
   Cell counts .................................................................................................................... 5
   Thesis objectives and Approaches ............................................................................. 7
   Chapter notes ................................................................................................................. 9

Chapter 2. Microeddy design on flow and microparticle trapping characteristics ..........10
   Summary ....................................................................................................................... 10
   Introduction .................................................................................................................. 11
   Experimental Details and Data Analysis .................................................................... 13
      Microdevice Geometry ............................................................................................... 13
      Microdevice Fabrication ........................................................................................... 14
      Flow conditions and visualization ......................................................................... 16
      Flow simulation ......................................................................................................... 17
   Results and discussion ............................................................................................... 18
      Oscillating flow and 2-D steady streaming ............................................................. 18
      Frequency dependence of the flow ....................................................................... 24
      Microparticle Trapping in Eddies .......................................................................... 27
   Conclusions and implications ..................................................................................... 32

Chapter 3. Microfluidic cell trapping array design rules to achieve desired detection and
determination limits ........................................................................................................33
   Summary ....................................................................................................................... 33
   Introduction .................................................................................................................. 34
   Experimental methods and statistical basis ............................................................... 36
      Hydrodynamic tweezer array fabrication and operation ....................................... 36
      Particle counting method ......................................................................................... 37
      Statistical Basis for the Analysis ............................................................................ 39
Results and Discussion ............................................................................................................. 41
Distribution of particle trapping in the load-then-trap hydrodynamic tweezer array............. 41
Confidence Interval for λ_{exp} (determination limits [136]).................................................. 45
A simpler treatment when λ_{exp} \sim O(1) or smaller......................................................... 46
Detection limits of rare events .............................................................................................. 49
Conclusions .......................................................................................................................... 50
Acknowledgements .............................................................................................................. 51
Appendix: Derivation of confidence interval.......................................................................... 51
Counting all particles ........................................................................................................... 51
Counting only empty traps .................................................................................................. 52

Chapter 4. Design and Implication of A High-throughput Microeddy Single-cells Co-culture Array .................................................................................................................. 55
Summary ................................................................................................................................ 55
Introduction ............................................................................................................................ 55
Experimental method and statistical analysis ....................................................................... 58
Microeddy array fabrication and operation ........................................................................... 58
Flow visualization and Particle trapping............................................................................... 59
Particle trapping and detection from bright field and fluorescent imaging......................... 61
Construction of binary, tertiary co-culture combinations in microeddies.............................. 62
Results .................................................................................................................................... 64
Binary system of particle trapping ......................................................................................... 64
Ternary system with fluorescent imaging ............................................................................. 68
Conclusions and Design Implications .................................................................................. 72
Conclusion .............................................................................................................................. 74
Acknowledgements .............................................................................................................. 75

Chapter 5. Translation of Hydrodynamic Tweezer Trapping Arrays – Commercialization of MTC (Microtrapping Cytometer) Translational strategy for bring microeddy trapping to the marketplace ............................................................................................................. 76
Problem .................................................................................................................................. 77
Solution ................................................................................................................................... 78
Market Analysis ...................................................................................................................... 80
Go-To-Market .............................................................................................................. 85
Traction ......................................................................................................................... 87
Finance .......................................................................................................................... 88
Exit Strategy .................................................................................................................. 89

Chapter 6. Conclusion, Recommendation, and Future Directions ................................. 90

Appendix A - Realization of 3D Steady Streaming Flow Using Three Dimensional Particle Tracking Velocimetry (3D PTV) ........................................................................... 94
  Material and Methods ................................................................................................. 94
  Results and discussion ............................................................................................... 100

Appendix B. Screening alternative systems - Trapping of Algal Single-cells and Optical Characterization ................................................................................................. 106

Appendix C. Screening alternative systems - Microeddy Trapping for Identification and Characterization of Bioenergy Endophytes ..................................................................... 108
  Introduction ............................................................................................................... 108
  Experimental Methods .............................................................................................. 110
  Result ......................................................................................................................... 114
  Discussion ................................................................................................................. 116

References ................................................................................................................... 122
LIST OF FIGURES

Figure 2-1. Schematic of the microeddy device layers.................................................................15

Figure 2-2. Cross-section view of nine different microchannel feature geometries.......................16

Figure 2-3. 2D eddy flow of cylindrical obstruction compared with simulation..............................20

Figure 2-4. 2D eddy flow of wall protrusion compared with simulation ........................................21

Figure 2-5. 2D eddy flow of wall cavity compared with simulation ................................................23

Figure 2-6. Computed eddy center distance as a function of Stokes layer thickness .......................26

Figure 2-7. Microspheres trapped in eddies generated by different geometries .............................27

Figure 2-8. Particle trapping distance as a function of Stokes layer thickness ...............................30

Figure 3-1. Identification of particles counts in trapping array ....................................................38

Figure 3-2. Correlation of measured sample mean and variance ................................................43

Figure 3-3. Computed probabilities compared to the averaged experimental frequencies from 11 particle concentrations ...........................................................................................................44

Figure 3-4. Concentration and λ value comparison of counting all particles or only counting empty traps ........................................................................................................................................49

Figure 4-1. Photograph of the device and hardware setup ............................................................59

Figure 4-2. Device schematic, geometry, and flow structure .........................................................61

Figure 4-3. Trapping of binary particles system ............................................................................66

Figure 4-4. Experimental and theoretical frequency of different particle count events for binary system ........................................................................................................................................68

Figure 4-5. Particle trapping and detection of a ternary mixture ..................................................70

Figure 4-6. Experimental and theoretical frequency of different particle count events for ternary system ........................................................................................................................................71

Figure 4-7. Mixed culture trapped in microeddy device ...............................................................72

Figure 5-1. Schematic illustrations of Micro Trapping Cytometer ..................................................80

Figure 5-2. Competitiveness analysis of SFA’s device ....................................................................85
LIST OF TABLES

Table 3-1. Calculation example of particles counts in array ..................................................39
Table 3-2. Confidence limits of experimental $\lambda_{\text{exp}}$ from Chi-square distribution ..................48
Table 3-3. Comparison of Confidence limits of $\lambda_0$ from Binomial-Normal distribution ............48
Table 4-1. Frequencies of different particle count events ..........................................................67
Table 5-1. Comparison of Competitor Products versus MTC Device ........................................84
Table 5-2. Summary Income Statement .....................................................................................89
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DEDICATION

To my parents.
Chapter 1. Introduction

Single-cell analysis

*Every cell is different.* Studies of cellular process are usually carried out with large populations of cells by measuring parameters in either whole cells or cell extracts, and reporting an average value. [1] However, genetically identical cells may display very heterogeneous behaviors; average signals from populations provide only partial information on cell dynamics.[2, 3] Such heterogeneity is commonly seen in bacteria and eukaryotic cells and can lead signaling pathways to disease.[1, 4-6] Also, because cell pools, physiology and gene expression respond in timescales of seconds or minute, the functional genomic results obtained from bulk population could be the response to experimental process rather than a response to the conditions under test.[1, 7-10] Current research suggested that this diversity can be stochastic, or as response to nutrient change, environmental stimuli; making the cells significantly different from the average population behavior.[4, 11-14] There has been much interest in biological responses of individual cells to various physiologically relevant stimuli as oppose to bulk population, and the cell-to-cell variability for cancer research, stem cell research and developmental biology. Analysis of cellular response to various physiologically-relevant environmental stimuli and the signaling biomolecules from single-cells is important for understanding not only cell-cell variation but also cellular dynamics and cell-cell interaction. Local and systematic cell-cell interaction networks modulate cell development, homeostasis, ageing, virulence production, and disease progression.[15-17] Dynamic cell culturing of single-specie or multi-species cell consortium has drawn great attention to understand the cell-cell interaction and microbiome activities in nature[18-22], human body[9, 16, 23-26] Cell dynamics of parasitism, mutualism
(allelopathy, symbiosis), commensalism in microbiome and cell consortia at single-cell level have prompt the research on novel tools for in real time, long term cell monitoring and diagnostics. Once the distribution of responses to stimuli have been characterized, information can be acquired for quantitative predictions of cell behavior in various situations.[8]

Numerous microdevices have been developed over the decade that respond to the need of single-cells analyses and diagnostics in biological and clinical settings.[27] Flow cytometry, or, specifically fluorescent activated cell sorting (FACS), is widely used to sort and count heterogeneous mixtures of cells into two or more containers, based upon measurements of specific light scattering and fluorescent characteristics of each cell.[28, 29] Gene expression microarrays are used to look for specific complimentary nucleotide base pairs from DNAs or RNAs samples, the fluorescently labeled probe bonds strongly to specific sequences and generate signals at different hybridization conditions.[30-32] However, these methods are not able to monitor the transient dynamics of cellular networks in real-time. Real-time fluorescent imaging provides tool to monitor cellular compartment within single-cells but lack of throughput.[33] The need of monitoring cell/population dynamics have prompt the lab-on-chip community to design novel cell diagnostic devices, with focus on microarrays, to efficiently monitor and characterize cellular activity as a function of time. [33-38] While single-cell microtrapping arrays can have diverse cell trapping mechanisms, the general guidelines aim to provide gentle cell manipulation and handling, mimicking in vivo conditions, instrument compatibility for imaging and optical property measurements, small effective sample volume, high-throughput, stability and sensitivity.[7, 27, 39]
Microparticle trapping

The ability to trap molecules, micrometer-scale particles, and biological cells through direct control of particle position and velocity is important for many biological and colloidal applications. Dynamic measurements of single-cell behavior require a method of holding cells in place while delivering nutrients and reagents. Contact based methods like microwells [40], microweirs, dynamic microarrays [41], can trap very large number of particles, albeit with limited ability for fine-scale manipulation of single particles. The direct physical contact can trigger undesirable response in biological cells. Non-contact trapping requires forces can also manipulate particles in suspension. Optical tweezers (OT) use a tightly focus laser beam to generate forces on a cell based on the difference in the refractive index between the cell and the medium. [42-46] Dieletrophoretic (DEP) traps create a trapping force by inducing dipoles on polarizable particles or cells subject to an non-uniform electric field. [47-49] Optoelectronic tweezers (OET) utilize direct optical image and photosensitive surface to create localized non-uniform electric fields for DEP, but the method increases the flexibility and spatial resolution for DEP.[50-52] Magnetic tweezers generate trapping force by using the magnetic properties of the trapping object, or by encapsulating magnetic particles into target object and manipulate in a magnetic field. [53-57] Acoustical tweezers create a trapping force via the difference in compressibility between the particle and the medium. The pressure forces generate by the absorption of high-frequency acoustic energy cause aggregation of particles at pressure nodes or antinodes. [57, 58] These trapping methods typically require sophisticated experimental systems and rely on the coupled physical properties of the trapping object and medium. Thus the trap control can be complicated by the variation of trapping object and medium. Moreover, these
methods utilized conditions that might be invasive or outside the physiological range for typical biological samples, affecting the cell behavior and viability. None of these methods are ideally suited for low cost, non-intrusive trapping analysis.

**Steady Streaming Flow and Hydrodynamic Tweezer**

We have previously shown that low Reynolds number (Re) steady streaming near cylindrical obstructions can be used to create microeddies that trap particles, mix, and carry-out homogeneous reactions.[59-61] We have explored flows that used high aspect ratio cylinders (where one expects a 2D flow) as well as microfluidic devices where the cylinder aspect ratio is \( \sim O(1) \). Even when using moderate aspect ratio cylinders, the frequency of operation can be tuned to make a 2D layer in the center of the channel where particles are trapped and held in suspension.[59, 62] We have used this trapping mechanism to capture, hold, and maintain microparticles (polystyrene and glass) and cells that range from algae, T-cells, and yeast.[59, 63, 64] We have come to call this non-contact suspension trapping “hydrodynamic tweezers”, by analogy with optical tweezers. In many cases, the forces acting on individual cells are low enough to maintain long-term cell viability during trapping, even when trapping motile cells. However, in some cell lines, we observed detrimental effects on cell viability. In fact, high Re streaming flows have been used to lyse cells.[65] We have recently shown the effects of device geometry on the flow and trapping traits of hydrodynamic tweezers as a critical step in expanding their application to single cell diagnostics.

Though not much research has been done on the statistical properties of trapping arrays or their application to single cell diagnostics, much is known about the physics of the flow. Steady
streaming flow is the steady (i.e. time-averaged) flow driven by Reynolds stresses created in unsteady oscillating flows of kinematic viscosity . [66-72] Fluid oscillations with frequency and amplitude $s$ generate a Stokes layer of thickness $\delta_{AC} \sim O(\sqrt{v/\omega})$ on all walls. For all but unidirectional oscillating flows, time averaging the Navier-Stokes equations produces average stresses (analogous to Reynolds stresses in turbulence) that drive a steady secondary motion called steady streaming. The most thoroughly studied of these flows is the steady streaming induced by an oscillating flow impinging on a circular cylinder of radius $a$. [73-77] In this 2D flow, four steady symmetric eddies are formed adjacent to the long cylinder, with the far field dependent on details of the outer boundary conditions. Steady streaming has also been studied for oscillating flows near wavy walls, where the eddy structure and strength is linked to details of the Stokes layer thickness, characteristic length of the wall features, and the steepness of the wall structure. [78-80] Less attention has been given to steady streaming in cavities. [81] In our research, we explore the role of geometry in detail to understand how geometry affects the flow, trapping, and the physiological impact of trapping on cells.

**Cell counts**

Cell counting is an essential and basic process for obtaining cellular information in medical and biological applications. [82] Turbidity, as by measuring the optical density (i.e. the amount of UV absorbance/scattering at a defined wavelength) of a cell suspension. [83] It is also commonly used to estimate microbial cell titer and monitor cell growth. [84] However, calibration curves for each strain have to be performed to define the relationship between the absorbance and cell counts. Results rely on the growth model and the calibration between OD and viable count data based on Lambert-Beer law. [85-87] As cell morphology changes over time and cell viability
changes, that affects OD measurements.[88] Most importantly, these population-based measurements do not give access to single cell data.

Flow cytometry or fluorescence-activated cell sorting (FACS) are versatile techniques widely used to study individual cells moving in a flow stream. [89] These methods count cells one-by-one or measure the fluorescent signal of small molecules or proteins in living single-cells while they pass through the interrogation point. The high cost, high maintenance, and complex sample preparation make these methods unapproachable for small scale labs and industry. Alternatively, Coulter counters use the change in electrical resistance of cells and the surrounding electrolyte inside microchannel to monitor and count cells by size. It can be used for counting large numbers of particles, and is cheaper than cytometry, but has the disadvantage that it cannot differentiate between living or dead cells. [90, 91] More broadly, these methods take a snapshot of the population (one cell at a time), but it is not possible to monitor the signal from the same cell over time, for instance, after the application of a stimulus. [92-97]

The alternatives to expensive instruments are simple counting methods that rely on statistical analysis of cells measured by human operators. Haemocytometry is commonly used for counting blood cells by applying cell samples to a chamber-embedded slide. By counting the cells in chambers under the microscope, the total number of cells can be determined. However, it is extremely labor intensive and slow when handling large numbers of cultures, and can be subject to inter-observer variation. A key feature of hemocytometry is the nature of the particle statistics. We will show below that understanding the trapping statistics of our devices is important for creating a low cost, high accuracy microfluidic cell counting and analysis system.
Overall, we seek to develop our steady streaming microeddy devices to have the throughput of a cytometer, the analysis capabilities of FACS, the cost of hemocytometry, and the new functionality of a device that holds the cells over time to monitor their temporal behavior.

**Thesis objectives and Approaches**

Steady streaming microeddy flows can be generated near any obstruction that causes streamlines to deviate from a pure unidirectional oscillating flow. Understanding how different obstruction geometries impact flow and trapping is critical for designing low shear stress, stable hydrodynamic tweezers. In chapter 2, we described the impact of design on microeddy devices for hydrodynamic tweezer applications. We examine a wide range of microfabricated channel and wall obstructions to show their impact on the flow and stability of particle trapping. Different materials were chosen to fabricate devices for different uses. While the geometric design is mainly based on 2D theory with certain 2D assumptions, we also explore the 3D structure of the real flow by the three-dimensional particle tracking velocimetry (3D-PTV) system.

Microeddy arrays allow one to examine many single cells and connect their individual properties to bulk population data. It is important to study many single cells simultaneously in order to avoid misleading or wrong conclusions. In order to acquire sufficient information via sampling, while maintaining high-throughput, we examine the statistics of populating traps in the microeddy array and connect that to the total cell titer. In Chapter 3, we demonstrate the statistical approach to examine trapping behavior in microeddy device based on particle distribution. Such an approach can be applied to different trapping array systems for efficient and
accurate analysis of particles. We also describe a simple analytical form of the Poisson
distribution for the unpopulated traps can be used to estimate cell titer. We then utilize these
design rules to further derived methods for multi component systems described in Chapter 4.
We demonstrate the design implications for microarray studies of multi-population samples,
specifically targeting the design for generating cell co-cultures. Software was developed for
auto-detection of the particles/cells through paired optical images during device in operation.

We seek to translate the microeddy trapping, counting, and sensing technology to a
commercial product for use in real world. In Chapter 5 we present the translational strategy for
bringing this microeddy cell trapping device to the market place. We present the
commercialization plans for StaticFlow Analytics, a UW student team that proposed using
microeddy trapping technology to help the algal biofuel industry grow. We have developed a
prototype device that demonstrates the basic features of cell trapping and counting on a low-cost,
portable platform with the microeddy technology.

Chapter 6 concludes the research results demonstrated here, and addresses
recommendation of experiments, and provides future directions required to further apply
microeddy devices for cellular researches.

Overall, this work describes the importance of careful experimental design and theoretical
scaling for developing steady streaming microeddies, as an approach tailor trapping conditions
and processes for single-cells researches.
Chapter notes


Appendix A. In preparation for publication.
Chapter 2. Microeddy design on flow and microparticle trapping characteristics

Summary

The two dimensional (2D) steady streaming induced by an oscillating flow is well studied for a long circular cylinder. The time-averaged inertial terms in the fast oscillating flow of frequency $f$ and amplitude $s$ generate a Stokes layer of thickness $\delta_{AC} \sim O(\sqrt{\nu/\omega})$ and drive a steady secondary motion called steady streaming that generates microeddies near the cylindrical obstructions. Microeddies (or “hydrodynamic tweezers”) can trap particles or cells, maintain viability, mix, and carry-out homogeneous reactions. While designed geometry is a key variable in low Reynolds numbers (Re) microfluidic systems, the role of device geometry on the flow and trapping traits of low Re steady streaming systems is little explored. Here we expand our studies to characterize 9 distinct geometries. The imaged eddy flows show that the device geometry affects the eddy number, shape, structure, and strength of the microeddies. Comparison of measured trap locations to computations of the eddy flow show that each trap is located near the eddy center. Trapping strength and location are controlled by the geometry and the oscillation frequency. The trapping behavior of different geometric shapes can be characterized as linearly proportional to the Stokes layer thickness. We show that steady streaming in microfluidic eddies can be a flexible and versatile method for non-contact microparticle/cell trapping as a platform for single-cell analysis. The trapping behavior can be predicted and better understood with the help of 2D computer simulation.
Introduction

In microfluidic systems, device geometry is a key design variable owing to the dominant role played by viscous drag when the Reynolds numbers (Re) is small. In low Re flow, the basic hydrodynamic traits of microfluidic devices are generally dictated by walls.[98] For example, in a micro-scale T-junction or pool/dam structure, the streamlines follow a smooth T, with none of the inertial traits normally associated with high Re (e.g., there are no boundary layers, eddies shed from obstructions, etc).[99-101] Thus, the fabricated device geometry is widely used to control local flow in both pressure and electrokinetic driven microfluidic devices. [102-104] Unlike most other microfluidic systems, low Re steady streaming flows have a microeddy structure that enables unique device functionalities.[60, 61, 66] However, the role of device geometry is little explored for steady streaming microfluidic devices, where small amplitude time-averaged inertial terms in the fast oscillating flow are responsible for driving the slow steady motion.

Much is known about low Re steady streaming induced by an oscillating incompressible fluid of kinematic viscosity $\nu$. [66-72] Fluid oscillations with frequency $\omega$ and amplitude $s$ generate a Stokes layer of thickness $\delta_{AC} \sim O(\sqrt{\nu/\omega})$ on all walls. Time averaging the Navier-Stokes equations produces average stresses (analogous to Reynolds stresses in turbulence) that drive a steady secondary motion around obstructions called steady streaming. The most thoroughly studied of these flows is the steady two dimensional (2D) streaming induced by an oscillating flow impinging on a long circular cylinder of radius $a$.[76, 77, 105, 106] Four steady symmetric eddies are formed adjacent to the long cylinder, with the far field dependent on details of the outer boundary conditions. Steady streaming has also been studied for oscillating flows
near wavy walls, where the eddy structure and strength is linked to details of the Stokes layer thickness, characteristic length of the wall features, and the steepness of the wall structure. [78, 79, 107] Less attention has been given to steady streaming in cavities. [81]

We have previously shown that low Re steady streaming near cylindrical obstructions can be used to create microeddies that trap particles, mix, and carry-out homogeneous reactions. [59-61] We have explored flows that generated with high aspect ratio cylinders (where one expects a 2D flow) as well as microfluidic devices where the cylinder aspect ratio is \(\sim O(1)\). Even when using moderate aspect ratio cylinders, the frequency of operation can be tuned to make a 2D-like layer in the center of the channel where particles are trapped and held in suspension. [59, 62] We have used this trapping mechanism to capture, hold, and maintain microparticles (polystyrene and glass) and cells that range from algae, T-cells, and yeast. [59, 108, 109] We have come to call this non-contact suspension trapping “hydrodynamic tweezers”, by analogy with optical tweezers. Others have performed elegant experiments where streaming flows generated by oscillating bubbles are used to steer particles in a mean flow or lyse cells. [65, 110, 111]

The role of device geometry on the flow and trapping traits of low Re steady streaming systems has not been explored for non-cylindrical geometries. Here we expand our prior studies of microdevices with cylindrical obstructions to characterize the effects of 9 distinctive geometries. We show that steady streaming in microfluidic eddies can be a flexible and versatile method for non-contact microparticle trapping. The trapping behavior can be easily predicted and better understood with the help of 2D computer simulation.
Experimental Details and Data Analysis

Microdevice Geometry

Figure 2-1a shows a schematic of the 4 layers that make up the microeddy device. The top layer is comprised of two piezo disks that are bonded to the glass coverslip beneath it. The coverslip has two holes that allow fluid to enter the channel of PDMS layer, which is bonded to a glass slide. The region labeled b in the PDMS channel layer is shown as a close-up schematic view in Figure 2-1b. Each channel has a series of geometric features that cause the oscillating flow to deviate from a simple unidirectional flow. A series of circular cross-section features are structured, including a cylindrical channel obstruction, a hemi-cylindrical protrusion from the wall, and a hemi-cylindrical cavity on the wall. The channel height is denoted $h$. Fluid oscillations are driven by displacement pumping with the paired piezo disks. An actual image of the device is shown in Figure 2-1c, with inlet and outlet fluidic ports attached to the top glass coverslip.

As noted, Figure 2-1b illustrates a channel with obstructions that are circular in cross-section. Figure 2-2 shows the cross-sections for all the geometries used here. Each cross-section has a characteristic length denoted $a$. For example, $2a$ is the diameter of the circular cross-section features or the length of a side on a square or diamond cross-section feature. The first column in Figure 2-2 shows the cross-sections for freestanding channel obstructions, the second column shows wall protrusions, and the third column denotes cavities on the wall. The actual dimensions of the device were measured using 3D imaging with a laser scanning confocal microscope (LSM 510, Zeiss) The rectangular channel had a height of $h = 116 \mu m$, width $w = 1000 \mu m$, and length of 30 mm. Typical feature dimensions were $a = 25 \mu m$. 
Microdevice Fabrication

The PDMS channel layer was molded from an SU-8 master following standard procedures described in the literature. [112, 113] A 150 μm thick layer of SU-8 Negative resist (SU-8 2100, MicroChem Corporation) was spin coated onto a pre-cleaned (Acetone/IPA/DI water, 180 °C for 2 min.) silicon wafer. After soft bake, the wafer was aligned and exposed using a direct-write laser pattern generator (µPG 101 Laser Pattern Generator, Heidelberg). Post-exposure bake was completed immediately after the exposure. Then the wafer was developed (SU-8 developer, MicroChem Corporation), followed by a hardbake at 150 °C for 5 min. The SU-8 master was then treated with trichlorosilane (GELEST, Inc.). This structured SU-8 master was the molding master for mass replication of PDMS channel layers.

The elastomer and curing agent (Sylgard® 184 Silicone Elastomer, Dow Corning Corporation) were mixed with a 10:1 ratio, and poured onto the wafer. The mixture was degassed under vacuum for more than 4 hours. To squeeze out excess material and generate a uniform PDMS layer, a laser printer transparency was taped to a flat quartz plate and then placed on top of the PDMS-coated wafer and pressed down until contact was made with the frame surrounded the master mold. After curing in an oven at 70 °C for 12 hr, the transparency-PDMS layer was peeled from the master. The channel side of the PDMS layer was then plasma-bond to a drilled coverslip where fluid ports could be added. The other side was bond to a glass slide for support. The device was then heated on a hotplate at 70 °C for 30min to ensure a stabilized, non-leak bond. Two single layer piezoelectric disks (Piezo Systems, USA) were then attached to the coverslip above the pair of cavities in the PDMS layer using conductive epoxy (SPI Supplies).
Nanoports (Upchurch Scientific) were attached to allow syringe connections for loading fluids into the device.

Figure 2-1. a) Schematic of the microeddy device layers. A thin PDMS microchannel layer is sandwiched between a coverslip and a glass slide. The channel side was bond to the coverslip. b) Close-up schematic view of the microchannel. A cylindrical channel obstruction lies at the center of the fluid channel, while wall features (protrusion and cavity) are also present. c) Picture of an actual device. Fluid displacement by a pair of piezoelectric disks creates the oscillations that drive steady streaming.
Figure 2-2. Cross-section view of nine different microchannel feature geometries, placed as illustrated in Figure 2-1b. The left column shows free standing channel featured, whereas the other columns show different types of protrusions and cavities on the PDMS walls. The characteristic size is $a$=25 μm.

Flow conditions and visualization

The device is transparent so flow visualization relied on optical methods such as small fluorescent polystyrene particles of nearly neutral buoyancy (Fluoresbrite® Multifluorescent Microspheres, $d_p$=1 μm, $\rho_p$=1.05 g/cm$^3$, Polysciences, Inc.) suspended in water ($\rho_f$ = 1.00 g/cm$^3$, kinematic viscosity $\nu$ = 0.0095 cm$^2$/s). Particles of this size have negligible Stokes number and tend to trace the fluid motion, rather than being trapped. Fluorescence microscopy (TE2000-U, Nikon) with 200-1500 ms exposure time revealed the time averaged particle streak lines. In regions of high streaming flow, particle streaks showed the circulating microeddy motion. When
imaged in regions of negligible streaming flow (far from features in the channel or walls), the
straight streaks revealed the oscillation amplitude $s$. Amplitude $s$ was set by the voltage applied
to the piezo oscillation and was limited to $(1 \mu m \leq s \leq 5 \mu m)$. The piezoelectric disks were driven
at audible frequencies from 2 - 20 kHz. All experiments were performed at room temperature.

To see particle trapping, larger particles (Polybead® Microspheres, $d_p=10 \mu m$, $\rho_p=1.05$
g/cm$^3$, Polysciences, Inc.) were used. These larger particles trap, in part, because the Stokes
numbers of particles scale as $\sim d_p^2$. Each of the flow geometry produced hydrodynamic tweezers
(microeddies) that held the larger particles in x-y-z space, but with different experimentally
observed trapping traits.

Flow simulation

Flow in the mid-plane of the microchannel has qualitative and quantitative traits that
accurately match 2D steady streaming.[62] To compute the 2-D steady streaming flow, we start
with the Navier-Stokes and continuity equations. All experiments employed small amplitude
oscillations, $s/a \ll 1$, allowing us to use a regular perturbation approach to separate the
periodically oscillating and steady flows. The perturbation parameter was defined as $\varepsilon = s/a$. The
primary oscillating flow occurred in the $\sim O(\varepsilon)$ equations and the steady secondary streaming
flow equations were $\sim O(\varepsilon^2)$. [66, 114] Finite-element method computations used COMSOL
Multiphysics to sequentially solve the $O(\varepsilon)$ oscillating equations which are then applied to the
$O(\varepsilon^2)$ steady streaming equations. MATLAB (Mathworks) was used to compile data from
subsequent COMSOL simulation loops. All equations were solved in the Eulerian frame of
reference. Because flow imaging relied on visualization of particle paths, The Stokes drift
corrections for the Lagrangian reference frame were computed from the Eulerian frame data. For a single cylinder in the center of a wide channel, simulations were validated by comparison with results from analytical solutions.[106] In all simulations shown here, the contours and grey table are identical so that each geometry shows the same absolute range in streaming strength. Lighter colors and low density contours denote weak streaming relative to darker colors and denser contours.

**Results and discussion**

*Oscillating flow and 2-D steady streaming*

Figure 2-3 to Figure 2-5 show steady streaming eddies imaged at the midplane of the channel along with comparable 2D simulations. In all of the flows evaluated here, there is no mean flow in or out of the device, so mass conservation requires that any steady flow has closed streamlines. In each case, the oscillation frequency was 4.1 kHz, giving $\delta_{AC}/a = 0.25$.

Figure 2-3 shows the case for obstructions placed in the middle of the channel: 3(a) is a cylindrical post, 3(b) a square cross-section post, and 3(c) a diamond cross-section post. Computed streamlines of these flows are adjacent to each experimental image. The microeddy character of steady streaming is clearly evident in all of the images. Four high symmetry eddies are generated in each quadrants of the cylindrical post. The center of each eddy is located along the 45° lines from oscillation axis. Eddies generated by cylindrical obstruction have the highest degree of symmetry ($D_4$) of all the geometries studied (reflections on the vertical, horizontal, and both 45° diagonal lines). For the square posts, eight eddies, two in each quadrant, are observed with $D_2$ symmetry. The four major eddies make an angle < 45° from the oscillation axis, whereas
the four minor eddies are nearly at 90° from the oscillation axis. This is particularly evident in the simulation result in Figure 2-3(b). For the diamond post, four eddies are formed with centers that are > 45° from the oscillation axis (Figure 2-3c). The simulation results suggest that the steady streaming flow gets stronger in going from (a) to (c). Not shown in the experimental images are the complex 3D flow features near the top and bottom channel walls of each obstruction, since our imaging was done at the mid-plane where 2D flow character is expected.[62]
Figure 2-3. Particle imaging experiments (left column) are compared to the computed Lagrangian streamlines (right column) for channel obstruction geometries: (a) circular cylinder, (b) square post, (c) diamond post. Arrow denotes fluid oscillation direction. Shading and contours for the computed flows are all on the same absolute scale to allow direct comparison of flow strength.
Figure 2-4. Particle imaging experiments (left column) are compared to the computed Lagrangian streamlines (right column) for protrusion geometries at the channel wall: (a) circle wall protrusion, (b) square wall protrusion, (c) triangle wall protrusion. Arrow denotes fluid oscillation direction. Shading and contours for the computed flows are all on the same absolute scale to allow direct comparison of flow strength.
Figure 2-4 shows steady streaming microeddies images generated from protrusion features at the wall, for (a) circular cross-section, (b) square cross-section, and (c) triangle cross-section wall protrusions, with computations of these flows adjacent to each image. All the images reveal an isometric reflection across the vertical line. Compared to freestanding features (Figure 2-3), the symmetry is somewhat disrupted by the wall. For example, the major eddies for each shape are pushed away from the wall compared to their freestanding counterparts, and are also elongated. The presence of a wall (vs. a symmetry line in Figure 2-3) results in a weak eddies adjacent to the wall. These weak eddies are not visible for these experiments or the selection of simulation stream functions, but the presence of these weak wall eddies are responsible for pushing the major eddies away from the wall. Also note in the experimental image of Figure 2-4b that a fabrication error to the left of the image has resulted in a nearby eddy that compresses one side of the flow.
Figure 2-5. Particle imaging experiments (left column) are compared to the computed Lagrangian streamlines (right column) for wall cavity geometries: (a) circle wall protrusion, (b) square wall protrusion, (c) triangle wall protrusion. Arrow denotes fluid oscillation direction. Shading and contours for the computed flows are all on the same absolute scale to allow direct comparison of flow strength.
The cylindrical, square, and triangular wall cavities shown in Figure 2-5 represent the inverse shapes for the wall protrusions in Figure 2-4. Comparing Figure 2-4 and Figure 2-5, we see that the eddy structure for wall protrusions and cavities are decidedly different, aside from isometric reflection symmetry across the vertical line. Each cavity has two moderately strong eddies that penetrate into the cavity, as well as two weak eddies external to the cavity. All of the visible eddies in the experiments and simulation are weaker than those generated from wall protrusions or freestanding structures. In fact, the simulation streamline intervals for the cavities are presented at half the interval of Figure 2-3 and Figure 2-4, to reveal the eddy structure.

**Frequency dependence of the flow**

For the small amplitude oscillations used in these experiments, the steady flow structure (i.e. eddy shape and eddy center) is controlled by the device geometry and the oscillation frequency. [66, 70, 71, 106, 115] Figure 2-3 to Figure 2-5 show that mid-plane experimental imaging and 2D simulations compare nicely for a single frequency. For each of the 9 device geometries, we evaluate how frequency affects the distance from the strongest eddy’s core to the closest wall, to look deeper at the role of device geometry on flow structure.

Figure 2-6 shows the distance from each major eddy’s core to the closes point on the obstruction or wall feature ($L_{\text{eddy}}$), plotted as a function of the inverse root of frequency. The nondimensional eddy size is $L_{\text{eddy}}/a$, and the nondimensional oscillation frequency is represented by the Stokes layer thickness divided by $a$, $\delta_{AC}/a$. This nondimensionalization shows that the eddy center location has a nearly linear proportionality to the Stokes layer thickness for channel obstructions and wall protrusions. Thus, the scaling relationship between frequency and eddy
size that we have seen in previous work on free standing microcylinders holds for other freestanding or protruding obstructions. However, the exact value of the eddy size is geometry dependent. Among the freestanding or protruding geometries, the most dilated eddy centers are found on the diamond post, and the most confined eddies are seen with the freestanding square, though at small Stoke layer thicknesses (high frequency), several of the geometries converge.

The frequency dependence of eddy sizes in cavities is significantly different from the other geometries. Not only are eddies in these experiments significantly closer to the surface because of the geometric constraints inside a cavity, but their sizes also grow at a sub-linear rate with increasing $\delta_{AC}/a$. These geometry dependent eddy sizes have implications for further design of experiments. For example, we have shown previously that large particles are often trapped near the eddy center.[59]
Figure 2-6. Dimensionless plot of computed eddy center distance as a function of Stokes layer thickness $\delta_{AC}/a$ for the 9 geometries evaluated.
Figure 2-7. 10 µm polystyrene microspheres trapped in steady streaming eddies generated by different geometries. All trapping was performed at very low particle densities, so we show an “x” to indicate symmetric, but unfilled, trapping locations. Image (i) displayed no stable trapping sites for the range of conditions used here. Arrow denotes fluid oscillation direction.

*Microparticle Trapping in Eddies*

One of the main functionalities of these low Re steady streaming devices is their ability to trap and hold in one fixed position. We have experimentally observed the trapping of both dense (*e.g.* polystyrene, glass) and buoyant (*e.g.* gas bubbles, oil droplets) particles.[59, 109] Figure 2-7 shows trapping of 10 µm polystyrene microspheres in the steady streaming flows generated
by each device geometry. The trapped spheres are completely suspended within the fluid, not touching walls or moving in x,y or z directions. Trapped spherical particles rotate but do not translate.

Each panel of Figure 2-7 shows one trapped particle. All other trapping sites with comparable symmetry are also observed to stably trap particles, denoted by the “x”. We chose dilute particle concentration for the experiments (making most traps empty) in order to prevent any of the traps from containing more than one particle. Qualitatively, Figure 2-7 shows that stably trapped particles are located at positions similar to the center locations for the main eddies seen in Figure 2-3 to Figure 2-5. Thus, the symmetry of the trapping closely reflects the symmetry of the eddies, as expected. Note that Figure 2-7(i) has no particle or “x” shown. The eddy flow for a triangular wall cavity is the weakest of all studied (cf. Figure 2-5c with all others). Evidently, this weak steady streaming flow leads to unstable trapping of particles within those eddies. Though trapping for the triangular cavity do occur sometimes, small disturbances commonly found in our lab conditions cause the trapping to be transient. Similarly, the weak minor eddies associated with square posts and square protrusions also display unstable trapping behavior. Thus, our results show that trapping stability is linked to the strength of the flow generated by a particular geometry.

Given the results in Figure 2-6, we expect the particle trapping location to be dependent on the device geometry and the dimensionless Stokes layer thickness. Figure 2-8 shows the characteristic trapping distance $L/a$, defined as the distance from the trapped particle center to its nearest surface as a function of dimensionless Stokes layer thickness. The particle trapping location has a near linear relationship between the trapping distance and the Stokes layer thickness $\delta_{AC} \sim (v/\omega)^{1/2}$ for the nine design geometries. We also place a characteristic line (heavy
dashed line) for the relationship $L/a = \delta_{AC}/a$. Particles that trap close to this line are approaching the boundary of the Stokes layer, where shear stress in the primary oscillating flow is high. This issue may be important for cell trapping, where shear sensitivity is commonly observed. Thus, the dashed line serves as a useful reminder that the slow steady flow features like trapping, remain connected to the physics (and hence, shear stress) of the fast oscillating flow. We are exploring in greater detail the issue of shear stress in these traps.
Figure 2-8. Dimensionless plot of particle trapping distance as a function of Stokes layer thickness $\delta_{AC}/a$ for the 9 geometries evaluated. Error bars represents one standard deviation from at least three replicates. Black dashed line indicates trapping at Stokes layer.

Trapping near wall protrusions has a similar trend as trapping near freestanding cylinders but the trapping distances are slightly farther from the surface due to the elongation of the eddies described in Figure 2-4. Note that trapping was not found for the triangular cavity for the conditions and geometries used here. Overall, these results show that steady streaming eddies...
generated from different geometries can trap particles and the trapping behavior of different geometric shapes can be characterized as linearly proportional to the Stokes layer thickness.

Our previous work with cylindrical geometries suggested that trapping occurred quite close to the computed eddy center.[59] Comparing Figure 2-6 and Figure 2-8, one can see that trapping appears to happen significantly closer to the solid surface than the eddy center, for the device and particle geometries an lengthscales used here. There are several differences between these experiments and previous results, leading to the following conjectures. Foremost, our current device fabrication is an order of magnitude smaller and uses elastomeric materials rather than rigid materials for construction. Though we non-dimensionalize appropriately to remove size and frequency effects, material compliancy has not been addressed previously. The rubbery PDMS and higher frequencies associated with smaller systems create opportunities for small but observable compliance effects and structural resonance in the device. Moreover, small defects in fabrication will give rise to larger effects as fabrication length scales shrink. We also know that trapping is dependent on the particle size and the surrounding media properties, via the particle Stokes number, $St = \frac{d_p^2 \rho_\phi \omega_l}{18 \mu}$, as described by the Maxey-Riley equation for particle transport [116-118]). For example, very small particles ($d_p \ll a$) are not observed to trap, but particles comparable in size to $a$ do trap (cf. Figure 2-3 to Figure 2-5 with Figure 2-7). In short, a more quantitative theory for the stability and locations of steady streaming traps, as well as their dependencies on particle Stokes number, is necessary to accelerate our understanding of these scaling effects.
Conclusions and implications

We have explored 9 different steady streaming microdevice geometries to evaluate the connection between geometry, steady streaming flow, and microparticle trapping. The overall features of flow and particle trapping adjacent to wall protrusions and freestanding posts are quite similar among geometric pairs. Cavities have a significantly different steady flow structure, flow strength, and particle trapping stability. At a finer scale, replacing a flow symmetry line in a freestanding feature with a wall slightly disrupts the symmetry and shape of the eddy. Though we have established a fast and valuable 2D-based computational design methodology for understanding flow near the device mid-plane, we describe the need for more quantitative methods to predict details of the particle trapping physics.

The scale of experiments demonstrated here, along with the ability of these devices to trap and position cell-sized objects at a fixed point in x, y, and z suggests that steady streaming traps are well suited for cell separation, filtering, and single-cell analysis. We are exploring these elements further.
Chapter 3. Microfluidic cell trapping array design rules to achieve desired detection and determination limits

Summary

Quantitatively linking a collection of single-cell measurements to population behavior is an emerging area for microfluidic arrays. Proper statistical design is a critical element in making that link. We describe a general procedure for evaluating data quality, detection, and determination limits when using arrayed trapping devices that load a homogenized mixture of micro-objects followed by trapping. In this case, the a priori assumption is that trapping follows a Poisson distribution. We demonstrate the application of two statistical tests (equality of mean and variance, and a Pearson’s chi-square goodness of fit) to evaluate the operational domain where micro-object trapping is adequately described by a Poisson distribution. It is shown that finite size effects and particle masking eventually cause deviations from a Poisson distribution when conditions are chosen that produce large clusters. Once the conditions for Poisson trapping are known, the detection limit for rare objects and uncertainty in quantitative properties measured on the array are readily determined. In the dilute trapping limit, we show that counting the empty traps is a quantitatively effective method for determining titer. The set of statistical approach we presented here can be applied to various microparticle trapping arrays and tweezers, to improve the design and experiments of cell-trapping microarrays.
**Introduction**

Every cell is different. Recent studies reveal a large degree of individual cell heterogeneity, even within populations of the same genotype, resulting in diverse phenotypic traits such as growth, morphology, production, protein expression, translation rate, virulence triggering and cell fate decision[7-10] Phenotype diversity can be displayed within a single genotype based on stochastic processes or environmental stimuli.[4, 11-14] Moreover, local and systematic cell-cell interaction networks can modulate phenotype traits such as cell development, homeostasis, ageing, virulence production, and disease progression.[15-17] Flow cytometry and fluorescence activated cell sorting (FACS) methods are widely used to sort and count heterogeneous phenotypes into two or more classes.[28, 29] However, cytometry and FACS provide information at a single time snapshot of the culture. Alternatively, microscopy-based cell tracking algorithms have a fraction of the throughput of FACS, but they enables monitoring of single-cell dynamics in real-time as a route to understanding population dynamics.[119]

The microfluidics community has been designing cell trapping arrays and tools to efficiently monitor and characterize cellular phenotypes and activity in both research and clinical settings. [27, 33, 35-38] Creativity has been poured into designs that decrease sample volumes, increase throughput, are compatible with established optical detection methods, and provide culturing conditions needed to ensure biologically relevant results. [7, 27, 39] Design of experiments and sampling schemes for statistically-reliable cell characterization has been well discussed for systems like hemocytometers[120], flow cytometers[121] and gene expression arrays[122], but much less so in the field of cell-trapping arrays. For example, it is common in the trapping array literature to validate the basic traits of how cells distribute on the array using
 qualitative goodness of fit evaluation.[36, 123-125] In contrast, the high throughput gene expression array community uses sophisticated statistical analysis to glean the maximum quantitative information.[11, 126] As cell trapping array designers push for smaller sample volumes and trap dimensions, in order to achieve higher throughput, we show that the statistical properties of the device can change, at times invalidating assumptions that underpin device use and cell quantitation.

For the purposes of this paper, we distinguish two major classes of suspension cell-trapping microarrays. One class of microarrays that we term “continuous trapping” arrays that are designed to continuously trap the suspension cells from a flowing sample. These arrays resemble a cell filter, with cells systematically loading from the upstream side.[25, 35, 127] Alternatively, the trapping devices we call “load-then-trap” arrays randomly distribute the cells into the traps, either by a two step process of loading from a random mixture then turning on the traps, or by physically distributing the random mixture of cells all at once onto the array prior to analysis. Examples of this class of trapping mechanisms include common cell counting and trapping devices such as microwells[24, 40] and counting chambers (hemocytometers)[120, 128], as well as cell manipulating “tweezer” systems such as optical tweezers[42-46], dielectrophoretic traps[47-49], optoelectronic tweezers[50-52], magnetic tweezers[53-57], and hydrodynamic tweezers[129, 130]. While “continuous trapping” can process large amount of sample in a high-throughput manner[25, 35, 127], we focus on “load-then-trap” devices that enables observation of cellular dynamics as a function of time. The general class of “load-then-trap” arrays are versatile and when properly designed, we show they providing a quantitative link between single-particle and population-based properties. We characterize and exploit the statistical properties of these arrays to illustrate the design of array size and sampling schemes, how this
design enables quantitative analysis, and how to detect the breakdown of assumptions that underpin quantitation.

**Experimental methods and statistical basis**

*Hydrodynamic tweezer array fabrication and operation*

The devices used here are fabricated following standard soft lithography, with details of fabrication and operation described earlier.[130] In brief, rectangular PDMS microchannels (100 μm high and 1000 μm wide) with a square array of cylindrical obstructions (50 μm in diameter, pitch-to-pitch distance 125 μm) were cured and peeled from an SU-8 master. The actual dimensions of the device used in all of these studies was measured using a laser scanning confocal microscope (LSM 510 Confocal Microscope, Zeiss) and Scanning Electron Microscope (FEI Sirion SEM). The rectangular fluid channel had a height of 116 μm, width 1000 μm, and length of 30 mm. Typical cylinder radii were $a = 25 \, \mu m$.

The placement of multiple cylinders in the channel enabled arrayed microparticle trapping when filled with a liquid sample and subjected to audible frequency fluid oscillations. Flow oscillations were driven at 5 kHz and small displacement amplitude (roughly 1-3 μm) by displacement pumping with paired piezoelectric disks on both ends of the channel (Piezo Systems, USA). The waveform generator is an Apple iPhone. Since the device is transparent, flow field and particle imaging was done with optical methods. All experiments were performed at room temperature.
Polystyrene particles with 5 µm radii (Polyscience, Inc.) were used in all experiments. The particles have small but finite Stokes numbers, and were shown to get trapped near the core of each microeddy when the fluid oscillation was turned on. To vary the concentration of particles, the solution was diluted from bottle strength to the desired concentrations with filtered, sterile DI water. Approximately 1 ppm of dish soap was added to the solution to prevent particle agglomeration. The dispersed particle solution was then loaded to the device with a syringe. After loading, fluid oscillations began and the trapped particles were trapped in the nearest eddy core. Each of our experiments sampled 336 microeddies where the particles trapped.

Figure 1 is a top-down image of 12 cylindrical posts that generate 48 square-symmetry microeddy traps (each trap, though not visible, is denoted by the perpendicular dashed lines along symmetry planes). Also seen in the image are a total of 47 particles distributed among the 48 traps. Particles trapped in each quadrant of the posts were imaged with an optical microscope (BX51, Olympus) and counted with auto-detection software that identified the post structures with green circles, and the particles in red. Within Figure 1, one can observe 19 microeddy traps with no particles (k=0), 16 micro eddy traps with one particle (k=1), 8 microeddy traps with two particles (k=2), and so on. Bright field image of the 336 eddies area were taken with a color camera (QImaging U-TV0.63XC, Olympus), and then processed by our particle counting software. Eleven different particle concentrations (0.09 - 5.40 x 10^6 particles/mL) were tested, with each concentration repeated in 24 or more replicates. The device was trapped an identical fixed sample volume every time.

The particle detection algorithm uses the optical image to automatically find the posts and all the particles. Detection is accelerated by assuming that the size of the posts and the particles
are constant and known *a priori*, and that the fabricated array is spatially periodic. The basic algorithm first applies circular template matching[131] to detect the posts in the image, followed by the use of the RANSAC algorithm[131] to identify the grids. Once the grid and trap geometry is defined, particles are detected using the same template algorithm, tuned to the size of the particles. Due to image noise or specific particle arrangements, the template matching might contain errors (false or missing objects), so we use the periodicity of the fabricated array system to identify this class of error and fix it. The source code and experimental data for all our experiments will be made available in Supplementary Materials.

Figure 3-1. Plan view photo of 12 posts after particles have been loaded then trapped. Software first identifies post structures (50 µm green circles) and uses symmetry to draw the microeddy trap boundaries (drawn over part of the image with dashed lines). Trapped particles in each quadrant of the posts were identified (small red circles) and counted, giving rise to the experimentally observed distribution of particles and their frequency. Actual arrays had 336 traps per sample.
Table 3-1. Example showing the counting particles seen in Figure 3-1 and the resulting calculated quantities used in subsequent analysis with large arrays. The quantity $\lambda_{\text{exp}}$ is proportional to particle concentration.

<table>
<thead>
<tr>
<th>Number of particles per trap ($k$)</th>
<th>Number of Occurrence</th>
<th>Frequency $f(k)$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>19</td>
<td>$19/48 = 0.3958$</td>
</tr>
<tr>
<td>1</td>
<td>16</td>
<td>$16/48 = 0.3333$</td>
</tr>
<tr>
<td>2</td>
<td>8</td>
<td>$8/48 = 0.1667$</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>$5/48 = 0.1042$</td>
</tr>
<tr>
<td>&gt; 3</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

$\lambda_{\text{exp}} = (0*19+1*16+2*8+3*5)/48 = 0.9792$

Statistical Basis for the Analysis

Knowing how data is distributed allows quantitation of uncertainties in measurements. Briefly, this section lays out the basis for our subsequent analysis. Superficially, load-then-trap experiments provide discrete and independent trapping events when the sample particles are fully suspended, the test volume is constant, and the particles are distributed randomly prior to turning the traps “on”. Under these conditions, it is reasonable to expect trapping to follow a Poisson distribution:[132, 133]

$$P(k, \lambda) = \frac{\lambda^k e^{-\lambda}}{k!}.$$  \hspace{1cm} (1)

where $P(k, \lambda)$ is the theoretical probability of finding an integer $k$ number of particles per trap (0, 1, 2…) when the average number of particles held in a trap is $\lambda$. Given a device with $n$
total traps, each yielding a measured values of $k_i$, we can experimentally estimate the maximum-likelihood value of the parameter $\lambda$ for the sample as

$$\lambda_{exp} = \frac{1}{n} \sum_{i=1}^{n} k_i .$$  \hspace{1cm} (2)

Table 3-1 illustrates this calculation for the small sample dataset given in the figure. The quantity $\lambda_{exp}$ is proportional to particle concentration, and is given by the bulk concentration (# particles/mL) multiplied by the volume (ml/trap) of an individual trap.

A common, but inadequate, method for assessing a Poisson distribution is to experimentally measured the frequency of occurrence, for example as tabulated beneath Figure 3-1, and compare it to Eq. (1) with $\lambda_{exp}$ substituted into the equation. This is the only test we have found in prior work involving cell trapping arrays.[36, 123-125] A better test for assessing if data obeys a Poisson distribution is to show that the mean $\lambda_{exp}$ is equal to the measured variance $\sigma^2$ in the data, and the best test for assessing data fit to a Poisson distribution is the Pearson’s chi-square ($\chi^2$) test.[134, 135] For Pearson’s chi-square test, the theoretical probability $P(k, \lambda_{exp})$ in each category $k$ is calculated using equation (1), and then converted into a theoretical number of occurrence, $\varphi = n* P(k, \lambda_{exp})$. The chi-square value of each category is computed as

$$\chi^2_{m-p-1} = \sum \frac{(\varphi_{exp} - \varphi)^2}{\varphi}$$  \hspace{1cm} (3)

where $\varphi_{exp}(k)$ is the experimentally observed number of occurrence tabulated in table 1. Here we use a significance level $\alpha = 0.05$ to define where the chi-squared test fails, leading to a rejection of the hypothesis “trapping behavior follows Poisson distribution”. Also needed for this test is the degrees of freedom (DOF) for the dataset, defined as $DOF = m-p-1$, where $m$ is the
number of categories or classes of data and $p = l$ is the number of parameters estimated from the data.

**Results and Discussion**

*Distribution of particle trapping in the load-then-trap hydrodynamic tweezer array*

Figure 3-2 shows the results of 299 separate trials for particle trapping in the microeddy device, using 11 different concentrations of particles. In general the data points scatter along the 45 degree line (slope = 1), where the sample mean $\lambda$ is equal to sample variance $\sigma^2$. Given that the mean nearly equals the variance for most of the 299 trails, it appears the data are Poisson distributed, though data quality seems to breaks down for $\lambda_{exp} > 1.2$. A better test is the chi-squared evaluation of each trail, using Eq. (3) when DOF≥1. The red circles (O) in Figure 2 denote trials that passed the chi-square goodness of fit test, whereas black crosses (X) denote trials that failed the test. There are 299 points in total with 207 pass, 49 fail, and 43 data points with too few degrees of freedom to evaluate by this test. The high failure rate at $\lambda_{exp} > 1.2$ is an indication that trapping does not meet one of the requirements for Poisson to hold, namely, randomly distributed particles that trap as independent events.

Every microfluidic load-then-trap array will have an upper limit where Poisson breaks down, owing to physical size constraints where particle-particle or particle-force field interactions mean adding the next particle harder or easier than the one before. At this point, the assumption of independence is violated. Figure 3-1 shows how physical size constraints of a trap lead to particle-particle interaction in hydrodynamic tweezers. For $\lambda_{exp} \approx 0.98$, the number of particles in any given trap varies widely. However, Figure 3-1 shows that any trap with a single
particle, has it located roughly on the 45° diagonal of the symmetry line, trapped stably. When there are two particles in the trap, they tend to stably rotate as a doublet along the 45° line, whereas 3 or more particles are less predictable in their location. These sorts of particle-particle interactions can lead to the 4\textsuperscript{th} or 5\textsuperscript{th} particle being significantly more difficult to trap. Physical constraints can be changed by redesigning the array with larger pitch, but this provides a trade-off in larger sample volumes for the same n. Another source of non-Poisson behavior can be particles that vertically stack up in trap, making them hard to see in 2D imaging. Moreover, if the particles are hard to disperse due to attractive interactions, then it would be more probable to find doublets and higher than predicted.

Each type of load-then-trap array employs a different trapping geometry and mechanism, but each is likely to have similar physics-based constraints and sample/measurement-based constraints that are likely to become more challenging as sample volumes decrease and sample particle concentrations increase.
Figure 3-2. Correlation of measured sample mean $\lambda_{\text{exp}}$ and variance $\sigma^2$ for 299 samples acquired at 11 different particle concentrations. One test for data fit to a Poisson distribution is the equality of mean and variance for a given sample. A more stringent test is whether the sample passes (O) or fails (X) a Pearson chi-squared test. Dilute-limit samples had zero degrees of freedom (∆) and were not tested for fit.

Data that failed our chi-squared test were removed from our subsequent analysis, which relies on the validated assumption that the data is Poisson distributed. Figure 3-3 shows the averaged experimental frequencies for each $k_i$ from the experiments (data points) at the 11 different particle concentrations, plotted along with their theoretical frequencies using Eq. (1) and $\lambda_{\text{exp}}$ (lines). As expected, our data shows excellent agreement with the theoretical approximation from equation (1). The inset for Figure 3-3 shows the average value of $\lambda_{\text{exp}}$ for each of the particle concentrations used, confirming the linear relationship between $\lambda_{\text{exp}}$ and concentration. The slope of the inset line is equal to the volume per trap, yielding 301 pL/trap.
Estimates based on measurements of the device dimensions suggest the volume per trap is roughly 340 pL/trap, showing good agreement with our measurements.

Figure 3-3 Computed Poisson distribution probabilities compared to the averaged experimental frequencies from 11 particle concentrations. Inset shows λ_{exp} plotted against the concentration of particles in solution. The experimental data points correspond to sample concentrations: (A) 0.09x10^6 particle/ml, yielding λ_{exp} of 0.0346; (B) 0.18x10^6 particle/ml, λ_{exp} = 0.067; (C) 0.36x10^6 particle/ml, λ_{exp} = 0.112; (D) 0.45x10^6 particle/ml, λ_{exp} = 0.1468; (E) 0.54x10^6 particle/ml, λ_{exp} = 0.1635; (F) 0.72x10^6 particle/ml, λ_{exp} = 0.2387; (G) 0.90x10^6 particle/ml, λ_{exp} = 0.3264; (H) 1.80x10^6 particle/ml, λ_{exp} = 0.5760; (I) 2.70x10^6 particle/ml, λ_{exp} = 0.7794; (J) 3.60x10^6 particle/ml, λ_{exp} = 1.0668; (K) 5.40x10^6 particle/ml, λ_{exp} = 1.4082.
Confidence Interval for $\lambda_{\text{exp}}$ (determination limits [136])

As we’ve shown above, the parameter $\lambda_{\text{exp}}$ is proportional to the particle concentration and can be used to estimate the population cell count. It is important to examine the confidence limits of the estimator, which is determined by the microarray design and sampling scheme. Ultimately, in this section, we seek to answer the question: “How do I design my experiment to determine the cell count (and any per cell property) with high confidence?”

The Poisson distribution parameter $\lambda$ values approximated from the dataset that passed the chi-squared test can be used to calculate the confidence limits. For Poisson distributed $k$, the upper and lower limits on the mean value $\lambda$ can be found using central confidence intervals at a level $1-\alpha$, we set $\alpha_{lo} = \alpha_{up} = \alpha/2$, producing to be (detail see appendix)[137-139]

$$
\frac{\chi^2(\frac{\alpha}{2}; 2Nn\lambda_{\text{exp}})}{2Nn} \leq \lambda \leq \frac{\chi^2(1-\frac{\alpha}{2}; 2Nn\lambda_{\text{exp}+2})}{2Nn}
$$

(4)

where $\chi^2(X; Y)$ is the chi-square deviate with lower tail area $X$ and degrees of freedom $Y$, $N$ is the number of replicated tests.

Equations (4) is the foundation for designing a load-the-trap array to count cells (and measure their properties) to achieve a particular level of confidence in the measurement, so that inferences about the population can be reliably made form array data. Equations (4) shows that the confidence interval is identical if one does $N$ replicates on an array with $n_1$ traps vs. a single replicate on a large array with $n_2$ traps, so long as $n_1N = n_2$. The method above requires that every cell/particle be counted in determining $\lambda_{\text{exp}}$ and the experimental frequency of occurrence.

Before showing how sampling choices and array size impact measurements, it is worthwhile developing a simplified method for estimating particle concentration and the uncertainties associated with that estimate when $\lambda_{\text{exp}} \sim O(1)$. 

A simpler treatment when $\lambda_{\text{exp}} \sim O(1)$ or smaller

Figure 3-3 provides a clue for a simpler way to estimate population traits. In Figure 3, one observes that roughly 25% or more of the traps remain vacant for all values of $\lambda_{\text{exp}}$ used here. A single measurement of empty traps is sufficient to estimate $\lambda$, since a Poisson distribution has a single parameter. From equation (1) we see the probability of having zero particles in a trap is

$$P(0, \lambda_0) = e^{-\lambda_0},$$

so that

$$\lambda_0 = -\ln P(0, \lambda_0).$$

Equation (5) and (6) show that one can get an estimate of $\lambda$, denoted as $\lambda_0$, by only counting the empty traps ($k=0$). What is the price one pays in uncertainty by estimating cell counts from the empty traps? Trapping either zero or more than zero particles is a binary process, allowing us to propose a normal approximation method (detail see appendix) to calculate the confidence limits as

$$-\ln\left(\frac{n_0}{n} + Z_{1-\alpha/2} \sqrt{\frac{n_0(1-n_0/n)}{n}}\right) \leq \lambda_0 \leq -\ln\left(\frac{n_0}{n} - Z_{1-\alpha/2} \sqrt{\frac{n_0(1-n_0/n)}{n}}\right)$$

where $Z_{1-\alpha/2}$ is the $1-\alpha/2$ percentile of the standard normal distribution, $n_0$ is the number of empty traps without any particle in present.

The results of this section are shown in Table 3-2 and Table 3-3 for the particle trapping array data presented in Figure 3-3. Table 3-2 presents the upper and lower confidence limits of average $\lambda_{\text{exp}}$ values from counting all the trapped particles using Equations (4). Table 3-3 shows
the upper and lower confidence limits of the approximated $\lambda_0$ values from counting only empty traps using the binomial-normal approximation of equation (7). One can see that the confidence interval of an experimental data set is dependent on the estimated parameter $\lambda_{\text{exp}}$, number of eddies $n$, and the number of trials $N$. In both cases, while average $\lambda$ is linearly proportional to sample concentration, increasing the number of replicates $N$ gives tighter limits for $\lambda$. Figure 3-4 graphically shows the linear relationship between sample concentration and the estimated $\lambda$ values from a single trail, using the full method laid out in Eq. (4) or the approximate method from Eq. (7). The tabulated and graphed data show that there is not too much penalty to pay for measuring only empty traps, when $\lambda_{\text{exp}} < 1$. Both methods give similar linear relationship with particle concentration. When estimating $\lambda$ by counting only the empty traps, confidence interval increases with the particle concentration since there are progressively fewer empty traps to count.

The equations given above provide design guidance for the device size ($n$) and the replicate sampling regime ($N$). If one knows the range of cell concentration to be expected, then the design will need to accommodate physical or fabrication constraints that set $n$, while planning for a replicate sampling scheme that reduces the confidence limits to an acceptable level for the experiment.
Table 3-2. Confidence limits of experimental $\lambda_{\text{exp}}$ from Chi-square distribution approximation ($\alpha=0.05$)

<table>
<thead>
<tr>
<th>Concentration (10^6 particles/ml)</th>
<th>N</th>
<th>n</th>
<th>average $\lambda$</th>
<th>single trial</th>
<th>N trials/observations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>lower limits</td>
<td>upper limits</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>lower limits</td>
<td>upper limits</td>
</tr>
<tr>
<td>0.09 x 10^6</td>
<td>24</td>
<td>336</td>
<td>0.034598</td>
<td>0.017394</td>
<td>0.060486</td>
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<tr>
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<td>0.111951</td>
<td>0.078783</td>
<td>0.153509</td>
</tr>
<tr>
<td>0.46 x 10^6</td>
<td>34</td>
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<tr>
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<td>0.163549</td>
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</table>

Table 3-3. Comparison of Confidence limits of $\lambda_0$ from Binomial-Normal distribution approximation ($\alpha=0.05$)

<table>
<thead>
<tr>
<th>Concentration (particles/ml)</th>
<th>N</th>
<th>n</th>
<th>average $P(0,\lambda_0)$</th>
<th>average $\lambda_0$</th>
<th>single trial</th>
<th>N trials</th>
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<td>upper limit</td>
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Figure 3-4. Comparison of the relationship between sample concentration and estimated values of \( \lambda \) when counting all particles or just counting empty traps in a single trail. Uncertainty in the two measured values are also given. The constant of proportionality between \( \lambda \) and concentration is the volume of an individual trap, determined to be approximately 301 pL based on the best fit slope. Based on our estimate of the device geometry, the trap volume is expected to be approximately 342 pL.

Detection limits of rare events

The previous sections described the strategy for dealing with large number of particles/cells, such as whole blood count samples. Now if we consider detecting a sub-population of rare event, say rare circulating tumor cells in blood stream, the design of the device and sampling regime to achieve a certain “detection limit”, i.e. probability of detecting one or more rare cell from the sample, is more straightforward than the determination limits described
above. (Note that the “detection limit” we discuss here assumes the process of measurement, like imaging methods, software, etc., is reliable with high capability.[136]) If the probability of finding a rare cell in the total population is $x$, it can be shown that the probability of not finding a rare cell in a trap is

$$P(\text{rare cell} = 0, \lambda) = e^{-x\lambda}, \quad (8)$$

where $\lambda$ is the average number of cells per trap based on the total cell population. Extending Eq. (8), it is clear that the probability of not finding any rare cells in an array of $n$ traps is

$$P(\text{rare cell} = 0, \lambda)^n = e^{-x\lambda n}. \quad (9)$$

This result means that the probability of find one or more rare cells is $1 - P(0, \lambda)^n$ and that means there is 95% confidence that one or more rare cells will be detected when

$$1 - e^{-x\lambda n} \geq 0.95. \quad (10)$$

This equation provides a realistic minimum boundary for the amount of sample a device or array should process to find a significant rare cell event, such as FACS or cell trapping arrays, when dealing with genuinely rare cells. If one hopes to detect circulating tumor cells in an array when they represent 1 out of every $10^8$ healthy blood cells, then $x = 10^{-8}$ and the array must be massive, per Eq. (21), and/or the number of replicates ($N$) must be massive to ensure $x\lambda N \geq 2$.

**Conclusion**

We demonstrate the design guidelines of a statistical analysis approach for cell-trapping microarrays. We presented a microeddy hydrodynamic trapping platform to perform particle counts and distribution analysis. We defined and examined the Poisson distribution behavior of
particle trapping in microeddies. Based on the statistical observation, we proposed and demonstrated a fast particle quantification method of counting empty traps at lower particle concentration, and showed the differences of two methods. We then addressed the logistics of counting/detecting rare cells from a bulk population. The set of statistical approach we presented here can be applied to various microparticle trapping arrays and tweezers, to improve the design and experiments on these device towards the practical use of single-cells research.

**Acknowledgements**

We thank Prof. Marcia Ciol, Ms. Ping Xu and UW statistics department for statistical advice. This research is funded by the National Science Foundation NSF IGERT DGE-0654252 and Boeing-Sutter endowment.

**Appendix: Derivation of confidence interval**

*Counting all particles*

The Poisson distribution parameter $\lambda$ approximated from the dataset that passed our chi-squared test can be to calculate the confidence limits. For Poisson distributed $k$, the upper and lower limits on the mean value $\lambda$ can be found to be [137-139]

$$
\lambda_{low} = \frac{1}{2n} F_{\chi^2}^{-1}(\alpha_{lo}; 2k) 
$$

(11)

$$
\lambda_{up} = \frac{1}{2n} F_{\chi^2}^{-1}(1 - \alpha_{up}; 2k + 2) 
$$

(12)
where the upper and lower limits are at confidence levels of \(1 - \alpha_{lo}\) and \(1 - \alpha_{up}\), respectively, and the quantity \(F^{-1}_{\chi^2}\) is the quartile of the chi-square distribution. Using central confidence intervals at a level \(1 - \alpha\), we set \(\alpha_{lo} = \alpha_{up} = \alpha/2\), producing

\[
\frac{\chi^2(\frac{\alpha}{2}; 2k)}{2n} \leq \lambda \leq \frac{\chi^2(1-\frac{\alpha}{2}; 2k+2)}{2n}
\]  

(13)

where \(\chi^2(\alpha; b)\) is the chi-square deviate with lower tail area \(\alpha\) and degrees of freedom \(b\).

Here \(k\) is the total number of particles, \(k = n\lambda_{exp}\).

\[
\frac{\chi^2(\frac{\alpha}{2}; 2n\lambda_{exp})}{2n} \leq \lambda \leq \frac{\chi^2(1-\frac{\alpha}{2}; 2n\lambda_{exp}+2)}{2n}
\]  

(14)

For experiments with \(N\) replicates, the equation can be written as

\[
\frac{\chi^2(\frac{\alpha}{2}; 2Nn\lambda_{exp})}{2Nn} \leq \lambda \leq \frac{\chi^2(1-\frac{\alpha}{2}; 2Nn\lambda_{exp}+2)}{2Nn}
\]  

(15)

where \(N\) is the number of trials. When quantiles of the chi-square distribution are not available, Byar’s approximation can be applied.[139-141]

**Counting only empty traps**

From equation (1) we see the probability of having zero particles in a trap is

\[ P(0, \lambda) = e^{-\lambda} \]  

(16)

so that

\[ \lambda_0 = -\ln P(0, \lambda_0) \]  

(17)
Equation (9) and (10) show that one can get an estimate of $\lambda$, denoted as $\lambda_0$, by only counting the empty traps ($k_i=0$). Trapping either zero or more than zero particles is a binary process, allowing us to propose a normal approximation method to calculate the confidence limits as

$$P(X = 0) = e^{-\lambda_0} = \theta$$  \hspace{1cm} (18)

$$P(X > 0) = 1 - \theta$$  \hspace{1cm} (19)

so the binary probability distribution function can be written

$$\ln P(k) = \sum \ln \left\{ \frac{\theta}{1 - \theta} \right\}^{k \text{ if } k = 0} \left\{ \frac{1 - \theta}{\theta} \right\}^{k \text{ if } k > 0}$$  \hspace{1cm} (20)

$$= n_0 \ln \theta + (n - n_0) \ln (1 - \theta),$$  \hspace{1cm} (21)

where $n$ is the total number of traps, and $n_0$ is the number of empty traps. To get the experimental maximum likelihood estimate of $\theta$, we let the differential of probability distribution function equal zero, yielding a maximum likelihood estimate of $\theta$

$$\theta_{MLE} = \frac{n_0}{n}. \hspace{1cm} (22)$$

The normal approximation of confidence intervals for the binomial distribution $\theta$ is then

$$\theta_{MLE} \pm Z_{1-\frac{\alpha}{2}} \sqrt{\frac{\theta_{MLE}(1-\theta_{MLE})}{n}} \hspace{1cm} (23)$$
where $Z_{1-\alpha/2}$ is the $1-\alpha/2$ percentile of the standard normal distribution. Since $\lambda_0 = -\ln \theta$ from Eq. (11), we estimate the confidence interval for $\lambda_0$ as

$$-\ln\left( \theta_{\text{MLE}} + Z_{1-\alpha/2} \sqrt{\frac{\theta_{\text{MLE}}(1-\theta_{\text{MLE}})}{n}} \right) \leq \lambda_0 \leq -\ln\left( \theta_{\text{MLE}} - Z_{1-\alpha/2} \sqrt{\frac{\theta_{\text{MLE}}(1-\theta_{\text{MLE}})}{n}} \right) \quad (24)$$
Chapter 4. Design and Implication of A High-throughput Microeddy Single-cells Co-culture Array

Summary

Cells tend to live in communities where cell-cell interactions are critical at every step of their life-cycle. Thus, microfluidic diagnostic tools need to create environments that accommodate cell-cell interactions and allow the dynamic monitoring of cellular phenotypic development. In this work, we presented a particle trapping platform using arrays of microeddies that can be paired with statistical design to assemble and hold mixed populations of micro-objects in predictable singlet, doublet, triplet and higher order combinations. Microeddy arrays paired with statistical design guidelines are shown to achieve the following goals: (1) create mixed populations with predictable sub-population ratios, (2) enable trapping in a hydrodynamically-isolated environment, (3) compatible with the optical tools commonly used to monitor expression, cell division, motility, and other cellular activity, (4) provide high-throughput. The statistical tools and experimental design we present here can be applied to different kinds of microtrapping arrays, thereby guiding the broader device system design to support the microbial co-culturing community.

Introduction

Individual cells of identical genotype can display phenotype heterogeneity within a population. Phenotypic characteristics such as growth, morphology, protein expression,
translation rate, virulence triggering and cell fate decisions can be heterogeneous owing to stochastic factors or in response to local environmental stimuli, resulting in individual cells that deviate from the overall population behavior. [4, 7-14] Moreover, in nature, cells do not exist in isolation but tend to live as communities. Cell development, disease progression, and cell death are largely associated with the local and systematic cell-cell interaction within the community networks they live in.[15-17, 126] Probing cellular response to local environmental stimuli and signaling biomolecules is important for understanding cell-cell variation and interactions, as well as cellular dynamics. Nonetheless, the tools needed to study single-cell heterogeneity and cell-cell interactions within microbial communities are still being developed.

A major issue that limits the study of microbial communities in vivo is an ability to co-culture the cells. Conventional laboratory cultivation mainly relies on pure culture of individual species, so the majority of microbes found in nature have not been cultured in the lab due to a lack of known co-culturing conditions.[19, 142] Thus, the current approaches for characterizing microbial communities [27-32] rely on microbial inference using data ranging from flow cytometry, ribosomal RNA[143] to metagenomic sequencing[144, 145]. Unfortunately, these methods are not well suited to monitoring the dynamics of cellular networks in real-time, as they involve complex multi-step procedures to extract the data [146] and lack the needed temporal throughput.[33]

The desire to probe cellular dynamics and interactions has prompted the lab-on-chip community to design or adapt devices for this purpose.[147] The main approach is “multiplexed trapping” so that large numbers of isolated, randomly distributed individual cells, or countable clusters of cells, can be monitored as a function of time. [33-38] [20, 36] Examples of “multiplexed trapping” include microwell arrays, classic hemocytometers, or the creation of
many microgel/droplet particles that each hold cells[20, 36]. The design goals are to increase throughput, produce a statistically representative sample so that individual and clustered cells can be related to via population measurements, provide physiologically comparable conditions to those found in vivo, and leverage established monitoring tools, especially optical methods found in many labs.[7, 27, 39] A critical question in this field is how to use the statistical distribution established during loading to tailor the local co-culture ratio, so that local cues and cell-cell interactions can be designed into the microtrapping experiment to create libraries for rapid screening of cell-cell interactions[123].

Here we show that microeddy arrays are an excellent platform for designing co-culture experiments. Our focus is on the distribution of individuals and countable clusters of mixed particles. Prior work has shown that microeddy devices can trap dense or buoyant particles, and can maintain viable diatoms, motile algae, yeast, macrophages and monocytes in a variety of different media[129, 130, 148, 149]. The microeddies stably trap individual cells under physiologically-relevant condition, where cells are maintained in suspension without perturbative external electrical, optical, or surface forces applied to the cells. The hydrodynamic trapping forces (and shear levels experienced by the suspended cells) are easily controlled by the frequency and amplitude of the oscillating flow.[130, 150] A critical, but subtle, feature is that each eddy is convectively-mixed within its volume, but relatively poorly mixed between eddies, because a dividing streamline separates each eddy. Transport across the dividing streamlines is limited by slow molecular diffusion because convective mixing $\nu \cdot \nabla c$ is identically zero. We’ve shown experimentally and with simulation that this means each eddy can be though of as a leaky, but substantially chemically isolated, volume for each cell or cluster of cells.[60, 61, 66] The statistical methods we demonstrate here provide the foundation of design-of-experiments that
will enable a wider range of microdevice-based measurements of the microbiome within diverse areas of microbiology and medical sciences.

**Experimental method and statistical analysis**

*Microeddy array fabrication and operation*

The device is fabricated following standard soft lithography, with details of the fabrication described previously.[130] Briefly, a rectangular PDMS microchannel was cured and peeled from a SU-8 master with a square array of 50 μm (diameter) cylinders arranged with a pitch distance of 125 μm. The PDMS channel layer is sandwiched between two glass slides with paired piezoelectric disks on both ends of the channel to drive fluid oscillations back and forth through the channel. Dimensions of the rectangular channel structure were measured and confirmed using a laser scanning confocal microscope (LSM 510 Confocal Microscope, Zeiss) and Scanning Electron Microscope (FEI Sirion SEM) with height \( h \) of ~100 μm, width \( w \) = 1000 μm, and length \( l \) of 30 mm. The completed device is thin, transparent, and about the size of a microscope glass slide, making it easy to operate with standard optical methods. A smartphone with waveform generator application drives an audio amplifier that is connected to the paired piezoelectric disks to actuate fluid oscillations. Sample is injected from a syringe to the inlet port with the outlet connected to a waste beaker. The compact physical arrangement is shown in Figure 1 on an optical microscope stage.
Flow visualization and Particle trapping

Flow oscillations are driven by displacement pumping with paired piezoelectric disks on each ends of the channel (Piezo Systems, USA) operating with a sinusoidal voltage. The fluid oscillations was operated at 5 kHz with a displacement amplitudes of 1-3 μm. All experiments are at room temperature. The completed device is small, light, transparent, and can be easily placed onto an optical microscopes like a standard microscopic slide, Figure 1. Figure 2 shows an image of one micron fluorescent polystyrene particles suspended in water (Fluoresbrite® Multifluorescent Microspheres, $d_p=1$ μm, $\rho_p=1.05$ g/cm$^3$, Polysciences, Inc.) with 850 ms exposure time that reveals the fluid path lines of the microeddy motion. These small particles of
nearly neutral buoyancy have negligible inertia and tend to trace the fluid motion. Larger particles with some inertial effects behave differently, and tend to be trapped near the center of each eddy. When imaging the regions of negligible streaming flow (far from channel features or walls), straight particle path-lines parallel to the fluid oscillation revealed the oscillation amplitude $s$. Trapping experiments used larger (5 - 10 µm) polystyrene particles that were diluted to desired concentrations with filtered, sterile DI water containing approximately 1ppm of detergent to prevent agglomeration. Mixtures of 10 µm non-fluorescent polystyrene particles (Polysciences, Inc), 10 µm green fluorescent polystyrene particles (Dragon Green, Bangs Laboratory), and 5 µm yellow fluorescent polystyrene particles (Polysciences, Inc) were prepared at different concentrations. The homogenized sample solutions were loaded onto the device with a syringe. As we turned on the fluid oscillation, particles were trapped in the nearest eddy where they distributed.
Figure 4-2. Illustration of basic device operation and geometry. (A) Schematic of the sinusoidal actuation of paired piezo disks used to induce a primary oscillating flow in the channel and a secondary steady streaming flow within the array section. (B) Schematic of the steady microeddy flow within the array region, and the symmetry-defined volume of each trap. (C) Top view of the microeddy flow structure experimentally imaged with 1μm fluorescent tracer particles. Each cylindrical post (non-fluorescent black circle) generates 4 identical recirculating microeddies separated from each other by dividing streamlines.

Particle trapping and detection from bright field and fluorescent imaging
Particles trapped in 336 adjacent microeddies were imaged with both bright field and fluorescent microscopy (BX51, Olympus) using a color camera (QImaging U-TV0.63XC, Olympus), and then processed with our custom auto-detection software. Detection is accelerated by assuming that the size of the posts and the particles are known \textit{a priori}, the emission colors of the particles are known, and that the fabricated array is spatially periodic with well defined symmetry (as confirmed in Fig 2 (C)). The basic algorithm first applies circular template matching\cite{131} to detect the posts in the image, followed by the use of the RANSAC algorithm\cite{131} to identify the symmetry lines (dividing streamlines) that define each particle trap. Once trap geometry is defined, particles are detected using the same template algorithm, tuned to the size of the particles and their color identified. Due to image noise or specific particle arrangements, the template matching might contain errors (false or missing objects), so we use the periodicity of the fabricated array system to identify this class of error and fix it. The source code and experimental data for all our experiments will be made available in Supplementary Materials. Results from the automated counting system were compared with replicate human counting and showed equal or better reliability.

\textit{Construction of binary, tertiary co-culture combinations in microeddies}

Depending on the particle concentration, each eddy can display one of the following events: (i) no particles are in the trap (denoted \(k=0\)), (2) one particle is in the trap (\(k=1\)), two particles are in the trap (\(k=2\)), and so on. Each particle or small cluster of particles detected in each trap has their color and size recorded. Detection in all 336 traps occurs simultaneously in software to determine the experimentally observed frequencies for the aggregate particle
trapping events, including the frequencies that different particle cluster combinations were observed. Replicate experiments were performed (14 for binary mixtures, 11 for ternary mixtures), providing a total eddy count of $n = 4704$ and $n = 3696$, respectively for binary and ternary experiments.

As we have described previously, the nature of microeddy array trapping is discrete and instantaneous, so it can be characterized by the Poisson distribution [chapter 3] if the particles are homogeneously distributed in the sample. In a given sampling interval,[132, 133] if the expected number of trapped particles per eddy is $\lambda$, then the probability $P$ that there are $k$ particles in any particular eddy is

$$P(k, \lambda) = \frac{\lambda^k e^{-\lambda}}{k!} \quad (1)$$

where $\lambda > 0$ is a real number and $k \geq 0$ is an integer. Poisson distributed sampling has the reproductive trait of combined populations, so when A and B independently follow a Poisson distribution described by $\lambda_A$ and $\lambda_B$, respectively, the mixed population of A and B will follow a Poisson distribution as well. The combined statistic is described by the sum of parameters where $\lambda_{A+B} = \lambda_A + \lambda_B$ [137, 151]. The probability of finding different combinations of particle clusters in any given microeddy trap when the mixture has $p$ components is

$$P \left[ \vec{k}, \vec{\lambda} \right] = \prod_{i=1}^{p} P[k_i, \lambda_i] . \quad (2)$$

Equation (2) is a key design equation that allows an experimentalist to tailor the trapping conditions to the individual cell titer in order to build the distribution of particle clusters one seeks to probe.
Results

Binary system of particle trapping

The theoretically predicted probabilities given in Eq. (2) can be compared to experimentally observed frequency data from array experiments. Applying equation (2) for a binary mixture ($p=2$) of A and B, provides the probability of finding a specific cluster in any eddy as

$$P[k_A, k_B, \lambda_A, \lambda_B] = P[k_A, \lambda_A]P[k_B, \lambda_B] = \frac{\lambda_A^{k_A} \lambda_B^{k_B} e^{-(\lambda_A + \lambda_B)}}{k_A!k_B!}.$$  (3)

Equation (3) provides a connection between the device design and cell concentrations. In our experiments, we observe how many particles ($k_A$ and $k_B$) are in each of the 336 traps. The experimental maximum likelihood estimate for A-type particles ($\lambda_{exp,A}$) is

$$\lambda_{exp,A} = \sum_{k_A=0}^{\infty} k_A f(k_A)$$  (4)

where $f(k_A)$ is the experimentally observed frequency that $k_A$ particles are found in traps on the array (number of traps with $k_A$ particles/total number of traps). A comparable equation holds for B-type particles, or any other type of particle in the array (if the mixture has $p>2$). We’ve shown previously that an alternative way to write Eq. (4) is

$$\lambda_{exp,i} = V_{eddy} \cdot C_i$$  (5)
where $V_{\text{eddy}}$ is the fluid volume per eddy (see Fig. 1(B)), and $C_i$ the sample titer (number per volume) for component $i$. Here, $V_{\text{eddy}} = 0.301 \text{ nL}$ is determined from a calibration run and is controlled by the geometry of our fabricated device, and thus is tunable [Chapter 3].

Figure 3 shows a typical optical image for 12 cylindrical posts that contain a mixture of 10 µm particles (one fluorescent, and one non-fluorescent) distributed among the 48 microeddy traps. Particles were loaded into the device from a homogeneous mixture, and after loading, the fluid oscillation was initiated to form the traps. Traps loaded with a single particle position the particle near the eddy center (c.f. Fig. 2(c) and Fig. 3). When more than one particle trap in an eddy, the cluster of particles tend to orbit one another in the vicinity of the eddy center.[130, 150] Particles trapped in an eddy do not exchange with neighboring eddies. Table 1 shows the automated particle count for each type of particle that was generated from many replicate experiments on the 336 eddy array. The frequencies of occurrence for each type of cluster in the traps was calculated from the number of occurrences divided by the total number of trapping events $n$ in all replicate experiments. The $\lambda_{\text{exp},i}$ values were calculated using Eq. (4), and the sum of the two individual $\lambda_{\text{exp},i}$ values from sub-populations are equals to the $\lambda_{\text{exp}}$ value for the total number of particles (if we do not differentiate between the sub-populations), showing the reproductive trait of the combined populations.

Figure 4 plots the experimental frequency for each particle clusters $(k_W, k_B)$ and compares it with the theoretically calculated probability, Eq. (3), using a value of $\lambda_i$ calculated from the manufacturer’s reported particle titer and Eq. (5). Different cluster combinations $(k_W, k_B)$ are also depicted graphically above the bar graph, and the actual number of occurrences for each cluster are given on top of the figure. The experimental result agrees well with the theoretical prediction. Based on previous chi-square testing of this device, we have shown that this device
follows a Poisson distribution at this particle concentration (i.e. \( \lambda \) value). As a result, the small cluster to cluster deviations seen between experimental frequency values and the theoretical probabilities is likely due to finite sample sizes. Detail of such effect is described in Chapter 3.

Figure 4-3. Non-fluorescent and fluorescent particles trapped from a mixed sample are distributed among all the microeddies, where they are held in suspension near the center of each eddy. The dividing streamline between each eddy (see Fig. 2(C)) isolates each particle cluster and prevents traveling between eddies. Software automates the process of identifying, labeling, and counting the particles in each trap (here, fluorescent particles are blue).
Table 4-1. Frequencies of different particle count events. Resolved $\lambda$ value of total particles count with no differentiation of colors, equals to the sum of the $\lambda$ values from the sub-populations.

<table>
<thead>
<tr>
<th>Number of particle per trap ($k_i$)</th>
<th>White</th>
<th></th>
<th>Blue</th>
<th></th>
<th>Total</th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Number of Occurrence ($n = 4704$)</td>
<td>Frequency $f(k_i)$</td>
<td>Number of Occurrence ($n = 4704$)</td>
<td>Frequency $f(k_i)$</td>
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</tr>
<tr>
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<td>4173</td>
<td>0.8871</td>
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<td>487</td>
<td>0.1035</td>
<td>1260</td>
<td>0.2679</td>
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<td>34</td>
<td>0.0072</td>
<td>307</td>
<td>0.0653</td>
</tr>
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<td>76</td>
<td>0.0162</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>0.0006</td>
<td>1</td>
<td>0.0002</td>
<td>10</td>
<td>0.0021</td>
</tr>
<tr>
<td>&gt;4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

$\lambda$ value

$\lambda_W = 0.3308$  
$\lambda_B = 0.1246$  
$\lambda_{total} = 0.4554$
Figure 4-4. Experimental measurements for the frequency of different particle clusters (red bars) compared with the theoretical probabilities (blue bars) as a function of the parameter ($k_W, k_B$). The cluster type is graphically illustrated above each data bar. The actual measured number of occurrences for the different particle clusters is also tabulated above each bar.

**Ternary system with fluorescent imaging**

We take Eq. (3) one step further by demonstrating a ternary sample, with differences in the fluorescent colors and particle sizes. For the experiments, 10 µm non-fluorescent polystyrene particles, were mixed with 10 µm green fluorescent polystyrene particles, and 5 µm yellow fluorescent particles. Figure 5 shows an example of the trapping, imaging, and output from the automated detection and counting software. As before, particles are trapped near the eddy core, with the smaller particles typically orbiting the larger one. It is straightforward for the software
to differentiate sub-populations of particles based on a pair of bright field and fluorescent images, Figs. 5(A) and 5(B), respectively. In this case, as in many biological imaging situations, there is a significant difference in fluorescent brightness between the green and yellow fluorescing particles. Nonetheless, both are easily and reliably detected by software, resulting in the particle labeling shown in Fig. 5(C). Because the brightfield and fluorescence images are taken in a sequence, clusters can move, though they remain within the same eddy. Thus, the software algorithm first uses the brightfield image to identify the positions of the posts, and hence the locations of the traps, and then counts the total number particles in the trap (undifferentiated by fluorescent color). It then uses the fluorescence image, taken in the same field of view, with the same trap geometry, to identify the number and distinct color of particles in each traps. The difference between total particles in the trap and the colored particles in the trap is the number of non-fluorescent particles.

Results for particle counts of the 3 component mixture are shown in Figure 6. All three populations are Poisson distributed, regardless of their size, at the particle titer used here (data not shown). The ternary case is similar to the binary case, where we see that the experimental frequency corresponds well to the theoretical probabilities calculated from Eq. (2). For both the binary and ternary cases, $\lambda_{\text{total}}$ is significantly less than unity, so the most frequent trap state is an empty trap. As a result, traps that do have particles most frequently possess a single particle. However, note that the total particle titer (which is proportional to $\lambda_{\text{total}}$, by Eq. (4)) in the ternary mixture is about half that of the binary mixture. Thus, the cluster distribution for the ternary case is totally dominated by pairs, with the frequency of triplet and quadruplet clusters being extremely rare compared to the binary case. As Eq. (2) shows, the relative frequency and mix of particles is directly related to the values of $\lambda_W$, $\lambda_G$, and $\lambda_Y$. 
Figure 4-5. Particle trapping and detection with a ternary mixture comprised of 10μm non-fluorescent polystyrene beads, 10μm green fluorescent polystyrene beads, and 5μm yellow fluorescent polystyrene beads: (A) bright field image of trapped particles; (B) fluorescent image of trapped particles. The software resolves particle counts based on the paired bright field and fluorescent images, differentiating between the three types of particles and out of focus objects that are not suspended in a trap (particles stuck to the top or bottom, or optical flaws on the device). (C) Shows the detected non-fluorescent particles (shown as pink circles), green fluorescent particles (shown in blue), and yellow fluorescent particles (shown in yellow). A particle near the bottom left corner post was declared a non-trapped object. The trapping detection threshold is set based on the shape, size, and color of the image objects.
Figure 4-6. Experimental measurements for the frequency of different particle clusters (red bars) compared with the theoretical probabilities (blue bars) as a function of the parameter \((k_W, k_G, k_Y)\). The cluster type is graphically illustrated above each data bar. The actual measured number of occurrences for the different particle clusters is also tabulated above each bar.

We demonstrate the use of our device and software with a binary mixture of the algae *Chlamydomonas* sp. and *Thalassiosira weissflogii*. *Chlamydomonas* is motile whereas *Thalassiosira* is non-motile; both are trapped. Figure 6 shows the bright field image from 80 traps on the array. The two species are clearly seen distributed among the traps, with *Chlamy* being more compact and dark green. The difference in color and size makes their identification possible directly from the single bright field image. From data on the cell count frequency, we can estimate the values \(\lambda_{Chlamy} = 0.1\) and \(\lambda_{Thala} = 0.0625\). Dividing these values by the trap volume
(0.301 nL) gives a quantitative estimate for the sub-population titers of $0.332 \times 10^6$ Chlamy/ml and $0.208 \times 10^6$ Thala/ml. We have used this device to maintain viable cells over long durations.

**Figure 4-7.** Mixed culture results for the algal species Chlamydomonas sp. and Thalassiosira weissflogii. Bright field image shows the clear size and color difference between the two algal species.

**Conclusions and Design Implications**

Here we have demonstrated the basic statistical properties of microeddy trapping using the Poisson distributed characteristics of the device. From our result in Figure 4, the mixture $\lambda$ values of $\lambda_W = 0.33$, $\lambda_B = 0.12$, and $\lambda_{\text{total}} \approx 0.45$ gives a reasonably high frequency of paired combinations, making it well suited for studying co-culturing of two particles (cells) in one trap, but less efficient for studying co-culture of 3 or more cells per trap. Our results show that the prediction of combinations is fairly accurate, so Eq. (2) and Eq. (4) provide an algorithm for designing multi-cell cultures where the device. If one knows the expected cell titer of each sub-
population, one can design the device so that the trap volumes produce $\lambda_{\text{exp-total}} \approx 1$ producing a nice mix of singlets, doublets, triplets, and so on. For example, if one wants to study triplet combinations, for a one-to-one ratio of A and B, setting up the system so that $\lambda_A = 0.6$ and $\lambda_B = 0.6$ yields the following triplet probabilities for 3 A-type particles or 3 B-type particles:

$$P[k_A = 3, k_B = 0, \lambda_A = 0.6, \lambda_B = 0.6] = \frac{0.6^3 \cdot 0.6^0 \cdot e^{-(0.6+0.6)}}{3! \cdot 0!} = 0.010843$$

while the probability of finding two A-type and one B-type, or two B and one A is

$$P[k_A = 2, k_B = 1, \lambda_A = 0.6, \lambda_B = 0.6] = \frac{0.6^2 \cdot 0.6^1 \cdot e^{-(0.6+0.6)}}{2! \cdot 1!} = 0.032529$$

Taking the device design one step further, if one wants to observe 100 occurrences of triplet particles in traps, then the necessary total number of eddies $n$ can be calculated as $100/(0.010843+0.010843+0.032529+0.032529)=1153$. Thus, the device should have at least 1153 eddies for a one-trial experiment, or one should perform more trials on the device to achieve a total eddy count of 1153.

Equation (2) shows that increasing $\lambda$ values shifts the distribution to higher order combinations. Nevertheless, there is the physical constraint on the maximum $\lambda$ value where a Poisson distribution will hold, namely, the ratio of particle size to eddy size, since over filling the trap can lead to size exclusion (making trapping dependent on the other particles trapped). The maximum $\lambda$ limit for 10 micron particles in the device used here was determined to be approximately $\lambda = 1.2$. In short, the parameters for designing combinations of co-culture groupings are: $\lambda$ values (i.e. cell concentrations) in the device, how many eddies are in the device, and how many trials are performed.
Our approach above provides the ability to create libraries for paracrine signaling mediated cell-cell interactions. The statistical process described here can be applied to cell counting and co-culturing [20, 36, 123] methods with microarrays or different cell trapping mechanisms. Once the distribution of responses to stimulus is characterized, the data can be used to predict cell behavior in varies situations.[8] One can also purposely mix populations and statistically determine the combination to study cell-cell interaction and cell consortia.

Conclusion

We demonstrate the use of a microtrapping array device that obeys Poisson statistics for creating particle combinations that are relevant for the study of microbial communities. The statistical design is general for any community possessing $p$ different sub-populations, though we demonstrate its use with binary ($p=2$) and ternary ($p=3$) component mixtures. Applying statistical design and sampling with our microeddy arrays allowed us to create mixed populations with predictable sub-population ratios. We also showed that mixing in the arrays produced particle traps that were hydrodynamically-isolated from one-another. We used both optical and multi-color fluorescent microscopy to demonstrate the device is compatible with the optical tools commonly used to monitor expression, cell division, motility, and other cellular activity. Finally, the arrayed nature of these experiments provides high-throughput. The statistical tools and experimental design we present here can be applied to different kinds of microtrapping arrays. The goal of these studies is to provide well-founded tools that enable the microbiology community to begin reconstructing ecosystem-wide association networks as a route toward global models of ecosystem dynamics.[126]
Acknowledgements

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Chapter 5. Translation of Hydrodynamic Tweezer Trapping Arrays –
Commercialization of MTC (Microtrapping Cytometer) Translational
strategy for bringing microeddy trapping to the marketplace.

Energy is the lifeblood of modern economies and global consumption is expected to grow 35% over the next 25 years, and even faster in developing countries. Meeting this global energy demand grows increasingly difficult as fossil fuel resources are decreasing and other energy resources are increasingly found in hard-to-reach places. In addition, the United States government worries about the economic impact of rising energy costs, America’s current dependence on foreign oil, and the greenhouse gas emissions from current energy production methods.

Algal biofuels offer the greatest potential to displace fossil fuels without adversely affecting the supply of food and other crop products. Algae produce biofuels by converting carbon dioxide and sunlight, converting it into natural oil-rich organic fuel. Algae fuels have significant advantages over other biofuel sources because of algae’s higher growth rates, tremendously higher yields, cost-effective cultivation inputs, and the potential for high efficiency production. The natural oils produced by algae can be refined into biodiesel, green diesel, green gasoline, and green jet fuel as well as used as a source of valuable pharmaceutical products. This fuel has already achieved significant momentum towards cost-efficient production and commercialization. At this time, a number of companies are developing demonstration scale production facilities which represent the stepping stone from experimental to commercial production.
Problem

Mass production of algae biofuel requires cost-effective, efficient and accurate culture monitoring systems. These systems do not currently exist.

Currently, algal biofuels produced in large volumes with current technology costs over $8 per gallon. Oil content inside the cells vary over the course of a day by roughly 20%, which equates to a difference in yield of up to 1300 gallons per acre per year or a difference between production costs of $8 per gallon and $6.67 per gallon. Inefficient biological productivity, which has the largest influence on fuel cost, is responsible for the significant risk and uncertainty that hinders commercialization of the process. According to the Department of Energy, “Continuous monitoring systems will be necessary since seasonal variation in competitors, predators, and pathogens is expected and requires early detection.” These invasive species and end-of-life-cycle declines can cause culture “crashes,” which are rapid reductions in culture reliability. Current methods for detecting unwanted organisms rely on microscopic analysis of samples taken to a central laboratory, which are too slow to be actively used to mitigate the potential crash of an algal harvest. In addition, the lipid, fatty acid, and chlorophyll content of algae is highly dependent on the physiological conditions such as light intensity, pH value, temperature and nutrients under which the algae are maintained. Rapid measurements are required for controlling the cultivation process with a selected algae species when scaling up to commercial production for producing a biofuel feedstock. Current methods for lipid analysis rely on taking samples to a central analytical lab, making these methods too slow for process control and optimization in large farms. Further, the lab devices used for this analysis requires trained technicians, which add to the already high cost of lab equipment and make the current methods too costly. Simply scheduling harvests based on average conditions puts too much lipid yield at risk, owing to
environmentally-induced drift in the optimal harvest time. To effectively maximize the algae biofuel production and monitor the many variables in order to control the growth process, continuous, instantaneous, and highly accurate monitoring capabilities are desperately needed by algae biofuel researchers and companies. In short, for the industry to transform from experimental demonstration to commercial production, robust process control and monitoring systems are required.

Improved algae monitoring technologies are also needed in research settings. At present, researchers (in companies, universities and research institutes) use laboratory tools, such as fluorometers and flow cytometers, of tools and processes to monitor algae cultures for commercial optimization and other research goals. These tools and processes are very expensive, have limited functionality, have limited accuracy, and are labor dependent. Many of the existing tools and technology were designed to inspect microorganisms in a low throughput laboratory setting and are not designed to quickly inspect large quantities of algae. Research institutions would benefit from access to a more accurate, economic, functional and efficient monitoring technology.

In all, there is an urgent need in both industry and research settings for low-cost, efficient, and accurate hand-held devices that can increase biological productivity by 5-15%, providing the momentum to advance the research in algae biology, and algae biofuels for clean and renewable energy.

Solution

The Micro Trapping Cytometer, a patented technology, provides a cost-effective solution for efficient and accurate monitoring of algae culture.
StaticFlow Analytics’ patented Micro Trapping Cytometer (MTC) provides the cost-effective, efficient and accurate monitoring systems that the algae biofuel industry requires and can potentially save the industry $80 million per year. Figure 2-1 demonstrates pictorially the monitoring cycle of the MTC from algae cell capture, filtering and detection, to digital readout and image projection. Illustrations of the MTC technology are shown in Figure 2-1(A-F), and a proposed prototype is demonstrated in Figure 2-1(G). The core of this technology is based on a cell trapping layer (B, UW patented technology, illustrated in E, F) that can separate and trap cells individually. This technology allows for high throughput culture analysis and near real-time monitoring of cellular lipid content, cellular chlorophyll content, cell concentration (population) and the presence of invasive species at single-cell level. MTC systems quickly, accurately and inexpensively accomplish the algae related monitoring functions of flow cytometers and fluorometers in a small and portable device. By selectively choosing harvest time under changing physiological conditions, this devise can advance quality control, ensure peak lipid content, measure cell concentration, and identify potential algal strains, thus optimize the maximum lipid yields for biofuel.

The first device for commercialization based on the MTC technology is a handheld device and will be followed by an auto-sampling device. Handheld devices are well suited to laboratory and mobile field use. Handheld MTC devices will also have applications in healthcare and other research fields because they will have the ability to perform cell counts, cell fluorescent assays, and real-time single cell diagnostics in a high throughput manner. The handheld device uses readily available, low cost materials and will cost ~$25 to manufacture. Autosampling devices will be developed further in the future once algae production companies establish scale production facilities. These devices automate the sample-taking and data-gathering by mounting
to the pond wall and transmitting the data to a central system, thereby allowing for continuous, automated, real-time monitoring of the algae cultures. Combined with custom-made software, the data from these devices can generate detailed algae culture telemetry, remote measurement and reporting of information, for analysis and tracking without any labor inputs. Robust systems of this kind can create enormous value for algae biofuel producers.

The MTC empowers the growing algae biofuel industry by providing the monitoring systems required to propel research and maximize production efficiency, in-turn supporting profitable, sustainable biofuels.

![Micro Trapping Cytometer Schematic Illustrations]

Figure 5-1. Schematic illustrations of Micro Trapping Cytometer. Left: A) fluorescence light source; B) trapping array layer; C) fluorescence filter layer; D) detector layer; Right: Schematic illustration of a hand-held prototype device.

Market Analysis

Currently, the biofuel market, which algae biofuel is a subset of, is estimated at $19 billion revenues in US and $76 billion globally with 300 companies in the biofuel sphere and over 50 in the specific algae biofuel sphere. The market has experienced rapid historic growth, but the swell
has yet to crest: large scale production is estimated to begin between 2012 and 2015. In short, this nascent market has only just prepared to take off. The biofuel market in the US is estimated to reach $25.4 billion revenue by 2015 and the global market to reach $247 billion by 2020. Specifically, the existing market for algae biofuel production technologies is $271 million and projected to rise to $1.5 billion by 2015 according to a 2010 market research study by Global Information Inc. Historically, government support has been key to the growth of this industry and the government support remains strong through the beginning of large scale commercial production. Government sponsored research labs and consortiums, research grants, mandated biofuel production levels, and a plethora of funding sources. Recently, funding levels have been increasing and come from multiple DOE departments, the Department of Agriculture, the Air Force Office of Scientific Research, and a host of others. Additionally, Exxon Mobil and Chevron each have formed partnerships of over $500 million in this space and private investment has begun to significantly outpace government funding. As this government supported industry nears large scale production potential, it is being weaned off of government support and attracting much private investment with its promising high growth rate. StaticFlow Analytics is positioned to participate in this growth as the industry moves towards commercialization. The market for the MTC device can be segmented into industry algae production companies and algae research labs.

Algae production companies are currently in pilot phases to determine the most economical commercial scale system and are constructing demonstration production facilities to produce hundreds of thousands of gallons of oil per year. The current time presents an opportunity to develop the MTC’s monitoring capabilities in parallel with their systems development and scale up with them over time. Because the industry is still in the scale up phase,
it is difficult to determine what the mature market size will be. To help estimate future market size, we looked at Renewable Fuel Standards (RFS) mandated by the Energy Independence and Security Act of 2007. This act mandates that by 2022, 21 billion gallons of non-ethanol based biofuels be blended into the liquid fuel infrastructure. The 21 billion gallons will be phased in with ever increasing requirements each year starting in 2009. Once the market reached the mandated 21 billion gallons, and assuming the price per gallon of fuel is $4, the MTC device would create a total market savings of roughly $80 million per year. Currently, StaticFlow Analytics has received strong interest from Phycal, a leading industrial company, and is exploring the possibility of a partnership in developing, demonstrating, and testing the device.

Algal researchers work in universities, research institutes, and government funded consortia. In total, these institutions represent approximately 100 well-funded potential customers working on algae biofuel commercialization. Currently, many use traditional lab devices and technicians to perform the analysis the MTC technology can automate. Based on an estimated savings of 5 man-hours per week multiplied by 50 weeks at a cost of $50 per man-hour, the MTC device would save research organizations a total of $1.8 million per year. In addition, a researcher confirmed the usefulness of the technology in determining which of thousands of strains to select for biofuel production since “no high-throughput methods are currently available for research to conduct such analyses.” Similarly, a consortium validated the opportunity and expressed their desire to purchase the device “if you had a product that quantitatively measured intracellular neutral lipids without disrupting the cultures AND the measurement was rapid.” Lastly, Dr. Thomas Dempster, Research Professor at the Laboratory for Algae Research and Biotechnology stated “the bottom line is that we are looking for a balance between the most accurate tool and a reasonable price.”
Since the nascent algae biofuel industry is still in its growth stage, upstream products are undeveloped. All algae researchers and businesses utilize labs to assess and monitor algae growth and lipid production. The MTC technology represents a significant improvement over existing tools such as flow cytometers (which are bulky, range in price from $100k - $200k and require an experienced operator) and fluorometers (which are bulky, cost approximately $100k, require expensive upkeep, and cannot determine cell concentration), as well as processes such as manual microscopy which is labor intensive and slow. Traditional laboratory analysis of lipid content in microalgae do not appear suitable for scaling to a field deployable, rapid sampling, low cost platform. Alternatively, Turner Designs, the leader in fluorometer devices manufactures a line of field-deployable fluorometer devices that purport to measure important algae cell characteristics. These devices can assess cell characteristics such as cell concentration and total chlorophyll of the culture; however, these functions do not accurately measure per cell characteristics for algal companies. Recently, Agrilife Research has also developed an optical-electronic sensor designed to be placed in the algal-production raceways to automatically measure algal growth stages in real-time. The probe also measures optical density of algae as a means of determining cell concentration. Thus, it has the same shortcomings as the Turner fluorometers, such as measuring samples in mass for average data instead of per cell data. Further, the device still hasn’t proved accurate in field-tests. Table 5-1 compares the features of the various competitor products with the MTC technology.
Table 5-1. Comparison of Competitor Products versus MTC Device.

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</tr>
<tr>
<td>Field-Deployable</td>
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<td>✗</td>
<td>✔</td>
<td>✗</td>
<td>✔</td>
<td>✔</td>
</tr>
<tr>
<td>Cell Concentration</td>
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<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
</tr>
<tr>
<td>Total Lipid Content</td>
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<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✗</td>
<td>✔</td>
</tr>
<tr>
<td>Total Chlorophyll Content</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✗</td>
<td>✗</td>
<td>✔</td>
</tr>
<tr>
<td>Presence of Contaminating Cells</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✗</td>
<td>✔</td>
</tr>
<tr>
<td>Impending Culture Crashes</td>
<td>✔</td>
<td>✔</td>
<td>✗</td>
<td>✗</td>
<td>✗</td>
<td>✗</td>
</tr>
<tr>
<td>Single Cell lipid Content</td>
<td>✔</td>
<td>✔</td>
<td>✗</td>
<td>✗</td>
<td>✗</td>
<td>✗</td>
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<tr>
<td>Single Cell Chlorophyll Content</td>
<td>✔</td>
<td>✔</td>
<td>✗</td>
<td>✗</td>
<td>✗</td>
<td>✗</td>
</tr>
<tr>
<td>High Throughput Measurements for Single Cells</td>
<td>✔</td>
<td>✔</td>
<td>✗</td>
<td>✗</td>
<td>✗</td>
<td>✗</td>
</tr>
</tbody>
</table>

<sup>a</sup> The use of flow cytometer requires additional labor cost of $60/h, plus the access to a flow cytometry system which costs around $60,000-140,000.  
<sup>b</sup> Data from Dr. Alex Thomasson and team. Other probes/channels are available for purchase. The one(s) chosen are the most applicable for algae producers.  
<sup>c</sup> Or a lab can be rented at $126 per hour.
Figure 5-2. Competitiveness analysis of SFA’s device. Functionality is measured as a composite of time required, accuracy, measurement capabilities, including total and per cell.

Go-To-Market

Phase I (2011): The near term sales strategy is to partner with researchers and production companies to coordinate our product development activities. The goal is to partner with them as they begin scaling up their production process in the coming year. Initially, this will be focused on the hand held device and determining what features they find most useful. Because the customer base is relatively small, no distribution network is needed and instead we will handle all accounts. It is estimated each research lab or production company will purchase, on average, two devices, which should last three to five years. Secondary sales can be made by supplying custom made cytometers which can be tailored to handle particles within a certain diameter range. While potential revenues at this stage are limited, large net income is not the focus.
Currently, SFA is in the process of finalizing the development of the handheld device, focusing on partnering with algae biofuel production companies in determining the key features of the device. Through these discussions and partnerships, we will create an initial device to be field tested at one of the algae biofuel production companies and/or research labs. In order to finalize the design, explore cost-reduction improvements, test the device, and obtain any necessary product certifications, SFA plans to work with Pacific Design Engineering, one of the largest independent contract product design houses in Western Canada and a member of the Texas Instruments Elite Design House Network as well as a member of the Arrow Consulting Engineering Services. After final development, PDE will facilitate a smooth connection with a local manufacturer.

Initially, SFA will order a sample batch of units to be produced by the domestic manufacturer Fluke. Fluke will conduct a comprehensive testing process, working out any kinks found in the design before SFA begins larger scale production. Since many local manufacturers in the Puget sound area have the capabilities and the excess capacity and since our sales volumes will be relatively low for industry standards, we intent to remain with a local manufacturer for at least the first few years of operation. Each device will cost $200 and will sell for $5,000.

Phase II (2013): As the industrial companies scale up, the importance of real time, automated monitoring will become crucial. To address this, SFA will develop a standalone, automatic monitoring unit that will be integrated into the algae growth system across numerous locations. We plan to develop a complete commercial system that employs a central hub and nodes, devices mounted to each algae pond for continuous monitoring. Each node will send information to the central system for lab technicians to monitor and analyze. The software package will involve algorithms to analyze the information from the nodes and a cloud service so
that field technicians can access the data in real-time when in the field and away from the central system. For example, if a company has a Hawaii site and a Kansas production site with 5,000 acre growth ponds each, we will install a standalone system in each acre and one to three nodes per acre to accommodate differences across locations. This will allow the producers to automatically obtain location dependent information on their crops in real time to help them determine when to harvest. The central system will be priced at $50,000 plus $500 for each node. System management, maintenance and cloud service will be priced at $12,000 a year plus $25 per node per year on a subscription.

**Traction**

This technology is founded on a University of Washington (UW) intellectual property (US Patent No.: US 7803599 B2, Sep. 28, 2010) and the patent is available for licensing. One of the co-inventors is advising StaticFlow Analytics. Meanwhile, a new IP related to this innovation has been disclosed (UW ROI No. 45555, Mar. 16, 2011) and filed by one of the team members. Static Flow Analytics will immediately take action to secure the license option as the first step and negotiate further license terms with UW center of commercialization with whom we are currently in discussion. In addition, we have a patent-pending on the use of BODIPY dyes for lipid level assessment.

We have contacted a handful of companies and researchers and the initial enthusiasm is high. We are currently in contact with the VP of R&D at a Phycal, major, venture backed algae company and working out plans for a technology demonstration. We are also in discussions with algae researchers to assist with product development.
Finance

We will seek one round of equity financing cover the costs of development of the handheld device and the commercial system. $1.3 million will be raised in year one to cover the business startup costs, initial marketing activities, one full-time and one-part time employee, the final development of the handheld device, development of the commercial system, cash hire additional staff in year 3, and the manufacturing of the commercial systems.

Assumptions:

- Growth will scale with the mandated renewable fuel standards governing algae biofuel production.
- Market share of the handheld device will steadily grow and then remain at 25%.
- Algae will represent 2% of the renewable fuel standard for advanced biofuels and this percentage will double each year until it reaches 16% in year seven and 20% in year 8.
- The number of demonstration sites purchasing the commercial systems will steadily increase until large-scale production begins and after which it will decrease. The commercial systems will then be sold to production facilities.
- Market share will grow to and remain at 25% for the commercial system.
Table 5.2. Summary Income Statement

<table>
<thead>
<tr>
<th>INCOME STATEMENT (Thousands USD)</th>
<th>2011</th>
<th>2012</th>
<th>2013</th>
<th>2014</th>
<th>2015</th>
<th>2016</th>
<th>2017</th>
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<tr>
<td>Handheld Revenue</td>
<td>388</td>
<td>388</td>
<td>843</td>
<td>879</td>
<td>1,061</td>
<td>1,958</td>
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<tr>
<td>System Base Revenue</td>
<td>63</td>
<td>131</td>
<td>182</td>
<td>434</td>
<td>882</td>
<td></td>
<td></td>
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<tr>
<td>System Node Revenue</td>
<td>6</td>
<td>1,717</td>
<td>3,307</td>
<td>8,192</td>
<td>21,266</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Subscription Revenue</td>
<td>15</td>
<td>133</td>
<td>342</td>
<td>855</td>
<td>2,044</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Revenue</td>
<td>388</td>
<td>472</td>
<td>2,823</td>
<td>4,709</td>
<td>10,542</td>
<td>26,149</td>
<td></td>
</tr>
<tr>
<td>COGS</td>
<td>15</td>
<td>56</td>
<td>569</td>
<td>1,048</td>
<td>2,537</td>
<td>6,368</td>
<td></td>
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<tr>
<td>Gross Margin</td>
<td>372</td>
<td>416</td>
<td>2,255</td>
<td>3,661</td>
<td>8,005</td>
<td>19,781</td>
<td></td>
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<tr>
<td>SG&amp;A</td>
<td>354</td>
<td>378</td>
<td>860</td>
<td>1,407</td>
<td>1,831</td>
<td>31,303</td>
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<tr>
<td>Depreciation</td>
<td>1</td>
<td>6</td>
<td>6</td>
<td>9</td>
<td>12</td>
<td>26</td>
<td>50</td>
</tr>
<tr>
<td>Operating Income (EBIT)</td>
<td>(355)</td>
<td>(12)</td>
<td>(451)</td>
<td>838</td>
<td>1,818</td>
<td>4,876</td>
<td>13,852</td>
</tr>
</tbody>
</table>

**Exit Strategy**

By 2018, year eight, we plan to sell the company to a large instrument manufacturing firm such as Fluke. The industry has been consolidating at a rate of 1.2% with a future expected rate of 0.9%, which equates to approximately 35 firms per year being acquired. StaticFlow Analytics is similar to Dynisco, who was sold to Roper Industries in 2006. Dynisco is a provider of high-performance and cost-effective sensors, controls, and analytical instruments for plastics extrusion processing. Dynisco garnered a price of $243million, a price 8.7 times EBITDA. Based on an 8 time valuation over EBITDA, we estimate StaticFlow Analytics can sell for approximately $111 million in year eight.
Chapter 6. Conclusion, Recommendation, and Future Directions

The design properties of hydrodynamic microeddy devices are critical for putting this technique to real use in biological settings. Researchers seek tools that can help them decipher biological information in a practical sense. This work was intended to fill the gap between microfluidic innovations and the practical experimental design issues needed to retrieve biological data. This is done in the context of the hydrodynamic tweezer technology developed in our group, but most of the analysis is independent of the trapping technology.

In Chapter 2, the design and prediction of 2D steady streaming flow structure, as well as the trapping properties were reported. The time-averaged inertial terms in the fast oscillating flow of frequency $\omega$ and amplitude $s$ generate a Stokes layer of thickness $\sim O(\sqrt{v/\omega})$ and drive the steady secondary steady streaming motion that generates microeddies near the cylindrical obstructions. The role of device geometry is shown to be a key variable in low Reynolds numbers (Re) microfluidic systems. Our study characterized 9 distinct design geometries. The imaged eddy flows show that the device geometry affects the eddy number, shape, structure, and strength of the microeddies. Comparison of measured trap locations to computations of the eddy flow show that each trap is located near the eddy center. The trapping behavior of different geometric shapes was linearly proportional to the Stokes layer thickness. Trapping strength and location are controlled by the geometry and the oscillation frequency. The 9 geometries provided data for comparison to steady streaming eddy flow modeling.

While the term “trapping” is defined as the object being at the same x-y plane, differences in the trapping stability were observed. Moreover, as we showed in later chapters, clusters of particles can grow and fill the trap. Thus, trapping is more complex than the behavior
in on plane of the 3D flow field. The next step is to identify the hydrodynamic parameters of controlling the stability of microeddy traps. The ability to characterize trapping stability can provide a variety of applications such as particle sorting, motile cell characterization. Understanding of the full 3D flow structure is also a key for predicting traps. The collaborative work with UW Aero/Astro Dabiri group (Appendix A) used 3D-PTV technique to provide real flow observation as a first step to understand the 3D steady streaming flow. Preliminary results have shown that we can reconstruct the flow field, however the 3D properties still need to be characterized. There is much to be done in characterizing the 3D flow.

Chapter 3 addressed a statistical approach to trapping in order to quantify traits of generalized array-based methods such as the determination limits in datasets, the detection limits of such a device, and to provide general guidelines for high throughput microtrapping array design. There have been numerous research studies on new devices for cell isolation and trapping, but very few have addressed the practical aspects of data quality based on cell distribution. The microeddy hydrodynamic trapping device demonstrated Poisson distribution behavior up to particle concentrations where large cell clusters size-excluded others and were visibly masked and hold to count. We showed that the Poisson parameters is proportional to particle concentration and counting empty traps at lower particle concentration is a fast and nearly as accurate way to determine cell numbers. From this work we also realize that cell properties (like size, density, etc.), trapping behavior/distribution, trapping force, and the device geometry connect tightly to the resulted outcome and should be consider while designing any cell trapping arrays.

Recommendation of future work on this chapter would be to validate the cell counting model system with real cell experiments. Among different cell types, near-globular cell strains
with size 5-20 µm, with the cell concentration of $\lambda < 1.2$ would be optimal for the current design described in Chapter 3. The detection software developed here can be tailored easily by adjusting the recognition threshold of size, color, and shape. Cell trapping behavior that deviates from a Poisson distribution can be used to detect cellular interaction (e.g. adhesion between cells will change the distribution).

In chapter 4, a statistical approach for creating co-culture combinations in hydrodynamic microeddies was presented. We demonstrated the design process for creating co-culture with predictable sub-population ratios and ready optical access. Similar to chapter 3, the experimental design and operational details presented here can be applied to different kinds of microarrays for co-culture design, allowing better usage of microdevices to provide tools for investigating cell dynamics of microbial communities.

Several different cell strains have been tested in the microeddy device as single strains. We have shown that algal strains can be cultivated while remain trapped in eddies. Chapter 4 demonstrated a co-culture of two oceanic algae in the microeddy trapping array. However, the experiments were for demonstration only, and real biological questions still need to be answered. Future work on this subject should start with a simple system to demonstrating the device function for co-culture. An extended time-course study of two interacting (symbiotic, allelopathic, etc.) strains can be used to demonstrate co-culturing design. A classic system for demonstrating the competitive exclusion principle is the interaction between *Saccharomyces cerevisiae* and *Schizosaccharomyces kefir*. One can also observe the chemical signaling of trapped strains. A good complement to such studies would be to perform trap-to-trap mass transfer calculations to see if traps are chemically isolated. This work could build from the mass transfer studies of Bowman and Schwartz.
Chapter 5 presented the business plan for bringing microeddy device to market. The application described in chapter 5 is a hand-held single-cell algae trapping device for monitoring algal growth and phenotypic traits. The results have been presented at several business competitions. The concluded feedback we received are mainly two responses: the microeddy device is a great innovation with a beautifully worked out prototype work, but the application to algal biofuel market is rather risky for its current state of development. Another cellular application with a more developed market will greatly increase the opportunity of funding and investment. Since bio-diagnostic devices require mid-to-high investment to achieve the engineering development and product design, it is essential to switch gears and look at higher value and better established markets such as medical diagnostic application.
Appendix A - Realization of 3D Steady Streaming Flow Using Three Dimensional Particle Tracking Velocimetry (3D PTV)

The following section described the investigation methods and results of understanding 3D steady streaming flow using 3D PTD. Experiments below examine four cases of device structures, (1) Large single post of M=5~20 , (2) small single post of M=3~9, (3) array of in-line arranged cylinders, (4) array of diagonally arranged cylinders.

Case (1) is design to test and compare the trapping behavior of our current PDMS based device with the previous design made with PMMA by Lutz [152]. Devices in case (2) have different aspect ratio as well as the operating regime and are specifically design to form only the trapping eddies for future particle trapping use. Case (3) is designed to examine the flow structure and of to understand the 3D flow variation of cylindrical post arrays. These cylinders have the same aspect ratio as case (2) and thus can be used to validate if the unit eddies structure in case (2) can be amplify by mass fabrication of array structures. Case (4) design an array of same cylindrical posts but arranged diagonally.

Material and Methods

Microdevice Fabrication

Device is fabricated with the fabrication method described in Chapter 2.

The PDMS channel layer was molded from an SU-8 master following standard procedures described in the literature.[130] Two different thickness of SU-8 Negative resists (SU-8 2100, MicroChem Corporation) were spin coated onto pre-cleaned (Acetone/IPA/DI water, 180 °C for 2 min.) silicon wafers. After soft bake, the wafers were aligned and exposed using a direct-write laser pattern generator (µPG 101 Laser Pattern Generator, Heidelberg). Wafers were developed
(SU-8 developer, MicroChem Corporation), followed by a hardbake at 150 °C for 5 min, then treated with trichlorosilane (GELEST, Inc.). The structured SU-8 masters were then used for molding each of the designed PDMS channel layers. The elastomer and curing agent (Sylgard® 184 Silicone Elastomer, Dow Corning Corporation) were mixed with a 10:1 ratio, and poured onto the wafer. The mixture was degassed under vacuum for more than 4 hours. To form a uniform PDMS layer, a laser printer transparency was taped to a flat quartz plate and then placed on top of the PDMS-coated wafer and pressed down until contact was made with the frame surrounded the master mold. After curing in an oven at 70 °C for 12 hr, the transparency-PDMS layer was peeled from the master. The channel side of the PDMS layer was then plasma-bond to a drilled coverslip where fluid ports could be added. The other side was bond to a glass slide for support. The device was then heated on a hotplate at 70 °C for 30min to ensure a stabilized, non-leak bond. Two single layer piezoelectric disks (Piezo Systems, USA) were then attached to the coverslip above the pair of cavities in the PDMS layer using conductive epoxy (SPI Supplies). Nanoports (Upchurch Scientific) were attached to allow syringe connections for loading fluids into the device.

Actual dimensions of the channel were measured using 3D imaging with a laser scanning confocal microscope (LSM 510, Zeiss). The characteristic length $2a$ is the diameter of the circular cross-section features. The rectangular channel had a height of $h$, width $w$, and length of $l$. The measured device properties are listed as table A.1.

<table>
<thead>
<tr>
<th>Case (1)</th>
<th>Case (2)</th>
<th>Case (3)</th>
<th>Case (4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$2a$</td>
<td>150 μm</td>
<td>50 μm</td>
<td>50 μm</td>
</tr>
<tr>
<td>$h$</td>
<td>~250 μm</td>
<td>~100 μm</td>
<td>~100 μm</td>
</tr>
<tr>
<td>$l$</td>
<td>μm</td>
<td>μm</td>
<td>μm</td>
</tr>
<tr>
<td>$w$</td>
<td>2000 μm</td>
<td>1000 μm</td>
<td>1000 μm</td>
</tr>
</tbody>
</table>
Flow conditions and 2D flow visualization

Flow visualization relied on small fluorescent polystyrene particles of nearly neutral buoyancy (Fluoresbrite® Multifluorescent Microspheres, $d_p=1$ μm, $\rho_p=1.05$ g/cm$^3$, Polysciences, Inc.) suspended in water ($\rho_f = 1.00$ g/cm$^3$, kinematic viscosity $\nu = 0.0095$ cm$^2$/s). These particles have negligible Stokes number and tend to trace the fluid motion, rather than being trapped. Fluorescence microscopy (TE2000-U, Nikon) with 200-1200 ms exposure time revealed the time averaged particle streak lines. In regions of high streaming flow, particle streaks showed the circulating microeddy motion. The straight streaks revealed the oscillation amplitude $s$ when imaged in regions far from features in the channel or walls. Typical amplitude $s$ was limited to $1\mu m \leq s \leq 3\mu m$. The piezoelectric disks were driven at audible frequencies from 1 - 10 kHz. All experiments were performed at room temperature.

3D-PTV setup for 3D flow visualization

The 3-D μPTV system is developed and tested based on the principles introduced in the previous work by Tien et al.[153]. Figure A.1 shows a schematic of the current 3-D μPTV system setup. The experimental apparatus consists of four major components: the infinity-corrected inverted microscope system, micro-channel flow system, calibration system and data processing software.
A customized inverted microscope system is designed and assembled to have more flexibility to test different optical configuration. As shown in Figure A-1, the lights are set on top and the objective lens is put underneath the flow model. Both the objective lens (Olympus UPLanFLN 20X) and the tube lens (U-TLU) are made by Olympus. The 3-pinhole plate is placed in the objective lens right after the lens elements. The diameter of each pinhole is \(d = 2.53\) mm, and the distance \(R\) (from the pattern center to each pinhole center) is 3 mm, results in a pinhole separation distance of 5.196mm. This configuration is chosen from a set of different pinhole configurations based on the depth of field and particle image intensities measured from the
images of a target plate at different depth locations. The current configuration results in a measurable image volume of $600 \times 600 \times 200 \, \mu m$.

High power LED (light-emitting diode) is used as the light source of the system. Compare to other incoherent light sources, the main advantage of LED is the narrow spectral width. The spectral width (full width at half maximum, FWHW) of a typical quasi-monochromatic LED varies from 20~40 nanometers, which is ideal to match the spectral response of those pinhole color filters. In the current system, three Cree XR-E series LEDs with Red (620 nm), Green (520 nm) and Royal Blue (460 nm) are used to provide continuous light source. Critical illumination configuration is used where each LED is aligned with a lens doublet to focus the light to the object plane. A 1024 pixel $\times$ 1024 pixel resolution color CCD camera (uniqvision UC-1830CL) of a frame rate of 30 frame per second is used to capture the images. The typical exposure time is set to 4.167 ms (1/240 s). For particle density high enough to perform two frame tracking, the data rate is 30 Hz. Images are captured and recorded by software (Video Savant® 4, IO Industries Inc.) through a frame grabber board (Road Runner R3 CL, Bitflow Inc.) in a PC.

A calibration apparatus is constructed to perform the calibration procedure. As shown in Figure A-2(A), the calibration target is a 50 mm $\times$ 25 mm $\times$ 1.52 mm glass plate with grid arrays of blue chrome dots. The size of each dot is 3 $\mu m$ with a grid spacing of 20 $\mu m$ (Figure A-2(B)). The target plate is connected to a 6-axis translation stage (APT 600, Thorlabs). The stage is used to align the calibration target with the optical axis of microscope system to provide the accurate position in Z-axis of the target for each calibration plane. A glass slide of the same size to the bottom glass plate used for the micro-channel model is put in between the objective lens and the calibration target. By putting water in between the glass slide and the target plate, the refraction
effect of water the glass material is matched this way during calibration. The focal plane is set to the mid-plane of the calibration planes and centered around the step so that the measurable volume covers the whole microchannel. Figure A-2(C) shows a typical calibration image taken during the calibration test.

![Figure A-2. System Calibration: (A) Experimental Setup (B) Target plate pattern (C) Example of a calibration image.](image)

The data processing software is coded in MATLAB (Mathworks, Natick, MA). Video captured by the data recording software is saved in individual frame in 24 bit color image format, and the final data output is the particle locations in physical space.
Results and discussion

2D Flow visualization

Figure A-3 shows a flow image of case (1) at oscillating frequency of 5 kHz. Note that each quadrant generates an inner eddy and a recirculating outer eddy. From the dense particle pathlines one can see that the inner eddy is much denser with a higher velocity. The size of the inner eddies are measured at different frequencies to compare with the PMMA device presented previously.[150]

Figure A-3. 2D flow image of case (1) at oscillating frequency of 5 kHz.

We compare the normalized DC boundary layer thickness to the normalized Stokes boundary layer thickness. Figure A-4 shows the comparison of DC boundary layer thicknesses of the PDMS elastic device and the PMMA rigid device. The resulted normalized DC boundary layer thickness shows deviation away from the 2D theory line and results from the rigid device, though
it reveals a similar trend of curvature. We suspect that this is due to the PDMS elastomeric properties.

For Case (2) and (3), we observed only the inner eddies generated from the cylinder. (Detail of this flow is described in Chapter 2 and Chapter 4). Without the outer eddies, inner eddies extended to the edge of the channel. The dividing streamlines were only observed at the vertical and horizontal edge of eddies.

The 2D flow structure of case (4) is shown in Figure A-5. While each of the post structure generates four inner eddy from the cylinder, each eddy is diagonally connected to the neighboring eddy and form a peanut shape flow. This “eddy fusion” effect suggests that there must be 3D flow element traveling in z-direction.

![Figure A-4. Comparison of the normalized DC boundary layer thickness $\delta_{DC}/a$ as a function of normalized Stokes layer thickness from the cylinder $\delta_{DC}/a$ from the two experiments. The solid curve was calculated from 2D theory for an unconfined flow including the Stokes drift correction.](image-url)
Figure A-5. 2D flow image of case (4) at oscillating frequency of 5 kHz.

3D PTV for 3D steady streaming eddies

In the above section we presented the “2D flow” imaged by the center plane, but the flow must have 3D characters generate at the wall. However it is hard to image as previous method (laser sheet). Lutz et al. have shown the 3D scaling for single large post case[150], but we learned from Chapter 2 that the scaling of arrays is set not solely by the frequency but also the device geometry, because these posts size and post-to-post distance are comparable to the DC boundary layer thinness. This is especially apparent in arrays. We use 3D PTD to resolve the 3D flow structure and quantify the 3D (z-component) properties. Figure A-6 shows the 3D PTV result image of a unit cell from case (3). Image at the top shows the view from x-y plane, image below shows the view from x-z plane. The view from x-z plane shows a complex 3 D flow structure with symmetry at the mid plane. Figure A-7 shows the 3D PTV result image of a unit cell from case (3). The mid-plane symmetry is also observed in diagonal array, indicate that the 2D flow images we shown in previous section is revealing the flow activity at the mid-plane.
Flow structure of case (4) is very different from case (3). Figure A-8 shows the interpolation of the velocity field from a quartered unit cell in A-7. The gridded interpolation reveals the flow velocity and direction. Color scheme indicates the vector change in z-direction. Normalized integration of z-component (3D character) is shown in Figure A-9. One can see that the flow is symmetric with maximum z-variation showing at close the post wall where eddies are. This work is still under progress and is in preparation for publication.
Figure A-6 show the 3D PTV result image of a unit cell from case (3), above: view from x-y plane; below: view from x-z plane.

Figure A-7 show the 3D PTV result image of a unit cell from case (4), above: view from x-y plane; below: view from x-z plane. (grids are at the same scale)
Figure A-8. Interpolated velocity field of a unit cell from case (4). Color scheme indicates the vector change in z-direction.

Figure A-9. Normalized z-component of a unit cell from case (4).
Appendix B. Screening alternative systems - Trapping of Algal Single-cells and Optical Characterization

We demonstrated in previous sections that algae cells can be trapped and maintain viable in microeddy device for more than 6 days. Figure B-1 shows an example of quantitative chlorophyll detection of observing the cellular properties of trapped cells. Low concentration of *Chlamydomonas* sp. was loaded and trapped in the device. Once individual cells are trapped in the device, it is possible to study and report the functions and phenotypes of cells, such as cell counts from the bright field image (Figure B-1(a)). The chlorophyll content in each cell can be determined by the auto-fluorescence assay (Figure B-1(b)).

![Figure B-1](image)

Figure B-1. Chlorophyll content of single cells trapped in device can be determined optically with auto-fluorescent assay. a) Bright field image of algae cell (*Chlamydomonas* sp.) trapped in the device. b) Fluorescent assay. Auto-fluorescence intensity indicates the chlorophyll content in each cell.

Label-based fluorescent assay was also performed in device to quantify the per cell lipid body content. However, due to the strong partition of lipophilic dye into the PDMS channel, fluorescent intensity is relatively diminished by background signals. Our approach to this issue is to fabricate the microeddy channel with silicon and glass-based material instead of PDMS.
Figure B-2 shows the picture of the device. Channel structures are deep-RIE etched onto the silicon wafer and then anodic-bonded to borofloat glass wafer. The device provides more rigid support and reduced oscillation compliances.

Figure B-2. Image of the silicon-based device. Seven channels were deep-RIE etched on the wafers and can function independently.
Appendix C. Screening alternative systems - Microeddy Trapping for Identification and Characterization of Bioenergy Endophytes

Introduction

Environmental, long-term economic and national security concerns have motivated research into renewable, domestic sources of fuels and chemicals now mostly derived from petroleum, to reduce greenhouse gas emissions, pollution, resource depletion, unbalanced supply demand relations.[154, 155] Ethanol has been recognized as an alternative to gasoline, with the lower exhaust emissions to the atmosphere and toxicity, and has been used as a transportation fuel in a number of countries.[156, 157]

Lignocellulosic materials constitute an abundant and inexpensive feedstock such as crop residues, perennial grasses and woody residues and offer the potential for large reductions in GHG emissions.[158, 159] Lignocellulosic feedstocks are composed predominately of cellulose (40-60% of the dry biomass), hemicelluloses (20-40%), and lignin (10-25%)[154, 160] Cellulose and hemicelluloses can be converted to sugars, and then fermented and distilled to ethanol. Lignin is generally assumed to be used as an energy source to drive this process[161] Despite the most commonly use microorganism in industrial fermentation, Saccharomyces cerevisiae can only utilize hexose derived from cellulose, and has poor utilization of pentoses derived from hemicellulose. Also, ethanol yield and productivity can decreased due to the presence of inhibiting compounds, such as weak acids, furan derivatives, and phenolic compounds.[162]

Several hundred yeast strains have been screened for their ability to metabolize pentoses. However, none have had the ethanol tolerance, fermentation rate, and tolerance to fermentation inhibitors needed for large scale industrial applications. Naturally occurring and genetically
engineered xylose-utilizing microorganisms have been investigated since the 1980s. Transgenic strains can simultaneously ferment both glucose and xylose. However, these strains are less tolerant to natural environment; problems including sensitivity to inhibitors in original plant material and pretreatment process, poor utilization of different complex, higher cost of specific media and patent protections. It is estimated that more than 99% of the microbes in nature have remained uncultured. [163] Among the wide variety of microbes, yeast endophytes showed a great potential for bioenergy production. Endophytes are microbes that colonize living, internal tissues of plants without causing any immediate, overt negative effects. Endophytes appear naturally adapted to their host, with a higher tolerance to plant phytochemicals and pretreatment degradation products that inhibit growth of common fermentation yeasts. Problem also lies with present screening process; bulk biochemical methods are too slow to evaluate the enormous amount of microorganisms in a timely manner, difficulties in cultivability also limit the process of screening. There is a continuing need for single-cell suspension trapping methods that are easy to implement, insensitive to cell and medium properties, and can be arrayed into highly parallel systems. [164]

A set of classical biochemical methods is presented to further understand the process of cell screening. Classical biochemical approach for identifying and characterizing organisms tend to be time consuming and involved several steps including collecting samples, cultivation, purification of single strain, screening assays and genotype identification, which highly relies on microbiology tools. Research goal is to develop a high-throughput device that will accelerate this process and improve bench scale screening. The general approach is to first learn about the classical on bench technique, and then to begin device design based on the in lab experience.
The research vision is to generate a high throughput platform that can accelerate the discovery of new and valuable organisms beginning with bioenergy endophyte yeasts.

**IDENTIFICATION AND CHARACTERIZATION OF BIOENERGY MICROORGANISM**

<table>
<thead>
<tr>
<th>Classical approach</th>
<th>Microfluidic approach</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collect samples from nature</td>
<td>Collect samples from nature</td>
</tr>
<tr>
<td>cultivate in nutrient-based media plate</td>
<td></td>
</tr>
<tr>
<td>Plating for 3-6 times to get pure single strain</td>
<td>2 months</td>
</tr>
<tr>
<td>Search for promising colonies based on morphology</td>
<td>2 days</td>
</tr>
<tr>
<td>Test for ethanol production</td>
<td>Test for ethanol production</td>
</tr>
<tr>
<td>Identify promising strains with DNA sequencing</td>
<td>Identify promising strains with DNA sequencing</td>
</tr>
</tbody>
</table>

**Experimental Methods**

**Microorganism and Media**

Strains used in this study are listed in table 1. Poplar endophytes WP1, PTD2, PTD3, WP21(3/27-1), PT10(13) were isolated from wild poplar; GRH and GRI were isolated from Giant reed; J5 was isolated from soil, J7 was isolated from rotten fruit from UW medicine garden. ATCC 6037, ATCC 24860, Brewer’s yeast and Champagne yeast were maintained on YPD (Yeast extract 10 g/L, Peptone 20 g/L, and Dextrose 20 g/L) plates at 30 °C after precultivation. Poplar endophytes WP21, WP1, PTD2, PTD3, Giant reed endophyte GRH GRI and J7 from rotten fruit were maintained on xylose plates (MS, 3% xylose) and were selected for studying xylose utilization and ethanol production. Isolates were grown on xylose and control plates (MS, w/o sugar) to confirm the utilization xylose. E. coli was grown in Luria–Bertani medium (10 g/l
tryptone, 5 g/l yeast extract, 5 g/l sodium chloride, adjust pH to 7.0 with NaOH, containing 100 mg/l ampicillin). LB agar plates with ampicillin/X-gal/IPTG (LB agar containing 100 mg/l ampicillin, 0.5mM IPTG, and 80 µg/ml) were used for E coli. transformation.

Extraction of yeast genomic DNA and PCR amplification of 18S, ITS, and D1/D2 region

Genomic DNAs were prepared from individual isolates using QIAquick spin kit following manufacturer’s protocol. The 18S region, ITS1-5.8S-ITS2 region and D1/D2 domain of LSU rRNA genes were amplified using three sets of primers (listed in table. 2.) A 1.8-kb fragment of 18S region, a 600-650 bp fragment of D1/D2 region at the 5’ end of the large-subunit rRNA gene and a 600-620 bp fragment of ITS1-5.8S-ITS2 region were amplified. PCR was performed on DNA extracts in 25 µl with final concentrations of 1× PCR Pre-Mix buffer E (Epicentre, Madison, Wisconsin), 100 nM of forward and reverse primers, 5 U of Taq DNA polymerase (Fermentas), and 2 µl of template DNA. The reaction mixture was held at 98 °C for 5 minutes followed by 34 cycles of amplification at 94 °C for 60 s, annealing at 50 °C for 60 s and 72 °C for 60 s, with a final step of 72 °C for 10 minutes in a Mastercycler thermalcycler (Eppendorf, Westbury, NY).

Molecular cloning and sequencing

PCR products were subjected to electrophoresis in 0.8% (W/V) agarose gel with SYBR safe DNA gel stain and dye. (Fig. 1.) Target bands were collected from the agarose gel and DNA were extracted from gel using QIAEXII gel extraction kit (Qiagen, Madison, Wisconsin) following manufacturer’s protocol. DNA fragments were cloned using pGEM T Easy kit (Promega, Madison, Wisconsin) following the manufacturer’s instructions. After gel extraction, 3 µL purified DNA were mixed with 1 µL pGEM-T-easy plasmid DNA, 1 µL ligase and 5 µL
ligation buffer in an 0.5 mL Eppendorf tube, then sat on bench overnight for ligation. Plasmid transformation was performed using competent E. coli cells with heat shock method at 42 °C for 30 sec. Cells were spread on LB agar plates (containing ampicillin/X-gal/IPTG), grown at 37 °C overnight. Two white colonies were picked per insertion, incubated in 2 mL LB broth with 100 μL Ampicilin in a 20 mL test tube and grown at 37 °C overnight. Plasmid DNAs were purified using the QIAprep Spin Miniprep Kit followed by manufacturer’s protocol, and checked with restriction endonuclease EcoRI. Restriction fragments were analyzed by electrophoresis as above. (Fig. 2.) Sequencing was conducted using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) and an ABI 3730XL sequencer (Applied Biosystems) at the Department of Biochemistry sequencing facility of the University of Washington. Nucleotide sequence were aligned with the program ClusterW (Thompson et al., 1994) using default gap penalties. Sequence similarity searches were performed using the BLAST network service of NCBI database.

Assays

Ethanol concentration was measured by gas chromatography (GC). All GC experiments were performed using a Perkin-Elmer Auto System XL GC coupled with a flame ionization detector (FID) equipped with an Rtx-624 (Restek) capillary column with a pre-concentration step using a Perkin-Elmer Turbo Matrix Headspace Sampler HS-40. GC was calibrated with ethanol standard samples. Cell biomass was determined by optical density at 600 nm (S2100 Diode Array Spectrophotometer, biochrom). Dry cell weight (DW) was determined as follows: Cultured broths were centrifuged at 5000 rpm (3000Xg) for 10 min and washed with DI water for 3 times, then adjusted to various OD600. 40 ml of each cultured solutions were pelleted in
pre-weighed 50-ml tubes by centrifuge at 7250 rpm (6000Xg) for 20 min. Dry cell weight (DW) was determined by drying the cells at 80 °C for 16hr in a convective-type hot air oven. DW versus optical density at 600 nm with linear regression value of over 0.9920 was plotted. (Fig. 3.) Corresponding calibration curve is given by following equation:

\[
\text{Dry cell weight (g/L)} = k \times \text{OD}_{600}
\]

**Cell Growth and Fermentation**

To study the viable growing condition, microbes were stricken out from fresh 2 days-precultivated plates and then incubated in 125-ml Erlenmeyer flasks containing 50 ml of growth media (MS medium with different sugar source, xylose 30g/l, glucose 30g/l) at 25 °C with agitation at 160 rpm. Initial cell density was adjusted to an OD600 of 0.1. Fermentation experiments were performed anaerobically using capped 22-ml vials with 10 ml medium and an initial cell density at OD600 of 5.0. (Samples may require pre-growth in YPD media for higher cell density followed by three times washing) Samples were taken from each vial to test the optical density using a 1ml disposable sterile syringe. Ethanol concentrations were tested from 0hr time point up to 34hr.

**Isolation of endophytes**

Plant stems were surface-sterilized[165] with 10% bleach (1.2 % active sodium hypochloride) for 10 min and 1% iodophor for 5 min, then rinsed for 3-5 min with sterile water. The ends of the explants were removed, and 60 stems were incubated in the light on Murashige and Skoog medium with sucrose (MS; Caisson 61 laboratories Inc., Rexburg, Idaho). Morphologically-distinct colonies were streak-purified several times on xylose plates (MS, 3%
xylose). Growth of colonies on MS plates (MS, no sugar content) was performed as control. Isolated endophytes were listed in Table 1.

Isolation of microorganism from nature

Twelve, 15 g samples of soil, fruits, leaves and flowers were collected at the UW Medicinal Herb Garden. One gram of each sample was suspended in 100 ml of distilled water, vortexed for 30 seconds and the supernatant used to inoculate three Petri plates containing isolation medium. The medium used was Murashige and Skoog medium (MSP 0009; without sugar, Caisson laboratories Inc., Rexburg, Idaho), pH 5.8, augmented to include 3% Xylose. In the case of the fruits, 2 g were used for isolations.[166, 167]

Result

Morphology of yeast isolates

All isolates were tested on xylose, glucose and control (sugar free) plates. Using nutrient-based screening process, isolates in this study can all utilize xylose as the only sugar source for growth. (Table 3.) In compare with control strain Saccharomyces cerevisiae, WP1, PTD2, PTD3, are all pink to orange colonies and grow best at 30 °C, however J5 and J7 showed no growth at temperature above 25 °C and preferred temperature around 20 °C. (Fig. 4.) J5 and J7 appeared filamentous growth after 3 days. (Fig. 5.) Color changes of J7 by time were observed during incubation, from pale pink, pink to yellowish colonies with mycelia and black dots. Figure 6. shows photomicrograph of J7 in xylose broth medium. The long cell shape is rather different from common yeast cells.
**Fermentation**

Yeasts were precultured in YPD broth to obtain a higher cell concentration for fermentation experiments. Table 4. shows that after washing with clean MS, the residual ethanol content in cells might differ from different strains, which indicate that it is necessary to record the initial ethanol content at the starting point of fermentation that was produced in YPD cultivation. DW versus optical density at 600 nm with linear regression value of over 0.9920 was plotted. (Fig. 3.) Yeast dry weight was calculated through corresponding k value. Gas chromatography was calibrated with ethanol standards with a linear regression value over 0.9999 (Fig. 7.). Ethanol concentration was converted from corresponding GC peak area. Note that ethanol concentration value under 50 mg/L should not be taken seriously due to instrumental limitation. Figure 8. Shows the ethanol production of different yeast strains. All of the strains are capable to grow on xylose, however only few of them could produce decent amount of ethanol from xylose, meaning that some strains might go for other pathways of xylose metabolism and produce other products such as xylitol.(Fig. 9.)[168] Note that Baker’s yeast does not utilize xylose at all. J7 produced a higher amount of ethanol from xylose, and with an OD value of 20, 6000mg/L of ethanol can be produced (Data not shown). As a result, J7 was selected for sequencing. Strains that are not listed in Figure 8. produced a minor amount of ethanol.

**Identification of J7**

Analysis of the 18S, ITS1-5.8S-ITS2, and D1/D2 regions suggested that isolates J7 was identical to *Aureobasidium pullulans* up to 99% with only 1 base difference, which was not identified by sequencing. The morphological description of J7 (color changed, hyphae) also supports that it is identical to *Aureobasidium pullulans*. 
Discussion

In this study we perform the classical approach of cell-screen process for bioenergy microorganism. Process of repetitive cell plating and sorting is very time consuming with a high risk of contamination. High throughput devices can help accelerating these processes and has potential implication on related research such as the study of cultivation media and phytochemical tolerance. The detection of Ethanol concentration at single cell-scale is especially difficult, attempts using Ramen spectroscopy or enzyme tagging can be possible solutions. In the long run, a high throughput bioenergy yeast screening platform should be created and accelerated the whole process to a short time, provide a more efficient approach for bioenergy research and industrial fermentation. Also, the idea can be applied to other microorganism and different research fields such as microbial cultivation, media studies, single-cell diagnostics.[169, 170]

Figure C-1. DNA fragments after PCR; lane M, size marker (1 kb DNA Ladder, GeneRuler); lane 1, 18S amplicon; lane 2, lane 3, ITS1-5.8S-ITS4 amplicon; lane 4, D1/D2 amplicon of LSU.
Figure C-2. Restriction fragment variability in the 18S, ITS and D1/D2 amplicons. (a) Lane Mb, size markers (1 kb DNA Ladder, GeneRuler); lane 1, 18S fragment not inserted; lane 2, 18S fragment inserted; lane 3, 4, ITS fragment inserted; lane 5, 6, D1/D2 fragment inserted, lane Ms, smaller size markers (100 bp DNA Ladder, GeneRuler). (b) Lane Mb, size markers (1 kb DNA Ladder, GeneRuler); lane 1, 18S fragment inserted; lane 2, ITS fragment inserted; lane 3, D1/D2 fragment inserted.

Figure C-3. Optical density versus DW at 600 nm
Figure C-4. Growth of J5 and J7 under different temperatures in glucose medium.

Figure C-5. Photograph of J7. (Scale bar 1 mm.)

Figure C-6. Photomicrograph of J7. (Scale bar 15μm.)
Figure C-7. GC calibration curve. ■ Instrumental estimation; ● alcohol standards.

Figure C-8. Ethanol production of yeast fermentation using different sugar sources.
Figure C-9. Xylose utilizing pathways in bacteria and yeast.

Table C-1. Strains used in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Isolation</th>
<th>Species or closest rDNA match</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCC 6037</td>
<td>Baker’s yeast, the Nethlands.</td>
<td><em>Saccharomyces cerevisiae</em></td>
</tr>
<tr>
<td>ATCC 24860</td>
<td>Chemostat. culture for xylulose fermentation</td>
<td><em>Saccharomyces cerevisiae</em></td>
</tr>
<tr>
<td>Baker’s yeast</td>
<td>QFC</td>
<td><em>Saccharomyces cerevisiae</em></td>
</tr>
<tr>
<td>AY</td>
<td>English Ale’s yeast, FERMENTIS-LESAFFRE</td>
<td><em>Saccharomyces cerevisiae</em></td>
</tr>
<tr>
<td>CY</td>
<td>Champagne yeast EC-118, LALVIN</td>
<td><em>Saccharomyces bayanus</em></td>
</tr>
<tr>
<td>ATCC 2527</td>
<td>Air</td>
<td><em>Rhodotorula glutinis</em></td>
</tr>
<tr>
<td>WP1</td>
<td>wild poplar (<em>Populus trichocarpa</em>)</td>
<td><em>Rhodotorula graminis</em></td>
</tr>
<tr>
<td>WP21</td>
<td>Yeast isolation from wild poplar</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(<em>Populus trichocarpa</em>)</td>
<td></td>
</tr>
<tr>
<td>PTD2</td>
<td>Hybrid poplar (<em>Populus trichocarpa X P. deltoides</em></td>
<td><em>Rhodotorula mucilaginosa</em></td>
</tr>
<tr>
<td>PTD3</td>
<td>Hybrid poplar (<em>Populus trichocarpa X P. deltoides</em></td>
<td><em>Rhodotorula mucilaginosa</em></td>
</tr>
<tr>
<td>PT10</td>
<td>wild poplar (<em>Populus trichocarpa</em>)</td>
<td></td>
</tr>
<tr>
<td>GRH</td>
<td>Giant reed</td>
<td></td>
</tr>
<tr>
<td>GRI</td>
<td>Giant reed</td>
<td></td>
</tr>
<tr>
<td>J5</td>
<td>Soil</td>
<td><em>Cryptococcus arrabidensis</em></td>
</tr>
<tr>
<td>J7</td>
<td>Rotten fruit</td>
<td><em>Aureobasidium pullulans</em></td>
</tr>
</tbody>
</table>
Table C-2. List of primers used in the study.

<table>
<thead>
<tr>
<th>Amplified fragment</th>
<th>Name</th>
<th>Sequence (5' -3')</th>
<th>Annealing temperature, °C</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S region</td>
<td>NS8 FP</td>
<td>TCC GCA GGT TCA CCT ACG GA</td>
<td>44</td>
<td>White et al., 1990</td>
</tr>
<tr>
<td></td>
<td>NS1 RP</td>
<td>GTA GTC ATA TGC TTG TCT C</td>
<td>44</td>
<td>White et al., 1990</td>
</tr>
<tr>
<td>ITS region</td>
<td>F63 FP</td>
<td>GCA TAT CAA TAA GCG GAG GAA AAG</td>
<td>45</td>
<td>Fell et al., 2000</td>
</tr>
<tr>
<td></td>
<td>LR3 RP</td>
<td>GGT CCG TGT TTC AAG ACG G</td>
<td>45</td>
<td>Fell et al., 2000</td>
</tr>
<tr>
<td>D1/D2 region</td>
<td>ITS1 RP</td>
<td>TCC GTA GGT GAA CCT GCG G</td>
<td>44</td>
<td>White et al., 1990</td>
</tr>
<tr>
<td></td>
<td>ITS4 RP</td>
<td>TCC TCC GCT TAT TGA TAT GC</td>
<td>44</td>
<td>White et al., 1990</td>
</tr>
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</table>

Table C-3. Growth of Isolates in different media.

<table>
<thead>
<tr>
<th>Isolate name</th>
<th>Morphology</th>
<th>Xylose medium</th>
<th>Glucose medium</th>
<th>Sugar free medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baker’s yeast (Control)</td>
<td>White, beige, dry surface</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>WP1</td>
<td>Pink to orange, wet</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>PTD2</td>
<td>orange, wet</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>PTD3</td>
<td>orange, wet</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>WP21</td>
<td>Pale pink, filamentous</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>PT10</td>
<td>Pale pink, filamentous</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>GRH</td>
<td>Orange, wet</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>GRI</td>
<td>White, wet</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>J5</td>
<td>White, filamentous</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>J7</td>
<td>Pale pink, yellow, black, filamentous</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

Table C-4. Ethanol left in solution after washing with MS medium.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Ethanol left in solution (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before wash</td>
</tr>
<tr>
<td>AY</td>
<td>100</td>
</tr>
<tr>
<td>CY</td>
<td>100</td>
</tr>
<tr>
<td>J7</td>
<td>100</td>
</tr>
</tbody>
</table>
References


Curriculum Vitae

Education
2007-present  Ph.D., Chemical Engineering & Nanotechnology dual degree program, University of Washington, Seattle, Washington
   Dissertation: Design, Analysis, and Translation of Hydrodynamic Tweezer Microeddies
   Supervisor: Daniel T. Schwartz
2003-2007  B.S., Chemical Engineering, National Taiwan University, Taipei, Taiwan

Working Experience
2007-present  Pre-doctoral Research /Teaching Assistant, University of Washington
2011.6-2011.9  Summer Intern, Lam Research Corporation
   Engaged in process development and control system of new etching product. Performed tests and data analysis on system characterization and monitoring process.
2005.9-2006.8  Undergraduate Student Assistant, National Taiwan University
2005.7-2005.8  Summer Undergraduate Research Assistant, Material Science and Engineering, University of Washington

Research Experience
2007-present  Electrochemical Materials & Interfaces Laboratory, University of Washington
   Microfluidics for single-cell diagnostics
2006-2007  Department of Chemical Engineering, National Taiwan University
   Inhibition of amyloid fibrillization
2005-2006  Institute of Molecular and Cellular Biology, National Taiwan University
   DNA ligation, yeast transformation for examining meiotic cell-cycle control.
2005.7-2005.8  Material Science and Engineering, University of Washington
   Organic synthesis of electro-optic materials.
Peer Reviewed Publications


**Patent**

Selected Conference Presentations


Professional Memberships, Awards and Honors

2011
Top 10 Travel Award (out of 135 competitors), National Science Foundation 2011 IGERT Poster Competition, Washington D.C.

2011.5
Synapse Design Best Clean-Tech Idea, University of Washington Business Plan Competition

2011.5
Best Poster Award in Energy, UW GPSS Science and Policy Summit

2011.4
Davis Wright Tremaine Award, UW Environmental Innovation Challenge

2008-2010
National Science Foundation Bioenergy Integrative Graduate Education and Research Traineeship (NSF-IGERT)

2007-2008
Chemical Engineering Graduate Student Fellowship, University of Washington
2009-present
American Institute of Chemical Engineers (AIChE)
2009-present
The Electrochemical Society (ECS)
2007-present
Nanotechnology and Nanoscience Student Association at University of Washington

Technical Skills

Fabrication: Photolithography, MEMS fabrication methods, Machining, Plasma processing


Biology: DNA/RNA extraction, Polymerase Chain Reaction, Cell incubation, Electrophoresis, Plasmid Transformation.

Computer: General processing of documents and graphics, MS Word, MS Excel, MS Powerpoint, AutoCAD, MATLAB, KaleidaGraph, Illustrator.

Languages: English: Native
Chinese: Native
German: Working proficiency
Japanese: Elementary
**Leadership and Outreach Activities**

<table>
<thead>
<tr>
<th>Year</th>
<th>Position/Activity</th>
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| 2011-2012  | Team Leader, *StaticFlow Analytics/Biotek*  
Davis Wright Tremaine Award, University of Washington Environmental Innovation Challenge  
Honorable Mention, Social Venture Plan Competition, Seattle Pacific University  
Semi-finalist 42/510, Rice University Business Plan Competition  
Synapse Design Best Clean-Tech Idea, University of Washington Business Plan Competition |
| 2011.2-2011.4 | Volunteer mentor, Bryant Elementary School Science Fair, Seattle, WA |
| 2006-2007  | Staff member, United Student Association, National Taiwan University |
| 2005-2006  | President, Chemical Engineering Student Association, National Taiwan University  
Director, 60th Anniversary of Chemical Engineering Department, National Taiwan University |
| 2004-2005  | Chair of Student Activities, Student Association of Chemical Engineering |