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UNIVERSITY OF CALIFORNIA, SAN DIEGO

Microfluidics for Optics and Quantitative Cell Biology

A dissertation submitted in partial satisfaction of the requirements for the
degree Doctor of Philosophy

in

Physics

by

James Kyle Campbell

Committee in charge:

Professor Alex Groisman, Chair
Professor Steven Buckley
Professor Patrick Diamond
Professor Yeshaiah Fainman
Professor David Kleinfeld

2008

The Dissertation of James Kyle Campbell is approved, and it is acceptable in quality and form for publication on microfilm:

Chair

University of California, San Diego

2008

TABLE OF CONTENTS

Signature Page.....	iii
Table of Contents.....	iv
List of Figures.....	vi
List of Tables.....	ix
Acknowledgements.....	x
Vita.....	xii
Abstract.....	xv
1. Introduction.....	1
1.1 General Fabrication Methods.....	3
1.2 Standard Experimental Setup.....	5
1.3 Organization of Dissertation.....	6
2. Microfluidics for Quantitative Cell Biology.....	8
2.1 Chemical Concentration Gradients for Cell Biology.....	9
2.1.1 Generation of Complex Concentration Profiles in Microchannels in a Logarithmically Small Number of Steps.....	10
2.1.2 Neutrophil Chemotaxis.....	36
2.1.2.1 Bound Attractant at the Leading vs. the Trailing Edge Determines Chemotactic Prowess.....	43
2.1.3 Yeast Chemotropism.....	68
2.1.3.1 MAPK-Mediated Bimodal Gene Expression and Adaptive Gradient Sensing in Yeast.....	71
2.1.3.2 Exponential and Two-Dimensional Gradients for Non-adherent Cells.....	87

2.2 Chemostat.....	91
2.2.1 A Microfluidic Chemostat for Experiments with Bacteria and Yeast.....	95
3. Optofluidics.....	107
3.1 A Microfluidic 2x2 Optical Switch.....	108
3.2 Pressure-driven Devices with Lithographically Fabricated Composite Epoxy- Elastomer Membranes.....	121
3.3 Pneumatically Actuated Adaptive Lenses with Millisecond Response Time...	135
References.....	145

LIST OF FIGURES

Figure 1. Micrograph and drawings of the microfluidic devices for complex concentration profiles.....	16
Figure 2. Splitting and redistribution of flux in a horizontal channel.....	20
Figure 3. Schematic diagrams illustrating the construction of a gradient-making network.....	21
Figure 4. Distributions of FITC in the test channels of the microfluidic devices and the respective plots.....	29
Figure 5. Concentration of FITC as a function of position across the test channel.....	31
Figure 6. Microfluidic devices used in the chemotaxis study.....	37
Figure 7. Flow in the central area of the 81:1 device.....	40
Figure 8. Injection of the gradient-carrying stream into the test channel.....	41
Figure 9. Chemotaxis in a linear concentration profile of fMLP.....	48
Figure 10. Chemotaxis and migration in exponential gradients.....	51
Figure 11. Design and operation of the microfluidic device producing an exponential profile.....	66
Figure 12. Pheromone dose response analysis using a microfluidic experimental setup.....	73
Figure 13. Quantification of Fus1 protein and gene expression.....	76
Figure 14. Computational modeling of transcriptional regulation in the pheromone response.....	78
Figure 15. Role of Kss1 in regulating the pheromone response.....	81

Figure 16. Quantification of pheromone gradient sensing.....	84
Figure 17. Microfluidic device with exponential concentration profiles.....	89
Figure 18. Microfluidic devices generating a 2D concentration profile in a test chamber.....	90
Figure 19. Design and operation of the two-layer microfluidic device.....	97
Figure 20. A representative experiment showing development of a JM109 <i>E. coli</i> colony.....	100
Figure 21. Response of cells to addition and removal of exogenous signal.....	102
Figure 22. A microphotograph of the microfluidic switch device.....	111
Figure 23. Schematic drawings showing construction and consecutive stages of fabrication.....	112
Figure 24. Images of reflected and transmitted beams.....	116
Figure 25. Power of transmitted and reflected beams as a function of time.....	119
Figure 26. Composite membrane devices, the stretcher and rotator.....	122
Figure 27. Fabrication of a composite membrane.....	124
Figure 28. Photographs of diffraction patterns generated at different pressures for the stretcher and rotator.....	126
Figure 29. Relative extension of the central strips as a function of the elevation of the membrane for composite and plain membranes.....	128
Figure 30. Angle of in-plane rotation as a function of pressure.....	131
Figure 31. Schematics of an adaptive compound camera lens with flexible membrane.....	137

Figure 32. Temporal response of the lenses as measured by a photo-detector.....	140
Figure 33. Variation of refractive power of the membrane lenses with time in different regimes.....	142

LIST OF TABLES

Table 1: Least-squares fits of CI to C_s	57
Table 2: Insertion loss and extinction ratio (in dB) for transmitted and reflected beams.....	118

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Chapter 2.1.1, in full, is a reprint of the journal article *Generation of complex concentration profiles in microchannels in a logarithmically small number of steps* in Lab on a Chip, 2007. Campbell, K.; Groisman, A., Royal Society of Chemistry, 2007. Dissertation author was the primary investigator of this paper.

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Chapter 3.1, in full, is a reprint of the journal article *A microfluidic 2x2 optical switch* in Applied Physics Letters, 2004. Campbell, K.; Groisman, A.; Levy, U.; Pang, L.; Mookherjee, S.; Psaltis, D.; Fainman, Y., American Institute of Physics, 2004. Dissertation author was the primary investigator of this paper.

Chapter 3.2, in full, is a reprint of the journal article *Pressure-driven devices with lithographically fabricated composite epoxy-elastomer membranes* in Applied Physics Letters, 2006. Campbell, K.; Levy, U.; Fainman, Y.; Groisman, A., American Institute of Physics, 2006. Dissertation author was the primary investigator of this paper.

Chapter 3.3, in full, is a reprint of the journal article *Pneumatically actuated adaptive lenses with millisecond response time* in Applied Physics Letters, 2007. Campbell, K.; Fainman, Y.; Groisman, A., American Institute of Physics, 2007. Dissertation author was the primary investigator of this paper.

VITA

- 2002 Bachelor of Science in Physics and Mathematics, New Mexico Institute of Mining and Technology
- 2002 – 2003 Teaching Assistant, Physics Department, University of California, San Diego
- 2003 Master of Science in Physics, University of California, San Diego
- 2003 – 2007 Research Assistant, Physics Department, University of California, San Diego
- 2008 Doctor of Philosophy in Physics, University of California, San Diego

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ABSTRACT OF THE DISSERTATION

Microfluidics for Optics and Quantitative Cell Biology

by

James Kyle Campbell

Doctor of Philosophy in Physics

University of California, San Diego, 2008

Professor Alex Groisman, Chair

Microfluidics is a quickly expanding field with numerous applications. The advent of rapid-prototyping and soft-lithography allow for easy and inexpensive fabrication of microfluidic devices. Fluid manipulation on the microscale allows for new functionalities of devices and components not available on the macroscale. Fluid flows on the microscale are laminar with chemical mixing defined strictly by diffusion allowing us to design microfluidic devices with precise control of fluid flow and chemical concentration. New microfluidic technologies can provide new functionalities for micro-total analysis systems for greater device integration and portability.

To expand the utilization of microfluidics, this dissertation discusses new microfluidic techniques and devices. Chapter 2 examines microfluidics for quantitative

cell biology. New techniques discussed in this dissertation allow us to use microfluidics to study cellular response on a cell-by-cell basis in stable environments. Part of this chapter includes new architectures for creating chemical concentration gradients in microfluidic devices and their applications cell biology. The rest of Chapter 2 introduces a device which allows for cells to grow to high densities in chemostatic conditions. Chapter 3 introduces the merger of optics and microfluidics named “optofluidics”. This subset of microfluidics uses the techniques and materials of microfluidics for optical applications. This dissertation describes optofluidic projects involving light manipulation such as a switch, actuator and lens.

1. INTRODUCTION

The field of microfluidics involves the manipulation of fluids and gases on the nanoliter scale, with fluid pathways sub-micron to millimeters in size. Due to the small scales, microfluidic flows have very low Reynolds numbers resulting in predictable laminar flows. Scaling devices down to the microscale allows for new experiments not feasible on a larger scale such as cellular biology studies, in which the behavior of individual cells rather than large cell populations can be monitored. Smaller size also contributes to greater integration with other components like lab-on-a-chip technologies which drive to have total analysis tools incorporated into a portable package. Last but not least, the use of microfluidics makes it possible to dramatically reduce reagent consumption, thus costly samples can be used more effectively in serial studies, or in parallel assays.

Standard device fabrication techniques are easy and inexpensive, requiring minimal fabrication space and equipment. All these factors have contributed to a boom in the area of microfluidic technologies in the past few years. One of the predominant materials for constructing microfluidic devices is polydimethylsiloxane (PDMS), an optically clear silicone elastomer. PDMS prepolymer is a viscous liquid and can be poured onto a mold. Once PDMS becomes cross-linked and solidifies, it takes the shape of a replica of the mold. PDMS is compatible with aqueous solutions and chemically inert, which makes it ideal for many biochemical and biological applications. Flexibility of PDMS facilitates bonding of the devices to a substrate, and allows for the integration of control valves.

Designing a microfluidic device for a specific application requires many parameters to be considered. Fluidic resistance of the microchannels along with the driving pressure determines the speed of the fluid flow. The rate of flow is a very important factor when dealing with live cells as the cells can detach from a substrate and get washed as well as become stressed or unresponsive when in a high-shear flow. Diffusive mixing can also place constraints on the fluid speed. In some cases one has to make sure that the solution is completely mixed when used, whereas in other cases it is important to minimize the diffusive mixing so a chemical is not slowly depleted by diffusion. If there are particles such as beads or cells suspended in the working fluid, a consideration must be given to their size and to whether they can be deformed. Since PDMS is a flexible material, excessive pressure can cause undesired deformation of channels, but can also be applied deliberately to temporarily increase the opening of specific junctions to allow particles of larger sizes or a greater volume of fluid through those junctions.

Microfluidics is an interdisciplinary area with applications in biology, chemistry, biochemistry, biotechnology, medicine and optics. Miniaturization of the flow setups allows for easy integration with other systems, easier parallelization of fabrication and use, and increased portability. To enhance the power of microfluidic techniques for commercial and research applications, new types of microchannel arrangements and functionalities need to be developed, tested and implemented. The work presented in this thesis is a collection of new techniques in microfluidics and PDMS devices for cell biology and adaptive optics applications.

1.1 GENERAL FABRICATION METHODS

Fabrication of the devices used for the research presented in this thesis was done with the standard soft-lithography techniques¹. Once an arrangement of the microchannels is designed and its parameters are calculated, a drawing is made in Macromedia Freehand, a vector-based graphic software. If the device has channels of different depths or multiple layers of channels separate masks are drawn for each step in the depth and each channel level. Usually, multiple versions of each device are designed with incremental changes to some parameters of the device, such as the resistance of a certain channel, or the size of a micro-chamber. Once the individual devices are drawn, they are mutually arranged in an 3x3” multi-layer drawing. The individual layers, which are the drawing of individual 3x3” photomasks are then separated from each other and arranged on a larger page (~8x10”) for printing. The file is sent to a printing shop for high-resolution plotting (16,000 to 40,000 dpi) on a plastic film. Printing of the photomasks is the only part of the fabrication process not done in-house. Individual 3” by 3” transparencies are cut from the plastic film and glued onto a 4” by 4” glass plates to form the masks.

Because the microfluidic devices are machined in PDMS by molding, there machining requires a master mold. The master mold is fabricated by contact lithography with the printed photomasks. A 4” or 5” silicon wafer is spin-coated with either a layer of a photoresist of a desired thickness, either a “negative” photo-curable epoxy SU8 (Microchem, Boston, MA) or a “positive” AZ100 (Clariant, Charlotte, NC). The wafer with photoresist is baked on a hot plate according to specifications. After the wafer has cooled, it is placed on a mask aligner with the appropriate photo-mask. If the microfluidic

device has channels of different depths (most commonly with the SU8 resists), the master mold is fabricated with a multi-level relief in a multi-step process. The photomasks are used in the order of increasing height of the relief features and before the UV-exposure, they are carefully aligned with the existing patterns on the wafer. The wafer is then exposed to UV light for a set amount of time depending on photoresist type and thickness. With the SU8 resists, after each UV-exposure, the wafer is placed on a hot plate and baked to cross-link the photoresist in exposed areas. To remove unexposed and uncured photoresist, the wafer is dipped and agitated in a bath of photoresist developer. The complete wafer has a positive relief of the microchannels for the device.

To help the wafer and photoresist pattern survive multiple castings, the surface is passivated (made non-sticky) by vapor treatment with TMCS (Trimethylchlorosiloxane). A PDMS pre-polymer is prepared as a mixture of two components, cross-linker and base. A 1:10 ratio of cross-linker to base is the standard recommended by the manufacturer. However, the ratio can be varied between 1:5 and 1:20 depending on the application and the desired hardness of the cured PDMS. The wafer with photoresist pattern is placed in an aluminum-foil cup and the liquid PDMS pre-polymer is poured onto the wafer. The pre-polymer covered wafer is degassed to remove any bubbles and then baked at 85 °C for 45 minutes to 1.5 hours. The wafer with cured PDMS is removed from the oven and individual devices are cut with an exacto-knife and peeled off the wafer. Inlet and outlet holes are punched with a sharpened hypodermic needle attached to a luer holder (luer stub). If the device is made of two layers of microchannels, a flow layer and a control layer, with integrated valves, the master mold (wafer) with the flow layer pattern is spin-coated with a thin layer of PDMS (~100 μm). The wafer is then placed in an 85 °C oven

and baked for 10 to 20 minutes to solidify, but remain sticky. The wafer with the pattern of control layer channels is used to cast PDMS chips of a usual thickness (~5 mm). The chips are then placed onto the first wafer with the thin, sticky PDMS layer and aligned with the pattern of the flow layer channels on the wafer. After baking at 85 °C for 1.5 hours the two layers of PDMS become a monolith with thin flexible membranes in the regions where microchannels in the two layers overlap. The individual devices are cut out and the inlet and outlet holes are punched in them. Once a single or double layer PDMS device is finished, the engraved (channel) side of the device is sealed with a glass coverslip and baked in an 85 °C oven overnight to promote bonding between the glass and PDMS.

1.2 STANDARD EXPERIMENTAL SETUP

Most of the microfluidic experiments were done with a Nikon Eclipse TE2000-U inverted microscope with a CCD camera attached to its video port. The microscope had a incandescent light source for brightfield and phase-contrast illumination, as well as a mercury light source with a set of filter cubes for fluorescence microscopy. The working liquids fed to the inlets and drawn from the outlets were kept in syringes that were connected to the device using tygon tubing lines and short segments of hypodermic tubing (capillary) inserted into the inlet and outlet holes punched in the PDMS devices. All syringes were attached to different moving stages sliding along vertical rails placed next to the microscope. The flow in the devices was driven by positive differential hydrostatic pressure between syringes connected to the device inlets (placed at relatively high levels) and syringes connected to the outlets (placed at relatively low levels).

Importantly, the syringes connected to the outlets were placed at a level of ~10 cm above the level of the device, thus creating a positive ambient pressure in the device and preventing formation of air bubbles.

If the device had integrated membrane valves, an array of three-way solenoid valves (Lee Company) was used with a custom control box that was operated either manually or controlled by a computer through a NI DAQ card and a code in LabView. One inlet of the solenoid valves was connected to a source of pressurized air (>8psi), and the other inlet was open to atmosphere. The common outlet of the solenoid valve was connected to a control layer inlet of the microfluidic were through a tygon tubing and a metal capillary. The membrane valve (or valves) connected to this control inlet was open, when the common outlet of the solenoid valve was vented to atmosphere and closed, when it was connected to the source of pressurized air.

1.3 Organization of Dissertation

Most of the projects I have been involved with are described in this dissertation. Collaborative projects are introduced and my contribution is stated. A copy of the published results follows. Chapters describing projects where I was the leading contributor only include reprints of the published articles without separate introductions.

Chapter 2 reviews the projects, where I built and tested microfluidic device for experiments in quantitative cell biology. The first project in this chapter is a method to create a concentration field of a reagent of interest with a shape of any desired monotonic function out of two solutions with the highest and lowest concentration in the field. The next two projects involve the use of chemical concentration gradients to study the

gradient and dose response of live cells. The last project is a microfluidic device that allows cells to grow under chemostatic conditions up to high densities. These cell biology projects were collaborations with other research groups. My major contribution to these projects was the design, fabrication and characterization of the microfluidic devices.

Chapter 3 includes the use of microfluidics and of devices machined of PDMS in optical applications. Termed “optofluidics”, these projects describe new adaptive optics technologies that are based on methods and materials from microfluidics. The first project in this section describes a 2x2 optical switch that uses a PDMS channel filled with water or an index-matching solution for switching between reflection and transmission of a laser beam. The second project comprises of a PDMS membrane with imbedded SU8 epoxy parts that influence the bending mode of the membrane when inflated. The last project is a PDMS membrane-based lens with a 4 diopter focusing range and millisecond switching time. I was a leading contributor to all three projects and had unshared first authorship of the published papers.

2. Microfluidics for Quantitative Cell Biology

Microfluidics has gone through explosive growth in recent years and is now generally recognized as a powerful technology for biological and medical research. Fluid flows through the microscopic channels are generally stable and laminar, and chemicals mix by diffusion only. This allows for the concentration of these chemicals to be both spatially and temporally controlled. Individual cells can be continuously exposed to a known concentration of a nutrient to control growth, or they can be exposed to a gradient of a chemical to study their gradient or dose response. Moreover, the small size of the microfluidic devices and their compatibility with high-resolution light microscopy make it possible to study cellular responses on the level of individual cells.

The soft lithography fabrication process and PDMS as a material are a very good match for the needs of the laboratory research in cell biology. The fabrication is fast and inexpensive. The devices are reusable can be readily made on the scales of 10-100 copies, which are usually sufficient to perform an extensive series of laboratory experiments. The flexibility of PDMS facilitates sealing the devices outside of the clean rooms allows for integration of the membrane valves for better flow control. The devices are air-permeable, so air bubbles are readily removed by applying high ambient pressures to the device interiors and cells growing in the device have access to the atmosphere oxygen. Working within these features, devices are designed and optimized for a specific experiment or application.

Sub-chapter 2.1 describes experiments using microfluidics to control chemical concentration profiles. Sub-chapter 2.2 will discuss a microfluidic architecture that allows cells to grow to high densities in chemostatic conditions.

2.1 Chemical Concentration Gradients for Cell Biology

Some cells are known to respond to external gradients of chemical stimuli. Adherent cells such as bacteria and neutrophils can migrate towards higher concentrations of chemoattractant. Non-adherent cells, such as yeasts, can develop mating protrusions towards a higher concentration of pheromones in an attempt to find another yeast cell. In order to develop an accurate quantitative model of these processes, the concentration gradients the cells are exposed to need to be stable and well-defined. Chemical gradients created by many current methods are ill-defined and slow to form. One such method uses a micropipette filled with the chemical of interest and is placed in contact with a solution of medium and cells. The chemical diffuses constantly through the tip of the pipette, causing a concentration gradient to form. This gradient can slowly change over the course of hours and lead to the eventual contamination of the region of interest. Using microfluidics, we can design a device that has a known chemical concentration gradient using only a low and high concentration source of liquids. This gradient will remain stable as long as there is a source of fluids. A constant flow of the source and sink solutions (high and low concentration respectively) guarantees that there will be no degradation of the shape of the concentration gradient over time.

Chapter 2.1 of the dissertation describes the microfluidic tools for generation of stable gradient profiles of different shapes and the use of these gradient profiles in experiments on two types of life cells, human neutrophil-like differentiated cells HL60 and budding yeast. Chapter 2.1.1 will discuss a new microfluidic architecture that can create a chemical concentration gradient of any monotonic function in a logarithmic number of steps. Chapter 2.1.2 will discuss the use of chemical gradients to study

neutrophil chemotaxis, including my contribution to the project and a published paper. Lastly, chapter 2.1.3 will discuss yeast chemotropism in a no-flow chemical gradient, including my contributions to the project and a published paper.

2.1.1 Generation of Complex Concentration Profiles in Microchannels in a Logarithmically Small Number of Steps

The engineering of controlled liquid environments with gradients of concentration of relevant substances is important for various chemical and biological applications, in particular, for the study of chemotaxis. Reasonably stable gradient profiles are created in simple chemotaxis chambers designed by Zigmund² and by Dunn *et al*³ that consist of two large wells connected by a narrow channel. A gradient profile is generated by the molecular diffusion through the channel, from the well loaded with a solution with a high concentration of the chemoattractant (source) to the well loaded with a low concentration solution or a plain buffer (sink). This method of gradient generation has the drawbacks of limited control of the profile shape, long time required for the gradient to build up, and eventual deterioration of the gradient due to depletion of the source and contamination of the sink.

Truly stable concentration profiles can be created by using solutions with different concentrations flowing at a constant speed through a microscopic channel. Since the microscopic flows are generally laminar and stable, a state of dynamic equilibrium is reached in the channel - the diffusive flux driven by a concentration gradient across the flow is balanced by constant replenishment of the solutions. The simplest implementation of this approach is with two source solutions injected into a microchannel side by side.

While useful for some applications^{4, 5}, this arrangement has multiple shortcomings. The concentration profile across the flow always has a characteristic sigmoidal shape⁶, which may not be optimal for the desired application, and creating a gradient profile of sufficient width may require an impractically long channel. In addition, the shape of the profile depends on the position along the channel and on the flow speed.

These problems were successfully resolved in the devices introduced by Whitesides *et al.*^{7, 8} Variations of these devices have been used for a variety of biological applications, including experiments on chemotaxis of human neutrophils⁹⁻¹³, cancer cells¹⁴ and amoebas¹⁵; on culturing of cells at different medium conditions; and on cell dose response¹⁶⁻¹⁸. Among other applications there have been titration¹⁹, differential patterning of surfaces²⁰⁻²², fabrication of materials with spatially modified properties^{23, 24}, and on-chip adjustment of refractive index²⁵. A key part of the devices is a gradient-making network of microchannels that typically has a recognizable pyramidal shape.^{7, 8} In its basic configuration, the network is supplied with two source solutions and delivers to its outlet a set of N solutions with a linear sequence of concentrations. Inside the network the source solutions flow through a series of stages, where they are repeatedly split and mixed in different proportions. At the network outlet, streams of the N solutions merge into a single stream with linear variation of concentration across the direction of flow.

The gradient-making network by Whitesides *et al.* can be readily adapted to generate concentration profiles with shapes of power functions and polynomials.^{7, 8, 26, 27} However, generation of a polynomial of a power m requires $m+1$ separate source solutions, becoming impractical at large m , and there is no straightforward way to

generate non-polynomial shapes such as an exponent. In addition, the number of splitting-and-mixing stages linearly increases with N , and while large N is beneficial for the accuracy and stability of the concentration profile, in most practical implementations of the network $N \leq 10$. An alternative gradient-making network allowing generation of complex concentration profiles has been recently proposed by Toner *et al.*²⁸ However, it was only shown to be operational at very low flow rates (20 $\mu\text{m/s}$), and just as in the network in Ref. 6, the number of steps of splitting and mixing was linearly increasing with N .

Here we present an alternative architecture for microfluidic gradient-making networks that has two advantages over the original design by Whitesides *et al.*^{7, 8} First, it allows generating a monotonic concentration profile of any given shape. Second, a logarithmically small number of splitting-and-mixing stages ($\sim \log_2 N$) is required to produce a set of N solutions with different concentrations, making the network more compact and substantially increasing the practical limit on N . We have designed, fabricated and tested three microfluidic devices that produce an exponential concentration profile, a linear profile, and a profile with a shape of two fused branches of a parabola.

Experimental Section

Microfabrication. Each of the three microfluidic devices described in this work (Figure 1) has two inlets, one outlet and is made of a single cast of polydimethylsiloxane (PDMS; Sylgard 182, Dow Corning, Midland, MI) bonded to a microscope cover glass. A master mold to cast the PDMS chips was fabricated by spin-coating a 40 micron layer of negative photoresist (SU-8 2015, Microchem, Newton, MA) onto a 5" silicon wafer and exposing it to UV-light through a specially designed photomask with a resolution of

20,000 dpi. PDMS prepolymer was poured onto the master mold, degassed and cured by baking in an 80 °C oven for 1.5 hr. Individual chips were cut from the cured PDMS, and inlet and outlet holes were punched with a 20 gauge luer-stub. The chips were soaked in 0.01M HCl at 80 °C for 1 hour to make the surface more hydrophilic. The devices were completed by attaching #1.5 cover glasses to the patterned surfaces of the PDMS chips and baking them at 80 °C overnight to promote adhesion between the PDMS and the glass.

Flow control. The working liquids were kept in 60 cc plastic syringes, which were held upright with the luer connector at the bottom and were open to the atmosphere at the top. Each syringe was connected to a microfluidic device through a blunt luer needle (gauge 23), a long piece of Tygon tubing with an internal diameter of 0.5 mm, and a short piece of hypodermic steel tubing inserted into an inlet or outlet of the device. The flow in the microchannels was driven by setting a difference in pressure between the inlets and the outlet, P_d . The differential pressure was generated hydrostatically by attaching the two syringes connected to the inlets to a moving stage sliding along a vertical rail with a precise ruler.²⁹ The surfaces of liquid in the two syringes were at the same height. The syringe connected to the outlet was held at a constant lower level. The difference in the levels of liquid between the syringes connected to the inlets and to the outlet was measured with a precision of about 0.2 mm, corresponding to 2 Pa in pressure.

Chemicals. To visualize concentration profiles in microchannels we used solutions of fluorescein isothiocyanate, FITC, (Sigma, St. Louis, MO) with a diffusion coefficient $D = 5 \times 10^{-6} \text{ cm}^2/\text{s}$. To stabilize its fluorescence level, FITC was dissolved in a pH = 7.5 phosphate buffer.

Microscopy and measurements. Microfluidic devices were mounted on an inverted fluorescence microscope Nikon TE2000U, which was equipped with a cooled digital CCD camera, Spot RT-SE18 (Diagnostic Instruments, Sterling Heights, MI), and an electronic shutter in the fluorescence illumination light train. The shutter was normally closed to prevent excessive photobleaching; its opening was synchronized with taking fluorescence images. The concentration of FITC in microchannels in a specific area of a microfluidic device was evaluated by measuring the intensity of light at that location in a fluorescence image. Before the fluorescent light intensity was measured, the image was pre-processed in two steps. First, the background light level, corresponding to known non-fluorescent areas, was subtracted from the image. Second, the image was normalized with a distribution of fluorescent light from a slab of a uniformly fluorescent material at the same illumination and imaging condition. This normalization (flat-field correction) was performed to compensate for non-uniformity in the fluorescence illumination, light collection and sensitivity of the CCD array. To test for linearity of the dependence of the fluorescence intensity on the FITC concentration, we took images of microchannels with different known concentration of FITC inside. In the working range of 0 – 15 ppm, the dependence was found to be linear within ~1%. Maximal flow velocity in channels, $v_{\max}$, at various driving pressures, P_d , was evaluated by seeding solutions fed into the device with 4.6 μm fluorescent particles (Interfacial Dynamics Corp, Portland, OR) and measuring the extension of the longest streaklines produced by the particles. The mean flow velocity, $\bar{v}$, in the 50×40 μm serpentine channels was computed as $\bar{v} = 0.48v_{\max}$, with the ratio between $\bar{v}$ and $v_{\max}$ calculated using equations from Ref.³⁰.

Design and operation of the microfluidic devices. Similarly to the devices introduced by Whitesides et al.^{7, 8}, each of the microfluidic devices described in this paper consists of two parts: a gradient-making network and a test channel (wide vertical channel in Figure 1a, b and c). The gradient-making network is fed by two source solutions with different concentrations injected into the device through separate inlets, and it generates N solutions of different concentrations. These latter solutions are directed into the test channel through N separate narrow channels (the lowest row of serpentine channels in Figure 1). In all three devices described in this paper $N = 17$ (Figure 1). It is convenient to number these latter channels from left to right, and designate the concentration in n th channel as c_n , where $1 \leq n \leq N$. The set of c_n is constructed to be a discrete representation of the desired concentration profile across the test channel, $c(y)$.

Streams of all solutions injected into the test channel have the same volumetric flux and approximately the same width in the test channel, $\Delta y \approx w/N$, where w is the width of the test channel. Exceptions are the two streams at the edges, which are somewhat wider because the flow velocity near the edges of the test channel is lower than in the middle. As the flow advances along the test channel, the initial discontinuity in concentration between adjacent streams gradually disappears due to molecular diffusion. The characteristic diffusion time is $t_D = \Delta y^2 / D$, corresponding to a characteristic distance $\Delta x = \bar{v} t_D$ from the stream merging point at the beginning of the test channel. Here D is the coefficient of diffusion of the solute, and $\bar{v}$ is the mean flow speed. (For the actual test channel, the analysis is somewhat more complicated because of variation of the channel width in the beginning.) At distances $x > \Delta x$, $c(y)$ is a smooth function.

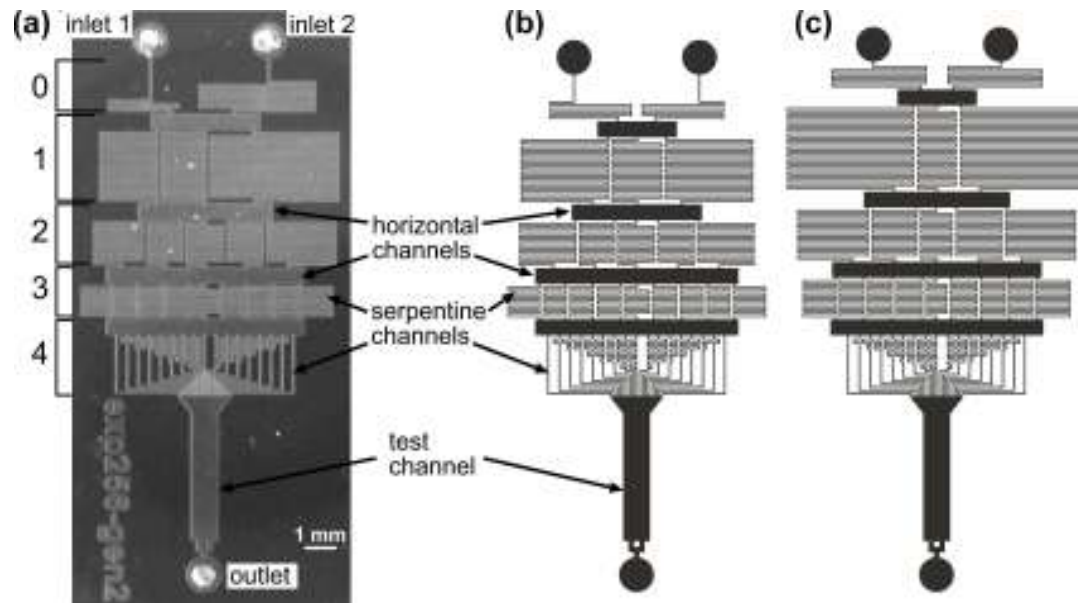


Figure 1. Microfluidic devices. (a) Micrograph of the microfluidic device generating an exponential concentration profile. (b) and (c) Drawings of the microfluidic devices generating a linear and double-parabolic concentration profile, respectively. Numbers on the left indicate individual stages of splitting-and-mixing.

Molecular diffusion across the test channel also levels off the non-uniformity of $c(y)$ on the scale of the whole channel, making $c(y)$ increasingly different from its desired shape as x and the residence time in the flow increase. Characteristic time of this unwanted leveling off can be estimated as $w^2 / D = (N\Delta y)^2 / D = N^2 t_D$, corresponding to a distance $N^2 \Delta x$ from the beginning. These simple arguments show that a suitable working range of x , at which the distribution $c(y)$ is both smooth and closely matching the set of c_n , is given by $\Delta x < x \ll N^2 \Delta x$. Furthermore, at $\Delta x < x \ll N^2 \Delta x$, the concentration profile has little sensitivity to variations of $\bar{v}$. Therefore, it is essential that N is sufficiently large, $N^2 \gg 1$, for the device to operate well. In addition to providing a long working region in the test channel, a large value of N also allows accurate definition of $c(y)$ by the discrete set of c_n . The latter is especially important for non-linear $c(y)$, because the molecular diffusion between the adjacent streams in the test channel tends to reduce the curvature of the concentration profile.

The proposed gradient-making networks have a layout similar to the networks introduced by Whitesides et al.^{7, 8}: they consist of consecutive stages, each comprised of a wide horizontal channel and of a set of narrow serpentine channels downstream from it (Figure 1). (The latter channels are called vertical channels in Ref.^{7, 8}). The horizontal channels distribute the flow emerging from the upstream serpentine channels over a larger number of the downstream serpentine channels. In addition, due to low flow resistance of the horizontal channels, all serpentine channels belonging to the same stage have equal pressures at their inlets and equal pressures at their outlets. The function of the serpentine channels is to provide diffusive mixing of solutions injected into them and to

generate a homogeneous solution of an intermediate concentration, which is fed into a downstream horizontal channel. The diffusive mixing is facilitated by the small width and large length of the serpentine channels, resulting in small characteristic diffusion time and large liquid residence time, respectively. Volumetric flux through a channel, $q = \Delta P / R$, is defined by the difference in pressure between the inlet and the outlet, ΔP , and by the channel flow resistance, R . The flow resistance is proportional to the channel contour length, $R = \kappa L$, where κ is the same for all serpentine channels, as they all have the same width and depth, $w_s = 50 \mu\text{m}$ and $h = 40 \mu\text{m}$, respectively.

The gradient-making networks in the devices shown in Figure 1 differ from those in Ref.^{7, 8} in two important regards. First, serpentine channels belonging to the same stage have different lengths, L , leading to different R and different q through the channels. Variability of q makes it possible to mix solutions in individually adjustable proportions and to generate concentration profiles with a variety of shapes. Second, the stream from an individual serpentine channel of the k th stage is split between 3 serpentine channels of the $(k + 1)$ th stage (Figure 2, 3), as compared to 2 channels of the $(k + 1)$ th stage in Ref.^{7, 8}. (An exception are streams from the channels at the edges, which are split between 2 channels of the $(k + 1)$ th stage just as in Ref.^{7, 8}.) The proposed gradient-making networks are built so that each odd-numbered serpentine channel of the $(k + 1)$ th stage is fed by a single channel of the k th stage and thus carries solution of the same concentration (Figure 2, 3). Each even-numbered serpentine channel of the $(k + 1)$ th stage is fed by two adjacent channels of the k th stage, and a solution with an intermediate concentration is generated in it. The numbers of solutions with different concentrations within the k th and

$(k+1)$ th stages, N_k and N_{k+1} , are connected by a recurrence relation, $N_{k+1} = N_k + (N_k - 1)$. Given $N_1 = 3$, we derive $N_k = 2^k + 1$ and $k = \log_2(N_k - 1)$, meaning that the number of stages in the proposed gradient-making networks increases only logarithmically with the number of distinct concentrations at the network outlet.

Construction of a gradient-making network of the proposed architecture begins with the definition of the desired shape of the concentration profile, $c(y)$, and the number of stages in the network. All three networks described here have 4 stages, corresponding to $N = N_4 = 2^4 + 1 = 17$ serpentine channels in the last stage. To keep the notation more compact, we will designate the n th serpentine channel (as counted from the left) of the k th stage as channel $\langle k, n \rangle$ (Figure 2b). The concentration and volumetric flux in channel $\langle k, n \rangle$ will be designated as $c_{k,n}$ and $q_{k,n}$, respectively. The resistance and length of channel $\langle k, n \rangle$ will be designated as $R_{k,n}$ and $L_{k,n}$, respectively. We will next consider in detail the construction of a network generating an exponential profile with a ratio of 256 between the highest and lowest concentration (Figure 3).

The concentrations in the serpentine channels of the last (4th) stage are chosen as a discrete set approximating the desired exponential profile, $c_n = c_{4,n} = [1, \sqrt{2}, 2, 2\sqrt{2}, 4, 4\sqrt{2}, 8, \dots, 128, 128\sqrt{2}, 256]$ (in relative units). The concentrations in successive channels differ by a constant factor of $\sqrt{2}$, so the sequence c_n is a geometric progression. The condition that each odd-numbered channel of the $(k+1)$ th stage be fed by a single channel from the k th stage uniquely defines the set of concentration in the serpentine channels of the 3rd stage:

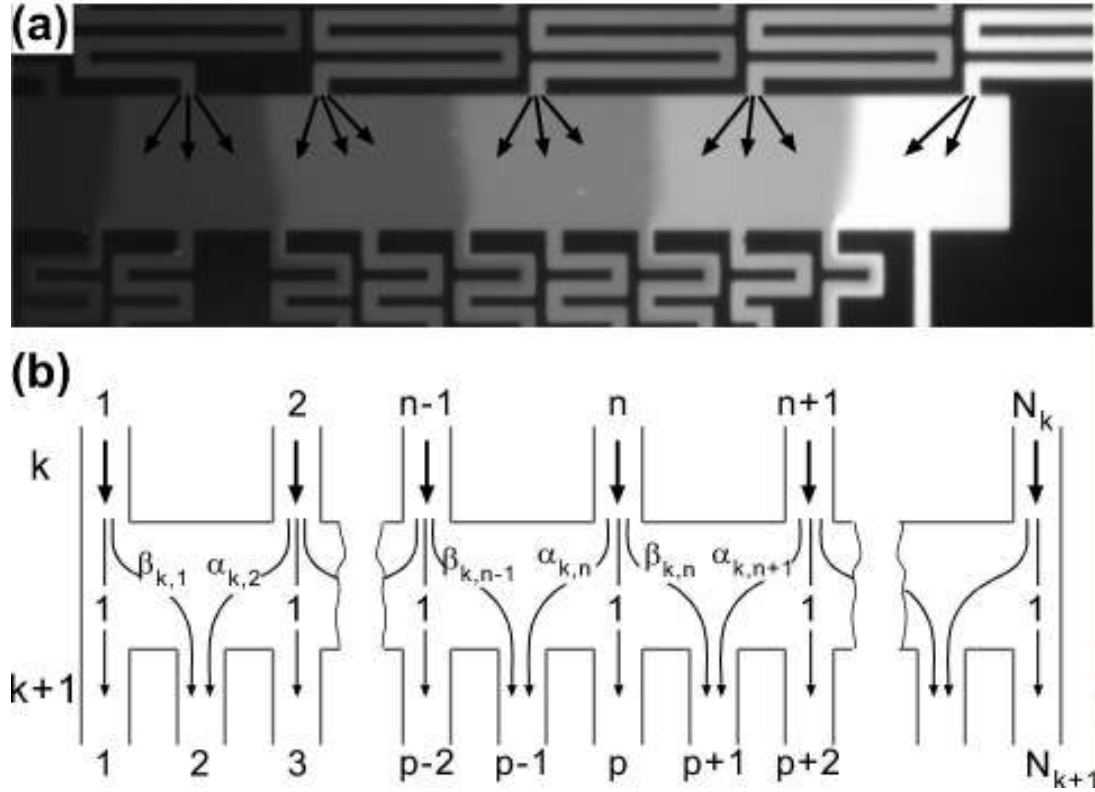


Figure 2. Splitting and redistribution of flux in a horizontal channel. (a) Fluorescence micrograph of the right hand side of the horizontal channel of the 4th stage in the gradient-making network generating an exponential profile, with serpentine channel of the 3rd and 4th stage at the top and bottom, respectively. The grayscale look-up table is non-linearly adjusted for better visibility. Arrows indicate the direction of flow. (b) Schematic diagram showing splitting and redistribution of flux between serpentine channels of the k th and $(k + 1)$ th stages and illustrating the meaning of the coefficients α and β . Serpentine channels of the k th and $(k + 1)$ th stages are shown as vertical channels at the top and bottom, respectively, and are numbered from 1 to N_k and from 1 to N_{k+1} , respectively. Coefficients at the arrows show portions of flux in the channel of the $(k + 1)$ th stage supplied by the channel of the k th stage.

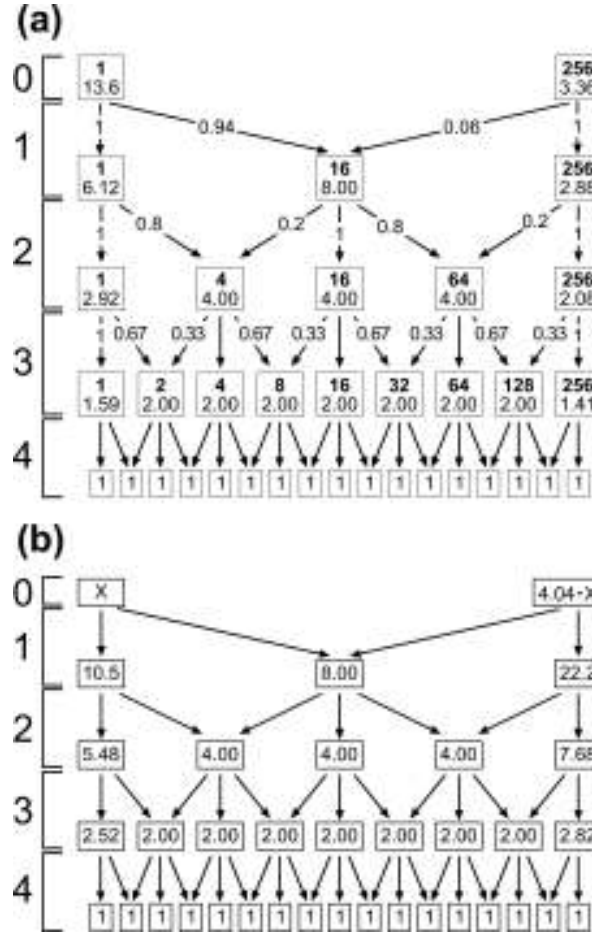


Figure 3. Schematic diagrams illustrating the construction of a gradient-making network generating an exponential concentration profile with the concentration increasing by a factor of 256 from left to right. Boxes represent individual serpentine channels (cf. Figure 1). Arrows show redistribution of flux in the horizontal channels (cf. Figure 1, 2). Numbers on the left indicate individual stages of splitting-and-mixing. (a) Numbers in the boxes show the concentrations (upper row in bold; not shown for the 4th stage) and volumetric fluxes (lower row) in the serpentine channels (both in relative units). Numbers on the arrows are portions of the flux in the channels at the arrow heads supplied by the channels at the arrow tails: coefficients $\alpha_{k,n}$ and $\beta_{k,n}$ for arrows pointing left and right, respectively, and 1 for vertical arrows (cf. Figure 2b). (b) Numbers in the boxes indicate lengths (and resistances) of the channels in relative units, as calculated from the equations $L_{k,n} / L_{k,m} = q_{k,m} / q_{k,n}$ and

$c_{3,n} = [1, 2, 4, 8, 16, 32, 64, 128, 256]$. Applied consecutively from bottom to top, the same condition defines the concentrations in the serpentine channels of the 2nd and 1st stages, as well as in the two channels connecting the inlets with the horizontal channel of the 1st stage, $c_{0,1} = 1$ and $c_{0,2} = 256$ (Figure 3a). We notice that $c_{0,1}$ and $c_{0,2}$ are the concentrations of the solutions injected into the inlets 1 and 2, respectively.

Volumetric fluxes in serpentine channels of the k th stage are specified by concentrations in channels of the k th and $(k+1)$ th stage and fluxes in channels of the $(k+1)$ th stage (Figure 2b and 3a). If channel $\langle k, n \rangle$ is neither the first nor the last of the k th stage, it supplies flux to three channels of the $(k+1)$ th stage: $\langle k+1, p-1 \rangle$, $\langle k+1, p \rangle$, and $\langle k+1, p+1 \rangle$, where $p = 2n-1$ (Figure 2b and 3a). Channel $\langle k, n \rangle$ is the sole source of flux supplied to channel $\langle k+1, p \rangle$ (vertical arrows in Figures 2b and 3a). Therefore, the flux in channel $\langle k, n \rangle$ is calculated as

$$q_{k,n} = \alpha_{k,n} q_{k+1,p-1} + q_{k+1,p} + \beta_{k,n} q_{k+1,p+1} \quad (1).$$

Here, $\alpha_{k,n}$ and $\beta_{k,n}$ are portions of the volumetric fluxes in channels $\langle k+1, p-1 \rangle$ and $\langle k+1, p+1 \rangle$, respectively, which are supplied by channel $\langle k, n \rangle$ (arrows pointing left and right, respectively, in Figures 2b and 3a); $0 \leq k \leq 3$ and $1 \leq n \leq 2^k + 1$. Once solutions fed to channel $\langle k+1, p-1 \rangle$ (from $\langle k, n \rangle$ and $\langle k, n-1 \rangle$) and to channel $\langle k+1, p+1 \rangle$ (from $\langle k, n \rangle$ and $\langle k, n+1 \rangle$) are mixed, the concentrations in these two channels become $c_{k+1,p-1} = \alpha_{k,n} c_{k,n} + \beta_{k,n-1} c_{k,n-1}$ and $c_{k+1,p+1} = \beta_{k,n} c_{k,n} + \alpha_{k,n+1} c_{k,n+1}$, respectively (Figures 2b and 3a). In order to find $\alpha_{k,n}$ and $\beta_{k,n}$, we further notice that $\alpha_{k,n} + \beta_{k,n-1} = 1$ and $\beta_{k,n} + \alpha_{k,n+1} = 1$ (Figures 2b and 3a).

Taken together, these equations give $c_{k+1,p-1} = \alpha_{k,n}c_{k,n} + (1 - \alpha_{k,n})c_{k,n-1}$ and

$c_{k+1,p+1} = \beta_{k,n}c_{k,n} + (1 - \beta_{k,n})c_{k,n+1}$. Finally, we derive

$$\alpha_{k,n} = \frac{c_{k+1,p-1} - c_{k,n-1}}{c_{k,n} - c_{k,n-1}} \quad (2),$$

$$\beta_{k,n} = \frac{c_{k+1,p+1} - c_{k,n+1}}{c_{k,n} - c_{k,n+1}} \quad (3).$$

For the first channel of a stage, the coefficient α is zero, and for the last channel of a stage the coefficient β is zero. The coefficients $\alpha_{k,n}$ and $\beta_{k,n}$ are readily calculated from the values of concentrations in the serpentine channels (which are uniquely defined by the concentrations in the channels of the last stage, $c_n = c_{4,n}$) and then plugged into equations 1. The system of equations 1 is closed by specifying the volumetric fluxes in the serpentine channels of the last stage, $q_{4,n}$, which are all equal 1 in relative units. The values of $q_{k,n}$ are calculated iteratively, stage by stage from bottom to top (Figure 3a).

To illustrate how individual fluxes are computed using Eq. 1-3, we consider a few examples (Figure 2b and 3a). For the leftmost serpentine channel of stage 3 (channel $<3,1>$), we have $k=3$, $n=1$, $p=2n-1=1$, and $\alpha=0$. We calculate

$$\beta_{3,1} = \frac{c_{4,2} - c_{3,2}}{c_{3,1} - c_{3,2}} = \frac{\sqrt{2} - 2}{1 - 2} = 2 - \sqrt{2} \approx 0.59, \text{ giving } q_{3,1} = q_{4,1} + \beta_{3,1}q_{4,2} \approx 1 + 0.59 \approx 1.59. \text{ For}$$

channel $<3,5>$, we have $p=2n-1=9$ and calculate

$$\alpha_{3,1} = \frac{c_{4,8} - c_{3,4}}{c_{3,5} - c_{3,4}} = \frac{8\sqrt{2} - 8}{16 - 8} = \sqrt{2} - 1 \approx 0.41 \quad \text{and}$$

$$\beta_{3,5} = \frac{c_{4,10} - c_{3,6}}{c_{3,5} - c_{3,6}} = \frac{16\sqrt{2} - 32}{16 - 32} = 2 - \sqrt{2} \approx 0.59, \quad \text{giving}$$

$$q_{3,5} = \alpha_{3,5}q_{4,8} + q_{4,9} + \beta_{3,5}q_{4,10} = \sqrt{2} - 1 + 1 + 2 - \sqrt{2} = 2. \quad \text{For channel } <1, 3>, \text{ we have}$$

$$p = 5, \quad \beta = 0 \quad \text{and} \quad \text{calculate} \quad \alpha_{1,3} = \frac{c_{2,4} - c_{1,2}}{c_{1,3} - c_{1,2}} = \frac{64 - 16}{256 - 16} = 0.2, \quad \text{giving}$$

$q_{1,3} = \alpha_{1,3}q_{2,6} + q_{2,7} \approx 0.2 \cdot 4 + 2.08 = 2.88$. We observe (Figure 3a) that all serpentine channels of any given stage have equal fluxes, except for the first and last channels of the stage. This feature is specific to gradient-making networks generating exponential and linear profiles (Figures 1a, b), and is not found in a general case (e.g. Figure 1c).

The calculated values of $q_{k,n}$ are used to select $L_{k,n}$. For all pairs of channels $<k, n>$ and $<k, m>$ belonging to the same k th stage, we have an equation

$$\frac{L_{k,n}}{L_{k,m}} = \frac{q_{k,m}}{q_{k,n}}, \quad (4)$$

setting coefficients of proportionality between the channel lengths. This equation gives a general recipe for building a functional network. Nevertheless, it does not specify the ratios between lengths of serpentine channels belonging to different stages. These ratios and the actual values of $L_{k,n}$ can be rationally selected based on the characteristic time of diffusive mixing in a serpentine channel and the mean flow velocity, $\bar{v}_{k,s}$, in the shortest channel of the stage, $<k, s>$, at typical operation conditions of the device. For any given stage, the shortest serpentine channel has the shortest residence time of liquid in it, $t_{k,s} = L_{k,s} / \bar{v}_{k,s} \propto L_{k,s} / q_{k,s} \propto L_{k,s}^2$, and $t_{k,s}$ has to be sufficiently long to allow complete diffusive mixing in the channel. In the network under consideration, the shortest

serpentine channel of a stage is always the channel in the middle. We further notice (Figure 3a), that the flux in the channel in the middle is multiplied by 2 as the number of the stage is reduced by 1. Therefore, for consistently thorough diffusive mixing, the lengths of the channels in the middle are to obey the equation $L_{1,2} = 2L_{2,3} = 4L_{3,5} = 8L_{4,9}$, completing equation 4 and offering a rule for a rational choice of length ratios between channels belonging to different stages (Figure 3b). Finally, we note that since pressures at the device inlets are equal, the ratio of $L_{0,1}$ and $L_{0,2}$ is given by $\frac{L_{0,1}}{L_{0,2}} = \frac{q_{0,2}}{q_{0,1}}$ (Figure 2b).

There is no diffusive mixing in the channels of 0th stage, however. Therefore, $L_{0,1}$ and $L_{0,2}$ do not have to be proportional to the lengths of the serpentine channels of the stages 1-4.

The network described above is constructed to generate an exponential profile of a shape $c(y) = \exp(ay)$, where the left and right edges of the test channel correspond to $y = 0$ and $y = 1$, respectively, and the maximum concentration is 256 in relative units [corresponding to $a = \ln(256)$]. The second gradient-making network (Figure 1b) is designed to generate a linear concentration profile in the test channel, $c(y) = by$. A set of the last-stage concentrations approximating this profile is $c_{4,n} = [0, 1, 2, 3, 4, \dots, 14, 15, 16]$ in relative units. The sets of $c_{3,n}$, $c_{2,n}$, $c_{1,n}$, and $c_{0,n}$ are found following the procedure described above and are used to calculate the values of $\alpha_{k,n}$ and $\beta_{k,n}$ according to equations 2-3. Finally, the system of equations 1 is solved using the condition of identical fluxes in the serpentine channels of the last stage, $q_{4,n} = 1$. The third gradient-making network (Figure 1c) is constructed to produce a profile with a

shape of two fused branches of a parabola taken with opposite signs. For brevity we will call it double-parabolic profile. Its functional form is $c(y) = 64 \cdot [1 - 4 \cdot (y - 0.5)^2]$ for $0 \leq y < 0.5$ and $c(y) = 64 \cdot [1 + 4 \cdot (y - 0.5)^2]$ for $0.5 \leq y \leq 1$, corresponding to a set of the last-stage concentrations $c_{4,n} = [0, 15, 28, 39, 48, 55, 60, 63, 64, 65, 68, 73, 80, 89, 100, 113, 128]$.

In the design of all three gradient-making networks (Fig. 1), we followed equation 4 and implemented the relation $L_{1,2} = 2L_{2,3} = 4L_{3,5} = 8L_{4,9}$, with $L_{1,2} \approx 31$ mm. In the networks generating exponential and linear profiles (Fig. 1a, b), the design resulted in identical residence times in the shortest serpentine channels of all stages. In the network generating the double-parabolic profile, the smallest residence time in the network was in channel $\langle 1, 2 \rangle$. In the two other networks, channel $\langle 1, 2 \rangle$ was one of the channels with the minimum residence time in the network. Therefore, the maximal flow rate in all three devices was limited by maximal $\bar{v}_{1,2}$ that allowed sufficient time for diffusive mixing in channel $\langle 1, 2 \rangle$ (Fig. 1). Characteristic diffusion time for an aqueous solution of FITC in a 50 μm wide channel can be estimated as $t_0 = w_s^2 / D = 5$ s, giving a condition $\bar{v}_{1,2} < \bar{v}_0 = L_{1,2} / t_0 = 6.2$ mm/s for the proper operation of the gradient-making networks.

Results and discussion

All three devices were tested with a 15 ppm (by weight) solution of FITC in pH = 7.5 phosphate buffer injected into inlet 2. The liquid injected into inlet 1 was plain phosphate buffer for the devices generating the linear and double-parabolic profiles and a 0.059 ppm solution of FITC (15 ppm solution diluted by a factor of 256 with the buffer)

for the device generating the exponential profile. The flow in all three devices was driven at pressure $P_d \approx 7.5$ kPa. (Because of imperfections in fabrication of the devices, the ratios of resistances of the serpentine channels were somewhat different from their intended values; the pressures at the inlets were individually adjusted to obtain the desired values of $c_{1,2}$ and were different from each other by up to 1.5%.) The volumetric flow rates of liquids injected into inlets 1 and 2, $q_{0,1}$ and $q_{0,2}$, are proportional to the difference in pressure between the inlets and the horizontal channel of the 1st stage. Based on our calculations of flow resistances in the networks, this difference in pressure was estimated at $\sim 27\%$ of P_d with an absolute value of ~ 2 kPa for all three networks. Therefore, the level of control of P_d (2 Pa precision) corresponded to controlling $q_{0,1}$ and $q_{0,2}$ within 0.1%.

In order to verify that the flow rate in a device was sufficiently low for thorough diffusive mixing, we measured the distribution of FITC in the stream emerging from channel $<1, 2>$. To perform the measurements, we took fluorescence images of the central part of the 2nd stage horizontal channel, where the stream expanded to a width of ~ 1 mm allowing for good lateral resolution. For all three devices, the standard deviations of the FITC concentration across the stream were less than 1% of the mean. The mean flow velocities in the test channel were 0.75, 0.75, and 0.57 mm/s for the devices generating exponential, linear, and double-parabolic profiles, respectively, which was 29 to 38 times higher than in Ref. ²⁸. The mean flow velocity in channel $<1, 2>$ was $\bar{v}_{1,2} \approx$

6.0 mm/s for all three devices. This value of $\bar{v}_{1,2}$ is consistent with the above estimate for $\bar{v}_0$.

Profiles of fluorescence and distributions of FITC across the test channels, $c(y)$, in all three devices closely matched the desired concentration profiles (Figure 4). Notably, in the device designed to generate an exponential profile (Figure 4a, d), $c(y)$ followed an exponential curve over more than two orders of magnitude in concentration. The measured concentration profiles differ from their desired functional shapes in marginal regions with widths on the order of w_s (Figure 4d-f). One of the reasons for this difference is the net diffusive mass transfer down the gradient, reducing the concentration at the high concentration edge and increasing it at the low concentration edge. This process occurs concurrently with diffusive smoothening of the discontinuities in concentration between the adjacent streams that have widths w_s and affects regions of similar widths at the margins. In the case of the linear profile (Figure 4d), there are plateaus of zero and maximal concentration of widths of about $w_c/2$ in the marginal regions. A better fit to a straight line would be obtained if the volumetric fluxes of the streams with zero and maximal concentration injected at the edges were reduced by half, $q_{4,1} = q_{4,17} = 0.5q_{4,2-16}$.

Because of relatively high flow velocities, variation of the concentration profiles in the test channels along the flow (in the x -direction) was rather slow (Fig. 5). In the case of the exponential profile (Fig. 5a), the profile shape was well preserved within the initial 1.5 mm of the channel (curves 1-3). Degradation of the exponential profile occurred because of the net diffusive transport from the high concentration edge to the low

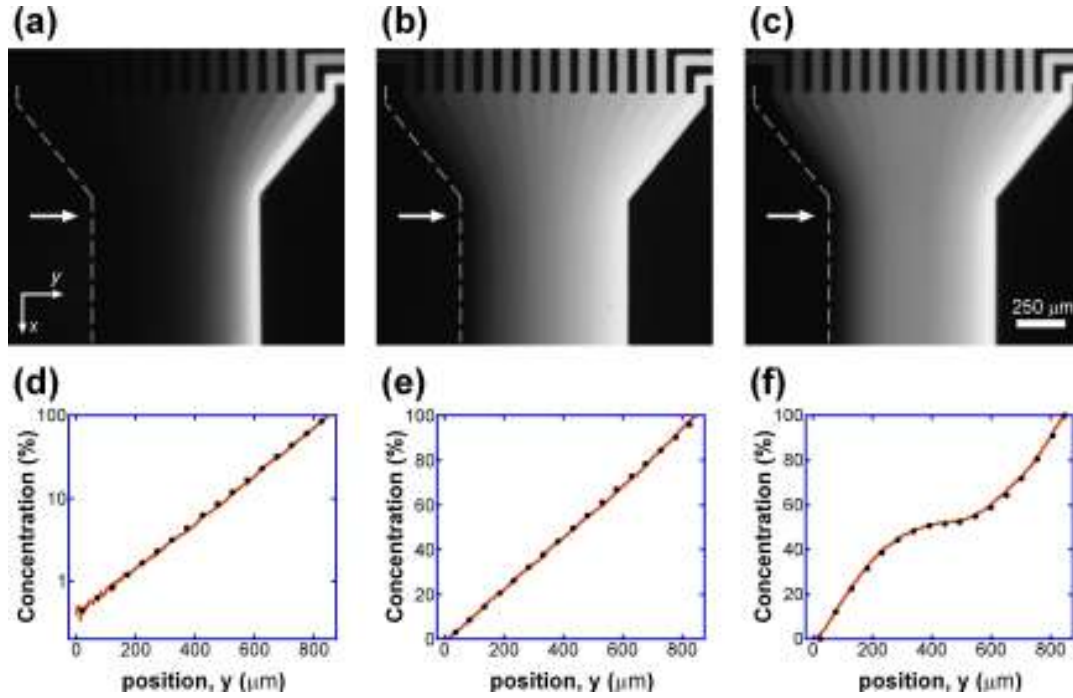


Figure 4. Distributions of FITC in the test channels of the microfluidic devices. (a), (b) and (c) Fluorescence micrographs of the test channels in the devices generating exponential, linear, and double-parabolic concentration profiles, respectively. Dashed lines are drawn along the left sides of the test channels. (d), (e) and (f) Concentration of FITC as a function of position across the test channel in the devices generating exponential, linear, and double-parabolic concentration profiles, respectively, is shown as a continuous line. The plot in (d) is in semi-logarithmic coordinates. The edges of the test channel correspond to $y = 0$ and $850 \mu\text{m}$. The concentration of 100% corresponds to the solution injected into the inlet 2, with 15 ppm of FITC. Round black dots in (d) – (f) show fits of the measured concentration profiles to an exponential, linear, and double-parabolic function, respectively. The fitting is made neglecting the data from the $50 \mu\text{m}$ wide regions at the edges. Horizontal arrows in (a) – (c) indicate the locations at which the concentration profiles in (d) – (f) are measured.

concentration edge and resulted in the development of flattened regions at both edges. Nevertheless, the concentration profile in an internal region between $y = 250$ and $700 \mu\text{m}$ remained exponential even at 3.5 mm from the beginning of the test channel (Fig. 5a) with an almost unchanged exponent of $\sim 6.5 \times 10^{-3} \mu\text{m}^{-1} = \ln(256)/w$ ($w = 850 \mu\text{m}$ is the width of the test channel). As expected for an exponential profile, the concentration in this internal region, $c(x, y)$, steadily grew with x . The increase in concentration over $\Delta x = 3 \text{ mm}$ (curve 5 vs. curve 2 in Fig. 5a) was $\sim 15\%$ on average. This number corresponded to an increase in concentration of only $\sim 0.5\%$ over $\Delta x = 100 \mu\text{m}$, which was more than two orders of magnitude lower than the twofold increase over $\Delta y = 100 \mu\text{m}$. The linear concentration profile (Fig. 5b) remained practically unchanged for the entire length of the test channel, apart from the development of flattened regions at the edges (with increased concentration at the low concentration edge and decreased concentration at the high concentration edge).

Concentration profiles in all three microfluidic devices were fully reproducible when the differences in pressure between the inlets and the outlet were the same. To test the sensitivity of operation of the devices to variations in the inlet pressures, we reduced the pressure at inlet 2 in the devices in Fig. 1a and b by different amounts, ΔP , and monitored the resulting changes in the concentration profiles. The difference between the concentration profiles was quantified in terms of its maximal and root-mean-square (RMS) values, both normalized to the maximum concentration. For the device generating a linear profile, the maximum and RMS values were 2.1% and 1.2% at $\Delta P = 125 \text{ Pa}$ and decreased to 1.0% and 0.5% at $\Delta P = 50 \text{ Pa}$. For the device generating an exponential

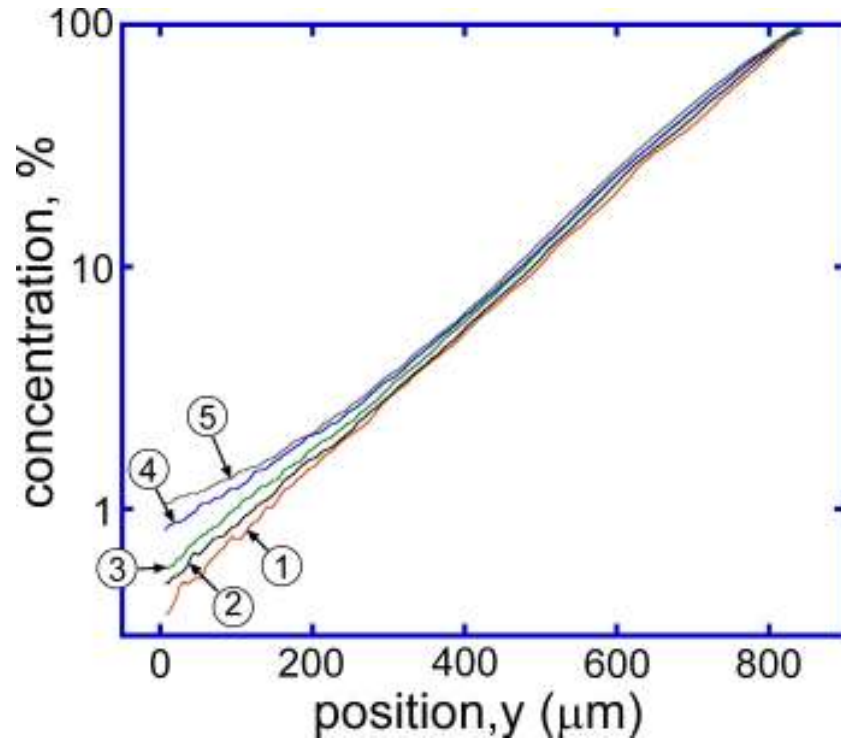


Figure 5. Concentration of FITC normalized to its maximum concentration as a function of position across the test channel, $c(y)$, at different distances, x , from the beginning of test channel in the device generating exponential profile. Curves 1-5 correspond to $x = 0, 0.5, 1.5, 2.5$, and 3.5 mm, with x measured from the beginning of rectilinear part of the test channel.

profile, the maximum and RMS values were 2.7% and 1.7% at $\Delta P = 125$ Pa and decreased to 1.0% and 0.8% at $\Delta P = 50$ Pa. (When normalized with the mean local concentrations, the maximal differences were 24% and 9% at $\Delta P = 125$ Pa and $\Delta P = 50$ Pa, respectively.) When extrapolated to a pressure difference of 2 Pa (the precision of pressure adjustment), both maximum and RMS concentration variations were below 0.1%, suggesting that the devices and the flow control set-up were more than adequate for generating well defined profiles of concentration.

For the device generating the exponential profile, the discharge of liquid through inlets 1 and 2 in 1 hr was ~ 72 μL and ~ 18 μL , respectively, corresponding to reductions by ~ 0.13 mm and ~ 0.03 mm in the levels of liquid in the syringes feeding the two inlets (and an increase of ~ 0.16 mm in the level of liquid in the outlet syringe). These liquid level variations were expected to change the ratio between $q_{0,1}$ and $q_{0,2}$ by less than 0.1%, which would have very little influence on the concentration profile. The effect of the liquid discharge was expected to be even smaller for the linear and double parabolic profiles because of equal $q_{0,1}$ and $q_{0,2}$. We tested stability of the concentration profiles in the devices producing linear and exponential profiles by comparing fluorescence micrographs of the same areas of the test channels taken with an interval of 1 hr. The root mean square of the difference of the fluorescence intensity between the two profiles was less than 0.5% and less than 1% of the maximal fluorescence intensity for the linear and exponential profiles, respectively.

The concentration profiles in Figure 4 illustrate the versatility of the proposed network architecture. The linear concentration profile has a constant first derivative,

dc/dy , and zero second derivative, d^2c/dy^2 . In the exponential profile, both derivatives are positive and grow exponentially across the test channel. In the double-parabolic profile, dc/dy is linearly decreasing on the left side, linearly increasing on the right side, and d^2c/dy^2 changes its sign in the middle of the test channel. The proposed scheme of building a gradient-making microchannel network can be used to generate any given concentration profile with one essential limitation: $c(y)$ has to be a monotonic function. This limitation originates from the fact that the concentration in any serpentine channel (except for channels at the edges) is a weighted average of the concentrations in the two adjacent channels, precluding local minima or maxima of concentration in the networks with two inlets. Concentration profiles with local maxima and minima can be produced by using networks with three or more inlets or by directing streams emerging from several independent networks into a single test channel.^{7, 8}

With the common planar architecture of the microfluidic devices, each channel can have only one or two channels immediately adjacent to it. Hence, a stream emerging from a channel can be directly merged with only one or two other streams, and by splitting and merging streams from a planar array of N channels, not more than $N-1$ new mixtures can be produced. (This limitation is lifted if a 3D microchannel network is used.³¹) Therefore, the growth of the number of distinct concentrations with the number of steps, $N_{k+1} = N_k + (N_k - 1)$ and $N_k = 2^k + 1$, which is realized in the proposed architecture, is the fastest possible under the constraint of a planar network.

The logarithmically small number of steps (stages), $k = \log_2(N_k - 1)$, needed to generate a given number of distinct concentrations allows significant reduction in area

occupied by the network and in pressure required to drive flow through the network compared with the design in Ref.^{7, 8}. For example, to generate a linear set of 17 concentrations with the same total flow rate, a gradient-making network with the architecture as in Ref.^{7, 8} would need to have 15 stages and a net area ~ 7 times larger than the network in Fig. 1b, with ~ 3 times higher pressure required to drive the flow in it. To make this comparison, we designed a network with the architecture as in Ref.^{7, 8} that had serpentine channels of the same cross-section as in Fig. 1b ($50 \times 40 \mu\text{m}$, separated by $50 \mu\text{m}$) and horizontal channels of the same width as in Fig. 1b (0.5 mm). All serpentine channels in the network had equal lengths, and the residence time in the middle serpentine channel of the 1st stage was equal to that in channel $\langle 1, 2 \rangle$ of the network in Fig. 1b. Resistances of 0th stages of both networks were disregarded. One can see that the values of N attainable with the proposed architecture, $N = 17$ in the present networks and $N = 33$ with just one more stage of splitting and mixing, would be rather impractical with the previous design because of the large footprint of the devices. Large values of N are important for accurate definition of complex continuous profiles $c(y)$ by discrete sets of c_n (Figure 4b, c, e and f) and for stability of $c(y)$ with respect to variations in x and $\bar{v}$.

The gradient-making networks could be further optimized by allowing uneven volumetric fluxes, q_n , in the serpentine channels of the last stage (which feed into the test channel). In particular, for accurate definition of a profile with large variations of dc/dy across the test channel (such as in Figure 4c, f), it could be beneficial to have a large number of narrow streams (low q_n) where dc/dy is large, and small number of wide

streams (high q_n) where dc/dy is small. (This would be somewhat analogous to the adaptive mesh used in numeric simulations.) The values of c_n would then be selected in accordance with the positions of the streams in the test channel, and the network would be constructed based on c_n and q_n .

Conclusion

We have described and validated an architecture of microfluidic gradient-making networks, which are more compact and versatile than the networks made using the previous design. A particularly useful feature of the proposed architecture is the ease of generation of exponential concentration profiles that can be applied to studies of chemotaxis and dose response of live cells. In an exponential profile of a chemoattractant, all cells are exposed to the same fractional gradient, $\frac{1}{c} \frac{dc}{dy}$, that remains constant as cells migrate, creating experimental conditions essentially different from those in linear concentration profile studied before^{9-13, 15}. For dose response studies, cells can be presented with solutions containing different concentrations of the substance of interest from the individual serpentine channels at the network outlet.^{16, 17} In successive channels of a network generating an exponential profile, the concentrations differ by a constant factor, making it possible to study the dose response in a broad range of concentrations with even fractional resolution, $\Delta c / c$.

Chapter 2.1.1, in full, is a reprint of the journal article *Generation of complex concentration profiles in microchannels in a logarithmically small number of steps* in Lab on a Chip, 2007. Campbell, K.; Groisman, A., Royal Society of Chemistry, 2007. Dissertation author was the first author and a leading contributor of this paper.

2.1.2 Neutrophil Chemotaxis

The use of the architecture for creating complex chemical concentration gradients described in chapter 2.1.1 allows for a thorough quantitative study of the chemotaxis of adherent cells. In collaboration with Henry Bourne's lab at University of California, San Francisco, microfluidic devices were used to study the chemotaxis response of neutrophil-like differentiated HL60 cells. The purpose of the study was to expose these cells to different concentration gradients and mean concentrations of a known chemoattractant and attempt to determine the dependence of the chemotaxis efficiency on the gradient and the mean.

The microfluidic devices used in this experiment were designed, fabricated and characterized in Alex Groisman's lab at UCSD. My contribution to the project, described below was fabrication, characterization and support for the devices.

The microfluidic devices used in the study are shown in Fig. 6. All four devices have the same general layout. They have two layers of channels, the main layer with 25 μm deep rounded channels and the control layer with 30 μm deep channels with rectangular profiles, and integrated membrane valves. The main layer of channels for the cells and media is adjacent to a #1.5 cover glass, whereas the channels in the control layer are connected to the membrane valves and are used for sealing and opening the main layer channels .

The devices mainly differ in the construction of the gradient-making network. The gradient-making network of the device in Fig. 6, adapted from an earlier work, has two inlets and generates a linear concentration profile with concentrations varying between

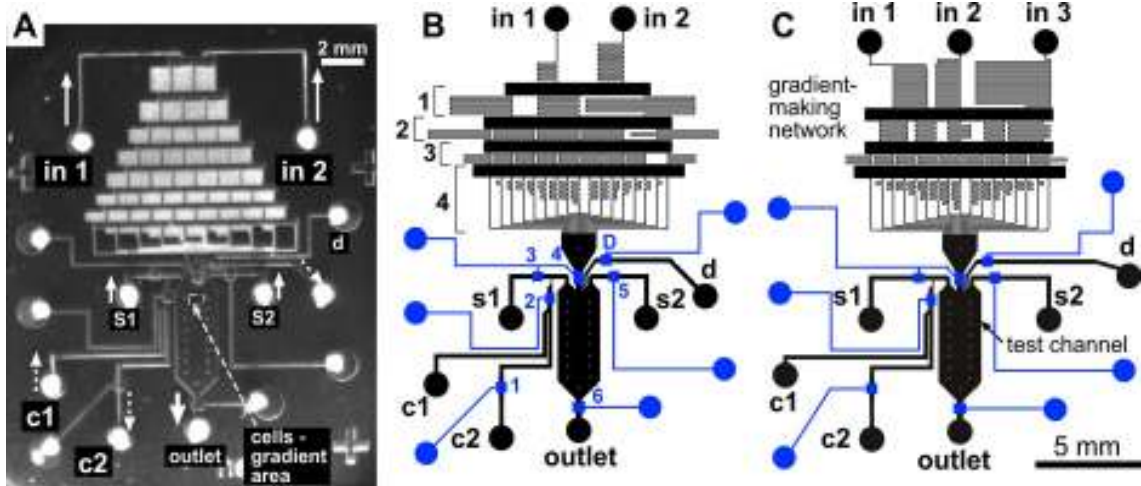


Figure 6. Microfluidic devices used in the study. (A) Micrograph of the device with gradient-making network generating linear concentration profiles. Solid/dashed arrows show the direction of flows, which are switched on/off during the chemotaxis experiments with cells in the test channel. (B) and (C) drawings of the devices with gradient-making networks generating exponential profiles with concentration ratios C_{high}/C_{low} of 16:1 and 256:1, respectively. Channels in the main and control layer are in black and blue, respectively. In panel (B), numbers in blue indicate different membrane valves, and numbers in black indicate different stages of splitting and mixing in the gradient-making network.

zero (plain medium injected into the inlet in1) and the concentration of the solution injected into the inlet in2 (50 nM fMLP in our experiments).

The gradient-making networks of the devices shown in Fig. 6B and 6C, designed according to principles previously described, generate exponential concentration profiles, in which the ratios between concentrations at the high and low concentration edges, $r = C_{high} / C_{low}$, are 16:1 and 256:1, respectively. The gradient-making networks are comprised of sequences of consecutive stages of splitting and mixing, which are marked by numbers from 0 to 4 in Fig. 6B and consist of a wide horizontal channel (500 μm in width) and a set of narrow serpentine channels (50 μm in width) of variable lengths. In all three networks that generate exponential profiles (Fig. 6B and C), the last stage contains 17 serpentine channels with concentrations varying by a constant multiplier between consecutive channels. The multipliers for the devices in Fig. 6B, and Fig. 6C are $3^{1/4}$, $2^{1/4}$, and $2^{1/2}$, respectively. The streams from the 17 serpentine channels merge at the exits of the networks to generate streams with concentrations varying exponentially across the streams. [The total variation of concentration between the edges of the streams, $r = C_{high} / C_{low}$, can be found as $(3^{1/4})^{16} = 81$, $(2^{1/4})^{16} = 16$, and $(2^{1/2})^{16} = 256$, respectively.]

Similar to the networks previously described, the gradient-making network in the device in Fig. 6B has 4 stages of splitting and mixing and 2 inlets, which are fed by solutions with concentrations C_{low} and C_{high} (with the concentration ratio $r = C_{high} / C_{low} = 16$). The networks in the devices in Fig. 6C both have 4 stages of splitting and mixing and 3 inlets, which are fed by solutions with concentrations C_{low} ,

$\sqrt{C_{low}C_{high}}$, and C_{high} (relative concentrations of 1, 16, and 256 for the device in Fig. 6C).

A third inlet is added to the networks with $r = 81$ and 256, to reduce the deviations of the concentration profiles from their designed exponential shape. These deviations mainly result from imperfections in the fabrication of the devices (in particular, from the variations of depths and widths of the serpentine channel that make the ratios of their resistances different from their desired values) and can be particularly large in concentration profiles with high r .

The two-layer construction of the device with 7 integrated membrane valves (labeled in blue as 1-6 and D in Fig. 6B) facilitates the loading of cells into the test channel, their exposure to gradient profiles with minimal transient times, and their incubation in the test channel without flow, prior to the application of the gradient. In particular, when cells are loaded into a device, valves 2, 3, 4, 5, and D are closed, valves 1 and 6 are open, and the test channel is purged of any residues of fMLP. A syringe containing a suspension of cells is connected to the port c1 and pressurized so that the cells flow from the port c1 to the port c2 (Fig. 6B). Once the concentration of cells in the microchannels connecting c1 and c2 reaches the desired high level, valve 1 is closed and valve 2 is opened, resulting in flow of the cells from the port c1 through the test channel to the outlet. After the suspension of cells fills the test channel, valves 2 and 6 are closed, and valve 2 remains closed till the end of the experiment. All valves remain closed for 5-10 min after loading cells into the test channel, allowing the cells to incubate and attach to the substrate with no disturbance by the flow.

The channel leading to the outlet d (and closed by the valve D) is connected to the gradient-making network downstream from the valve 4 that controls the connection

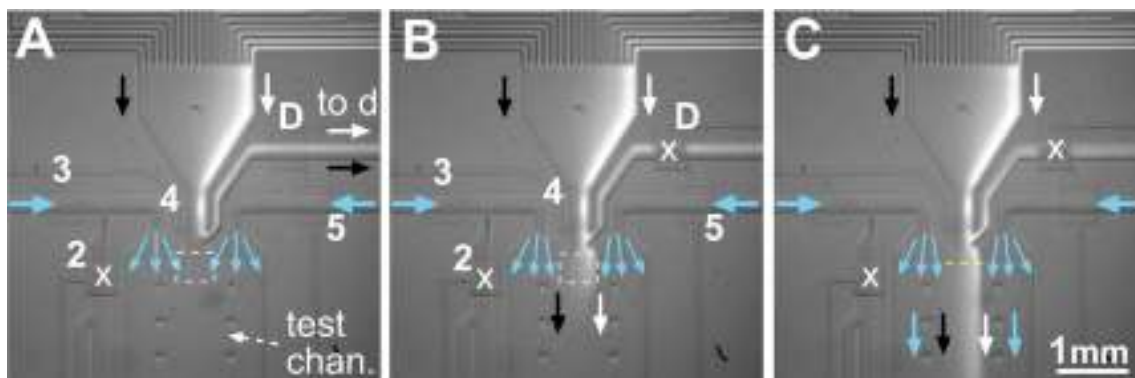


Figure 7. Flow in the central area of the 81:1 microfluidic device (shown in Fig. 3A in the main text) (A) before, (B) during, and (C) after the injection of the gradient stream into the test channel. Micrographs are taken with mixed fluorescence-brightfield illumination, with 50 ppm fluorescein fed to in3. Direction of the sheath flow is indicated with blue arrows. White and black solid arrows indicate the high and low concentration edges of the gradient stream, respectively, and its flow direction. Dashed-line white box in the middle of (A) and (B) indicates the region where the chemotaxis is studied. Valve 2 is always closed (indicated with a cross). Valves 3-5 are always open. Valve D is open in (A) and closed in (B) and (C). The micrograph in (B) is taken ~3 sec after closing the valve D.

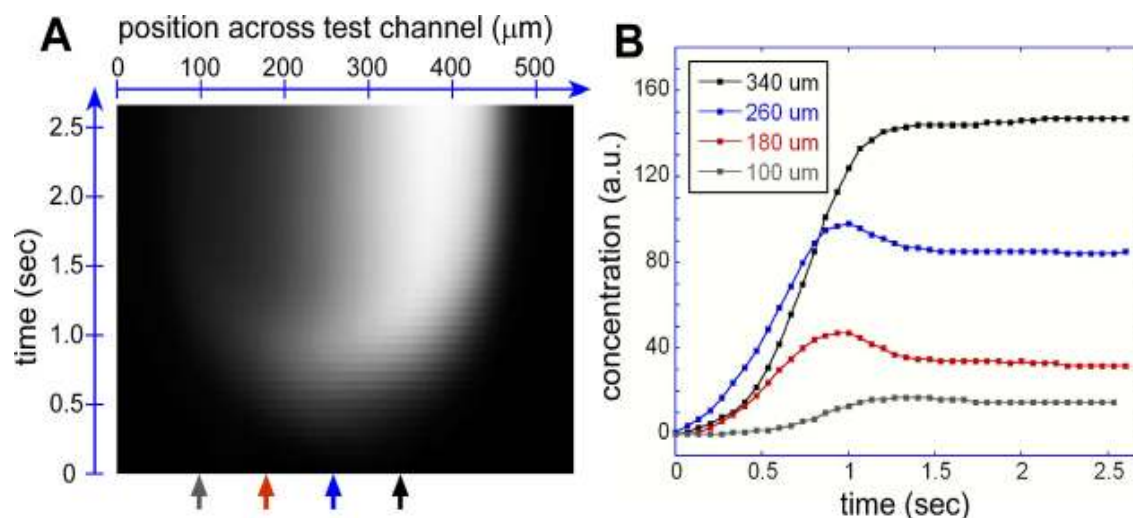


Figure 8. Injection of the gradient-carrying stream into the test channel. (A) A space-time plot of concentration of fluorescein in the test channel across the flow direction at $\sim 200 \mu\text{m}$ from the inlet (along the yellow dashed line in Fig. S6C). Distributions of fluorescence along this line in consecutive video frames were plotted from bottom to top. The mean flow velocity in the test channel was $200 \mu\text{m/s}$. (B) Concentration of fluorescein (in arbitrary units) at the positions indicated by arrows in (A), 100, 180, 260, and 340 μm , as a function of time. The inception of the gradient profile – transition from the initial zero to the final steady concentration – is accomplished within 1-1.5 s.

between the network and the test channel. This design minimizes the distance between the gradient stream emerging from the gradient-making network and cells in the test channel in the “off” state of the device (Fig. 7A). The small distance between the gradient stream and the cells reduces to a minimum the inception time of exposure of the cells in the test channel to the gradient (Fig. 7 and Fig. 8). For a region $\sim 200\ \mu\text{m}$ from the beginning of the test channel (Fig. 7), where chemotaxis was usually studied, the transition from the initial zero to a stable final concentration took $\sim 1\text{--}1.5\ \text{s}$ at the usual flow rate of $200\ \mu\text{m/s}$ in the test channel (Fig. 8).

Because of the curved shape of the front of the gradient stream advancing into the test channel (Fig. 7B), areas in the central part of the stream are exposed to fMLP somewhat earlier than areas on the margins (Fig. 8A). The delay in the exposure reaches $0.4\ \text{s}$ for areas separated by $80\ \mu\text{m}$ in the cross-stream direction (Fig. 8B). Nevertheless, the delay in the exposure to fMLP for two sides of an individual cell ($10\ \mu\text{m}$ in diameter) is always less than $0.05\ \text{s}$, suggesting that the temporal factor in the stimulation of the cells is minimal.

Lastly, during the entire course of the project (from Spring 2003 to Summer 2007) I regularly communicated with Paul Herzmark at UCSF to provide support for the microfluidic devices including operational procedures and the handling of occasional faulty devices.

The results of the biological experiments on HL60 cell chemotaxis performed with my devices were published in the Proceedings of the National Academy of Sciences and are republished with permission in this dissertation.

2.1.2.1 Bound Attractant at the Leading vs. the Trailing Edge Determines Chemotactic Prowess

Introduction

An essential property of eukaryotic cells is their ability to orient in response to spatial cues. Only by correctly interpreting spatial changes in external stimuli can yeast cells mate, soil amoebae form spores, progeny of a fertilized egg form an organism, or neutrophils crawl toward their prey. Cells are known to respond to gradients of external stimuli, such as chemoattractants, but how they sense and interpret gradients remains mysterious. The mystery goes beyond our ignorance of the biochemical basis of gradient sensing. More fundamentally, we have not even definitively identified the external cues sensed and interpreted by the cells and the respective roles of these cues in determining their responses to gradients.

Bacteria migrate up gradients of chemoattractants, in a process called chemotaxis. Chemotaxing bacteria assess gradients *temporally*, by moving through the attractant concentration field, sensing the local ambient concentration, comparing the concentration at a given moment with concentrations at previous times, and changing swimming behavior accordingly. It has been proposed that the larger cells of eukaryotes, in contrast, sense gradients by comparing the current attractant concentrations at different positions on their surfaces and orient themselves to crawl in the up-gradient direction by interpreting the spatial cues present in their current location. In other words, the eukaryotic cells assess the gradient *spatially* and respond to purely spatial cues by directed chemotactic migration. It has been shown that both neutrophils and *Dictyostelium discoideum* amoebae can sense relatively steep gradients of

chemoattractant, supplied by a micropipette, without moving and therefore without comparing ambient concentrations in different locations. In both cases, immobile cells, paralyzed by treatments that block actin polymerization, accumulate phosphatidylinositol-3',4',5'-*tris*-phosphate (PIP3) at the up-gradient edge. In the micropipette experiments, however, the side of a cell that is first exposed to the attractant is also the side that will eventually be exposed to a higher attractant concentration. Thus, the spatial pattern of PIP3 accumulation might be influenced by this temporal pattern of application of attractant. Moreover, it remains unclear whether actual migration of these cells up a gradient can occur in response to purely spatial cue or requires that they move to different positions and compare the local concentrations of the attractant. This is particularly true, when the gradients are relatively shallow as in traditional chemotaxis chambers. For example, Zigmond or Dunn chambers establish gradients quite slowly, over a period of ~ 20 min, which is longer than the time required for cells to polarize and orient their initial polarity (1-3 min) and long enough to allow them to migrate far from their starting positions in order to compare attractant concentrations at multiple locations.

In addition, we do not know how responses to gradients depend on the two most obvious potential external cues: the magnitude of the gradient at a given point *vs.* the ambient attractant concentration at that point. This problem was recognized by Zigmond more than 30 years ago, who studied gradient responses of neutrophils in a chemotactic chamber and quantified them by the degree of cell orientation in the up-gradient direction. She showed that orientation was most efficient for cells at ambient attractant concentrations near the apparent dissociation constant (K_d) of the cells' receptor for the attractant. Importantly, however, the ambient attractant concentration in those studies was

only defined up to a 3x or 10x range, and the shape of the gradient varied between experiments and was not well controlled . Moreover, cell orientation was measured 30 minutes after the gradient was first applied, a period during which the shape of the gradient underwent substantial changes. Therefore, the results are hard to interpret or reproduce. In addition, as with all experiments in traditional chemotaxis devices, they do not discriminate between spatial and temporal modes of gradient interpretation.

To address both problems, one needs to generate concentration profiles with well-defined shapes and to apply them quickly to cells. Generation of stable concentration profiles with linear or polynomial shapes has been enabled by recently developed microfluidic gradient-making networks , and by the use of integrated microvalves , which allow such gradients to be imposed within a few sec . Nevertheless, reports on chemotaxis in microfluidic devices have not addressed the issue of temporal vs. spatial sensing, while dependence of the efficiency of chemotaxis on attractant concentration, although discussed , has not been carefully studied.

The presence of a gradient leads to a difference in attractant concentrations, ΔC , across every cell in the gradient (for convenience we calculate ΔC in terms of a “standard” cell, assumed to be 10 μm in diameter). Most previous studies of chemotaxis in microfluidic devices required cells to interpret linear concentration profiles, where ΔC is constant at every point, while the ambient attractant concentration (C) increases linearly as a cell migrates up the gradient. As a result, the ratio of ΔC to C — that is, the fractional difference in concentration across the cell, hereafter referred to as $D_c (= \Delta C / C)$ — decreases. Because recent experiments on yeast cells , neurons , and *D. discoideum* suggest that these cells sense fractional rather than absolute differences in concentration

($D_c = \Delta C / C$, rather than ΔC), we felt it would be instructive to study chemotaxis in gradients in which D_c remains constant, regardless of ambient attractant concentration. This condition is met in exponential concentration profiles, in which the magnitude of the gradient is directly proportional to the mean concentration at every point, so that the fractional difference of concentration across cells ($D_c = \Delta C / C$) is constant.

Here we present experiments on chemotactic responses of differentiated HL60 (dHL60) cells, a neutrophil-like cell line, to gradients of a tripeptide attractant, f-Met-Leu-Phe (fMLP). Like blood neutrophils, dHL60 cells can polarize not only in fMLP gradients but also in response to fMLP applied at a uniform concentration, lacking any spatial cue. We studied chemotactic responses of dHL60 cells in microfluidic devices that created either linear or exponential concentration profiles. The results show that dHL60 cells can detect and respond to purely spatial cues, and that their chemotactic prowess depends critically on both the gradient and the ambient attractant concentration. In a linear concentration profile, where every increment in distance up the gradient produces the same absolute increase in C (that is, ΔC is constant), chemotactic prowess steadily decreases with increasing concentration of attractant at the cell's starting position in the gradient, C_s , and prowess is maximal at the lowest fMLP concentration tested. In contrast, in exponential concentration profiles, where ΔC increases in proportion to C (D_c is constant), chemotactic prowess increases with C_s at low starting concentrations, decreases with C_s at higher starting concentrations, and is maximal at concentrations close to those reported for the dissociation constant of the fMLP-binding receptor ($K_d \sim 10$ nM). We tested exponential gradients with different fractional concentration differences (D_c). For cells that start at a given concentration in these experiments,

chemotaxis is more efficient when D_c is larger — that is, when the spatial cue, ΔC , is larger at any given C_s . Our results suggest that dHL60 cells translate the gradient and ambient concentration into chemotactic responses by using saturable attractant receptors to assess the difference between the numbers of receptors occupied by attractant at the cell's leading vs. its trailing edges.

Results

Chemotaxis in a linear gradient

We began by investigating chemotactic behavior of cells exposed for 10 min to a linear fMLP gradient, in which the concentration difference across the 10- μ m diameter of an average cell is constant, $\Delta C = 1$ nM. Fig. 6A shows the results, interpreted in terms of the chemotactic index, CI, which we define as the ratio of the distance traveled in the correct up-gradient direction to the total length of the cell migration path during the same period. The CI value for each individual cell is shown in relation to that cell's starting fMLP concentration (C_s) over the linear concentration range of 5-45 nM fMLP. To our knowledge, no previous study of chemotaxis has assessed behavior of each individual cell in relation to its starting position in the gradient.

CI is at its highest mean value (0.4) at the lowest testable C_s ; no ascending portion of the curve was detected (Fig. 9A). From that point CI steadily decreases as C_s increases, becoming virtually indistinguishable from zero for cells that start at concentrations of fMLP above 30-40 nM. Because ΔC remains constant in the linear profile, while ambient fMLP concentration increases, we suspected that the progressive decrease in CI reflected a decrease in the cells' ability to sense and interpret ΔC at higher ambient fMLP concentrations.

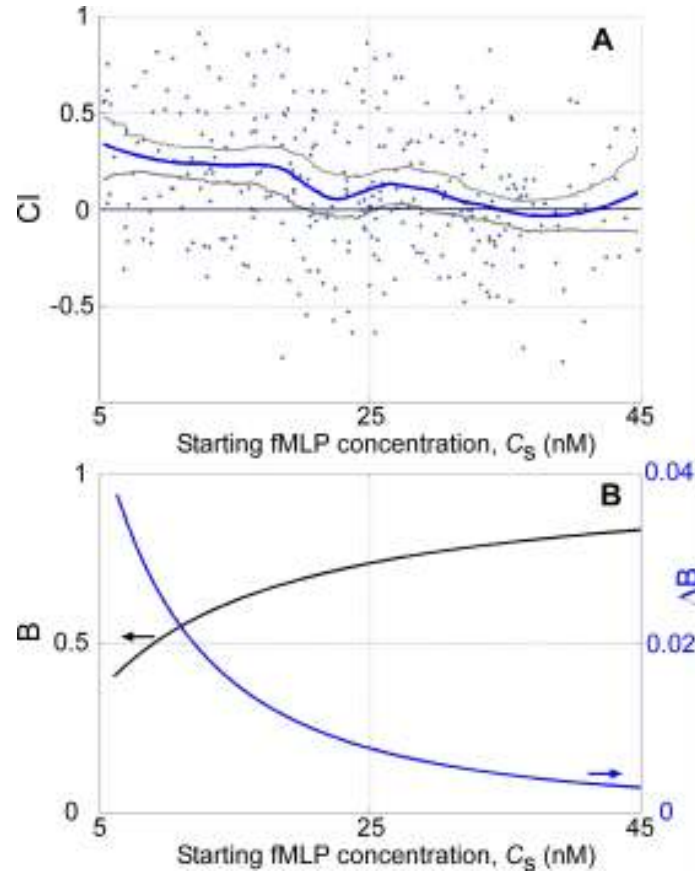


Figure 9. Chemotaxis in a linear concentration profile of fMLP.

(A) Chemotactic index (CI) is shown as a function of the starting fMLP concentration, C_s . Blue dots indicate the CI values of individual cells, and the blue curve depicts the mean CI, computed from the CI of individual cells using the smoothing algorithm described in Materials and Methods and Supplementary Information). Grey lines indicate the limits of 95% confidence intervals (see Materials and Methods and Supplementary Information). (B) The mean fraction of occupied receptors, (B, black line), and difference in the fraction of occupied receptors across a cell (ΔB , blue line) as functions of C_s for $K_d = 10$ nM, according to the proposed theoretical model. C_s is assumed to vary linearly from 5 to 45 nM fMLP over a distance of 400 μm , with a slope of 0.1 nM/ μm . ΔB is calculated for a cell 10 μm in diameter.

The most likely reason for such a loss of sensitivity is that dHL60 cells use saturable receptors on their surfaces to detect and interpret both the local ambient fMLP concentration and differences in fMLP concentration across the cell's diameter. Binding studies have reported dissociation constants, K_d , for fMLP receptors in the range of 1-15 nM. With such K_d values, receptors become increasingly saturated as C_s increases from 5 to 45 nM, thereby reducing their sensitivity to variations of fMLP concentration. The resulting high degree of receptor saturation as C_s reaches 30-40 nM would explain the reduction of chemotactic prowess to an undetectable level.

We thus propose — and later test — a straightforward model in which occupancy of fMLP receptors at any point on the cell's surface obeys the equation, $B = \frac{C}{C + K_d}$, which describes the relation between the fraction of receptors bound to the ligand (B), the concentration of free ligand (C), and the dissociation constant (K_d) for the ligand-receptor interaction. In this model, the difference in the occupancy between the front and back of a cell ($B_f - B_r$) is $\Delta B \approx \frac{K_d}{(C_s + K_d)^2} \Delta C$ (see Supplementary Information). If we further assume that CI increases steadily with ΔB , then this equation predicts that ΔB , and therefore CI, will decrease as C_s increases at constant ΔC — exactly the behavior observed in the linear profile (Fig. 9A).

To illustrate predictions of this model, we can assume K_d at 10 nM, a value well within the reported range (1-15 nM; see above). Then, at $\Delta C = 1$ nM over the range of fMLP concentrations (5-45 nM) shown in Fig. 9A, ΔB would decrease 13.3-fold, from approximately 0.04 to approximately 0.003 (green line, Fig. 9B). If the model is correct, a

decrease of ΔB and CI with C_s will always be seen for cells exposed to a given linear concentration profile.

Exponential gradients

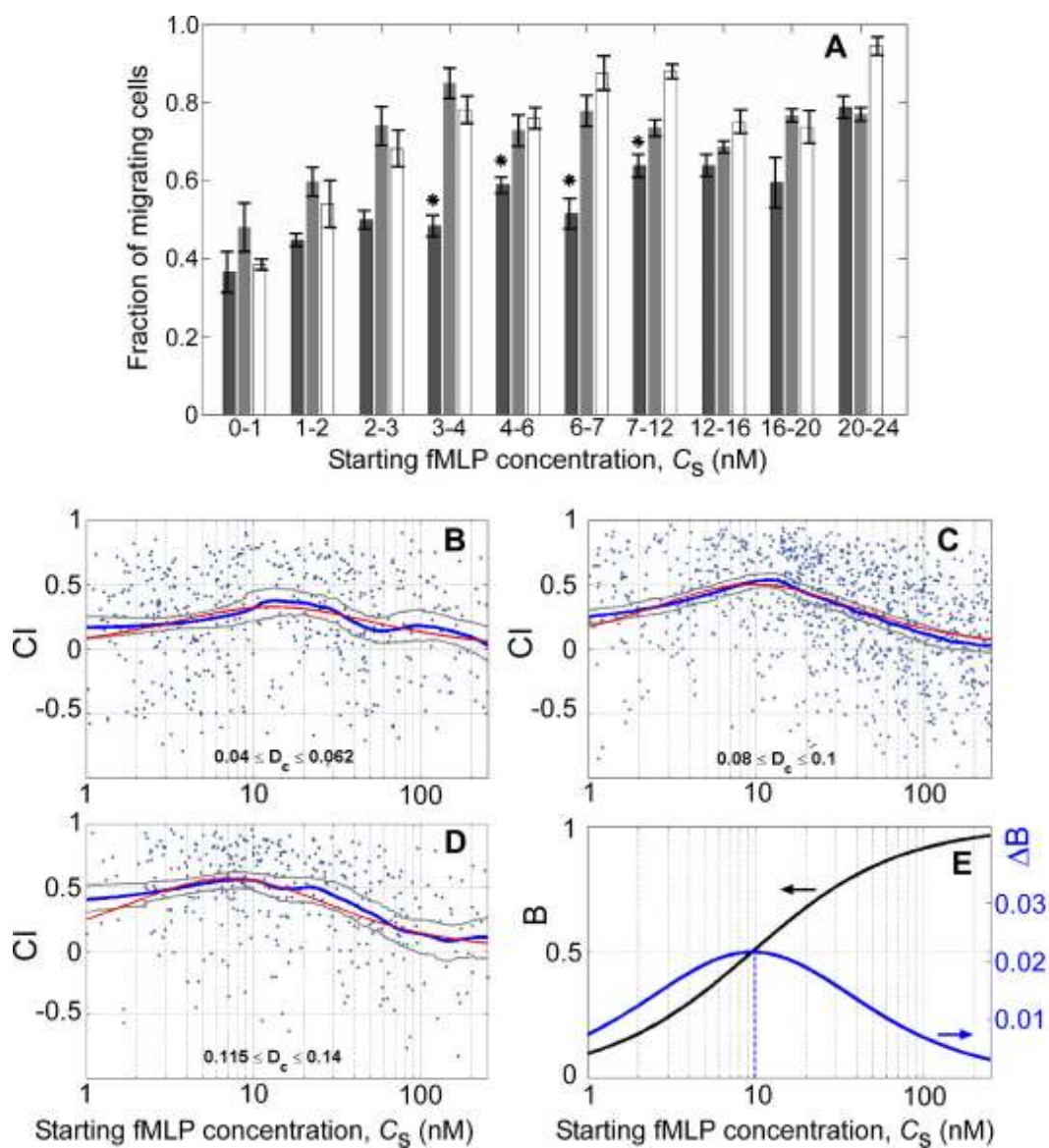
The ΔB -based model makes quite different predictions for exponential profiles, in which D_c ($\Delta C/C$) is constant, while C_s and ΔC increase exponentially as a function of position in the gradient. To study dependence of chemotaxis on the gradient and ambient concentration, we performed chemotaxis experiments in exponential concentration profiles with different values of D_c (Fig. 10). The exponential profiles were created using three different microfluidic devices (described in Materials and Methods and Supplementary Information) with specially designed gradient-making networks .

The difference in receptor occupancy across a cell at its starting position in the gradient is given by $\Delta B \approx \frac{K_d}{(C_s + K_d)^2} \cdot \Delta C = \frac{K_d C_s}{(C_s + K_d)^2} \cdot \frac{\Delta C}{C_s} = \frac{K_d C_s}{(C_s + K_d)^2} \cdot D_c$. Because exponential gradients maintain D_c constant over the entire range of C_s values, ΔB can be approximated by $\Delta B \approx \frac{K_d C_s}{(C_s + K_d)^2} \cdot \text{const}$, which is a non-monotonic function of C_s . Indeed, ΔB given by this relationship increases between $C_s = 0$ and K_d , reaches its maximum at K_d , and steadily decreases at higher C_s values (Fig. 10E). Dependence of chemotactic prowess (measured as CI) on C_s should have the same general form, providing that, as the model suggests, CI is an increasing function of ΔB .

In 73 separate experiments with exponential profiles at different D_c values (see Materials and Methods), we tracked migration trajectories of cells exposed for 10 min to a range of attractant concentrations (1-250 nM fMLP). Because D_c varied somewhat

Figure 10. Chemotaxis and migration in exponential gradients.

(A) Effect of D_c on the likelihood of cells to migrate, as a function of starting fMLP concentration, C_s . Black, gray, and white bars indicate the fraction of cells that migrated at D_c values of 0.05, 0.09, and 0.13, respectively. Numbers on the abscissa indicate the range of C_s values of cells represented by each set of three bars. Cells in each D_c regime were separated into bins of different C_s and the fraction of migrating cells (i.e., cells that moved more than 20 μm from their initial positions) was calculated for each bin. Each bin spanned a C_s range of at least 2 nM and included data from multiple experiments (5-22 experiments, all performed on different days), with at least 7 cells from every individual experiment. Error bars are ± 1 SEM. For bins centered between 3 and 10 nM, the fractions of migrating cells in the low D_c regime were significantly less ($p < 0.05$; indicated by asterisks) than the corresponding migrating cell fractions in experiments in either of the two higher D_c regimes in that concentration range. (B, C, and D) CI as a function of starting fMLP concentration, C_s , for cells that move more than 20 μm . Curves are plotted for the three D_c regimes: respectively, 0.05 ($n = 476$ cells; panel B), 0.09 ($n = 1023$ cells; panel C), and 0.13 ($n = 372$ cells; panel D). Each blue line depicts the average trend in the data, computed from the CI of individual cells (blue dots) using a smoothing algorithm. Gray lines show the 95% confidence intervals computed for the bootstrapped, then smoothed data (300 bootstrapping runs). The red line is the postulated theoretical relationship between CI and fMLP, $CI = k\Delta B$ (see text). K_d and the linear proportionality constant (k) are computed from fitting the equation to the CI of the smoothed data for each D_c regime, as described in Supplementary Information. (E) Theoretical fraction of occupied receptors, B (black), and difference in the receptor occupancy across one cell diameter, ΔB (blue), in an exponential gradient, as functions of starting fMLP concentration (abscissa). In this theoretical example, K_d is set at 10 nM, C_s changes from 1 to 100 nM



between experiments, even with the same device and the same concentrations of fMLP fed to inlets in the device, we grouped experiments at different D_c values into three clusters: low D_c (mean = 0.05, range 0.04-0.062, 476 tracked cells; Fig. 10B); medium D_c (mean = 0.09, range 0.08-0.10, 1023 tracked cells; Fig. 10C); and high D_c (mean = 0.13, range 0.115-0.14, 372 tracked cells; Fig. 10D).

To quantify chemotactic prowess, we first divided cells into two groups, “migrating” and “non-migrating”, depending on whether they moved within 10 min by more or less than 20 μm (two cell diameters) from their point of origin. As expected, the fraction of migrating cells strongly depended on the ambient fMLP concentration (Fig. 10A). At low fMLP (less than 2 nM), only 40-50% of the cells migrated more than 20 μm , regardless of D_c (or ΔC). At fMLP concentrations greater than 20 nM, higher proportions of cells (75-90%) of cells migrated farther than 20 μm , again regardless of D_c or ΔC . Surprisingly, however, migration of cells at intermediate C_s values (3-10 nM) was strongly augmented by higher values of D_c and ΔC . In particular, in the low D_c regime ($D_c = 0.05$) only 50-60% of cells starting at C_s between 3 and 7 nM migrated further than 20 μm , but 80-90% of cells in the two higher D_c regimes did so, at the same C_s concentrations. Tracking individual cells revealed another difference (Supplementary Information, Fig. S1): many non-migrating cells at the low D_c failed to move at all; in contrast, most of the non-migrating cells at the medium and high D_c were detectably displaced from their starting positions, although usually by less than a single cell length.

Cells make the decision to migrate before they have a chance to explore their environment on scales larger than their diameter and to detect the presence of a gradient by sensing temporal changes in the fMLP concentration as they move. Consequently, the

decision to move can only depend on the properties of the field of concentration on the scale of the cell, C_s and D_c (or ΔC). Enhancement of cell motility by higher D_c at a given C_s thus indicates that cells directly sense variations of fMLP concentrations over their diameter. In other words, their gradient sensing can be purely spatial. The fact that the dependence of motility on D_c is strongest at C_s near K_d is consistent with the mathematical model introduced above, $\Delta B \approx \frac{K_d C_s}{(C_s + K_d)^2} D_c$. Indeed, the effect of variation of D_c on ΔB is strongest when $C_s = K_d$, because at this concentration the factor by which D_c is multiplied, $\frac{K_d C_s}{(C_s + K_d)^2}$, is largest.

Once cells do initiate migration, how do they direct their course relative to the direction of the gradient? Figs. 10B-D show mean chemotactic index (CI) as a function of C_s at low, medium, and high D_c values, over a range of 1-250 nM fMLP. In each D_c regime, CI rises to a peak and falls progressively thereafter. These peaks are seen within a rather narrow range of fMLP concentrations: 13, 13, and 8.6 nM for low, medium, and high D_c regimes, respectively. The mean CI value at the peak increases progressively with D_c , from 0.38 at $D_c = 0.05$ (Fig. 10B) to 0.56 at $D_c = 0.13$ (Fig. 10D). [Note: data in Figs. 10B-D exclude all non-migrating cells, on the ground that short trajectories of these cells make it impossible to assess CI accurately (see Materials and Methods).]

At each value of D_c , the pattern of dependence of CI on C_s (blue lines in Figs. 10B-D) agrees with the ΔB -based model, in that CI increases at low C_s , decreases at high C_s , and is maximal at values of C_s that are within the range of K_d values reported in binding studies (1-15 nM). Thus the experimental results support the central element of

the model — i.e., that chemotactic prowess, CI, depends on the increment in receptor occupancy, ΔB , from the back to the front of the cell at the cell's starting concentration (C_s).

Our model need not make specific assumptions about exactly how CI depends on ΔB , apart from it being an increasing function. When spatial cues (ΔC and D_c) are weak and both ΔB and CI are small, it is natural to suggest that CI may increase linearly with ΔB . Such a linear regime could prove difficult to establish experimentally, however, because small directional biases in cell migration (that is, low CI) are difficult to assess accurately. In contrast, when chemotactic prowess is high, dependence of CI on ΔB should be a non-linear function, which becomes increasingly saturated as CI reaches values close to unity. Indeed, for CI = 0.38 at $C_s = K_d$ and $D_c = 0.05$, a linear dependence of CI on ΔB would produce CI values at $C_s = K_d$ of 1.04 for $D_c = 0.15$ and of 2.08 for $D_c = 0.3$. Neither of these can be true, because CI by definition is always less than 1.

Nonetheless, approximating chemotactic prowess, CI, as a linear function of ΔB ,

$$CI = k\Delta B \approx k \frac{K_d C_s}{(C_s + K_d)^2} D_c, \text{ is useful for testing the general agreement of the model}$$

with the experimental results. We fitted the experimental data obtained in each D_c regime with this function (red curves in Fig. 10B-D), considering k and K_d as independent fitting parameters. The best fits were obtained for K_d at 13.4, 8.4, and 7.5 nM fMLP for $D_c = 0.05, 0.09$, and 0.13 , respectively (Table 1). All three values of K_d fall within the range of K_d reported in binding studies (1-15 nM) and are close to the values of C_s at which the corresponding experimental dependencies have their maxima.

The convention of a linear relation between CI and ΔB also makes it possible to estimate how well the experimental data correlate with the ΔB -model. For instance, the values of the coefficient of determination, R^2 , show that the model accounts for 72, 90, or 79% of the trends relating CI to C_s , at D_c regimes of 0.05, 0.09, or 0.13, respectively (Table 1). Moreover, fits based on the approximation $CI = k\Delta B$ result in small values of the root mean square error, RMSE (Table 1), indicating that the fits are quite close to the experimental curves, despite the incorrect assumption that CI increases linearly with ΔB . The limitations of the linear approximation are manifested in the variation of the values of the proportionality constant, k , providing the best fit: k decreases from 26.8 to 19.3 as D_c increases from 0.05 to 0.13. (The decrease of k with D_c is consistent with the expected non-linear dependence of CI on ΔB and with the saturation of CI at large ΔB .)

We further note that the experimental data suggest that chemotactic prowess can also depend on factors that are not included in the current model. The curve of ΔB vs. C_s is symmetric with respect to K_d when plotted in semi-logarithmic coordinates (Fig. 10E), so the shapes of CI vs. C_s curves obeying the ΔB -model (both linear and non-linear) should be symmetric with respect to K_d as well. The experimental data in all three D_c regimes show an apparent asymmetry, however, with CI declining faster at $C_s > K_d$ than at $C_s < K_d$. A likely reason for this asymmetry is that the mean receptor occupancy, $B = \frac{C_s}{C_s + K_d}$, is increasing with C_s , and the detection of the difference in the occupancy across the cell, ΔB , becomes less efficient and reliable as B increases. (This subject is further discussed in Supplementary Information.) The asymmetry of the experimental

Table 1. Least-squares fits of CI to C_s .

The experimental data shown in Fig. 2B-D was fitted to the function

$$CI = k \cdot \Delta B = k \cdot \left(\frac{K_d C_s}{[C_s + \Delta C + K_d][C_s + K_d]} \cdot D_c \right) = k \cdot \left(\frac{C_s(1 + D_c)}{[C_s(1 + D_c) + K_d]} - \frac{C_s}{[C_s + K_d]} \right).$$

Fitting parameters, K_d and k , were chosen by the algorithm independently for the three D_c ranges. The d includes only cells that move more than 20 μm from their origin.

D_c	k (95% Confidence bounds)	K_d (95% confidence bounds)	R^2	Adjusted R^2	RMSE
0.04-0.065	26.8 (26.4-27.3)	13.4 (12.9-13.8)	0.7201	0.7195	0.04162
0.08-0.10	23.2 (22.9-23.5)	8.5 (8.2-8.7)	0.904	0.9039	0.04918
0.115-0.14	19.3 (19,19.63)	7.5 (7.2,7.9)	0.7925	0.7919	0.07024

curves probably accounts for the fact that the putative K_d values obtained from the fitting are all smaller than the values of C_s at the experimental curves.

The chemotactic index, CI, is the ratio of two independent parameters of the cell's migratory path: the mean displacement of cells in the direction of the gradient and the contour length of the migratory path. The displacement parameter, which can be viewed as an alternative measure of chemotactic prowess, behaves like CI in its relation to C_s (Supplementary Information, Fig. S2), in that it is higher at $D_c = 0.09$ and 0.13 than at $D_c = 0.05$ and in that the displacement is maximal at C_s near the putative K_d . Peaks of the mean displacement curves, however, are broader than those of CI and are somewhat shifted somewhat toward higher C_s values. The second parameter, length of the cell's migratory path, reflects the overall motility of cells. Variations in this parameter with C_s and D_c (Supplementary Information, Fig. S3) do not resemble the patterns seen with CI (Fig. 10) or with displacement in the correct direction (Supplementary Information, Fig. S2): mean path length for $D_c = 0.05$ or 0.09 fails to show a distinct peak; it does peak for $D_c = 0.13$, but at a C_s value (~ 50 nM) much higher than the putative K_d . Both the displacement in the direction of the gradient and the length of the migratory path are intrinsically of interest in their own right, and identifying their individual roles is a task for future experiments.

Discussion

The first important new observation in our experiments is simple: chemotactic prowess depends both on the variation in attractant concentration in space (represented by the difference across the cell's diameter, ΔC) and on the ambient concentration of attractant (represented by the concentration at the cell's starting point, C_s). To observe the

variation of chemotaxis with C_s , it was crucial to perform quantitative experiments using a fluorescent marker mixed with fMLP: this allowed us to measure the actual shape of the gradient and to determine C_s for each individual cell trajectory we analyzed (see Materials and Methods). Chemotactic prowess (measured by the chemotactic index, CI) should depend on C_s , of course, because a cell's attractant receptors are saturable and limited in number. As the ambient concentration of fMLP increases, an increasing number of receptors becomes occupied, thereby limiting the cell's ability to detect further increases in fMLP concentration.

Three decades ago, Zigmond showed that neutrophils exposed to a slowly forming gradient of attractant orient themselves — over a time period of 30 minutes — in the up-gradient direction, and do most efficiently at attractant concentrations near the K_d . The results of those experiments, however, were limited by the performance of the chemotaxis chamber used to generate the gradient. The ambient attractant concentration was specified with a low precision (up to a 3x range at best), and the shape of the gradients was poorly defined and varied in the course of the assays. More recently, chemotaxis of various eukaryotic cells was studied in well-defined concentration profiles in microfluidic devices . Those studies, however, have usually reported chemotactic responses of large cell populations exposed to relatively broad ranges of ambient attractant concentrations, without reference to the local concentration for individual cells.

Our second key conclusion is that neutrophil-like dHL60 cells are capable of sensing differences in attractant concentrations across their diameters in relatively shallow gradients and of translating this purely spatial cue into a decision of whether or not to migrate. At concentrations close to the apparent K_d for fMLP binding, higher ΔC

values (as produced in concentration profiles with higher D_c) made cells much more likely to migrate from their starting position (Fig. 10E). At a given ambient fMLP concentration, a cell's decision to migrate significantly depended on a small difference in the spatial cue to which it was exposed: the change from 5% to 9% (from $D_c = 0.05$ to $D_c = 0.09$) in the increment of attractant concentration between the back and the front of the cell.

The ability of dHL60 cells to interpret gradients *purely spatially*, without needing to compare ambient attractant concentration at different times, is in accord with the demonstrated capacity of immobilized dHL60 cells to accumulate PIP3 at the up-gradient edge . It is nonetheless important to show that cells can not only sense spatial cues in a gradient, but can also translate those cues into an essential response, the chemotactic migration. It is, moreover, not certain that PIP3 accumulation is the sole, or even the predominant, determinant of polarity and spatial orientation: pharmacologic inhibitors of PIP3 accumulation in dHL60 cells and transgenic knockouts of PIP3 degradation in mice exerted modest effects on chemotactic prowess.

We further note that the dependence of motility on C_s is similar to the dependence of polarization on concentrations of fMLP, when the attractant is applied uniformly (that is, in the absence of any spatial cue). For instance, 100 nM uniform fMLP induces 80-90% of dHL60 cells to polarize within 3 min, while 3 nM fMLP induces only 20% to do so; an intermediate fMLP concentration (10 nM) induces an intermediate proportion (~50 %) of cells to polarize (results not shown). This correlation between polarization and motility responses suggests that in addition to improving motility, application of larger

gradients ($D_c = 0.09$ and 0.13 vs. $D_c = 0.05$) probably also increases the fraction of cells that polarize.

Our third major conclusion is that chemotaxing cells behave as if they interpret gradients primarily by using saturable receptors to assess differences in the attractant concentration across their own diameters. More specifically, the major features of the chemotactic response of cells are well described by a simple model, in which chemotactic prowess is an increasing function of the absolute difference in receptor occupancy, ΔB , at the front of the cell vs. the back of the cell. As the model predicts, chemotactic prowess (assessed by the chemotactic index, CI) in a linear concentration profile steadily decreases with the ambient concentration of the attractant at the cell starting position, C_s (Fig. 9B). Results with exponential concentration profiles (Fig. 10B-D) provide even stronger evidence supporting the model. In all three tested ranges of D_c , the dependences of CI on C_s agree with the model prediction: CI increases at low C_s , decreases at high C_s , and reaches a maximum near at C_s values very close to K_d values determined in binding studies.

The experimental results show that chemotactic prowess depends on the ambient attractant concentration in gradients that maintain either ΔC or D_c constant — that is, in linear or exponential gradient profiles. Hence, while each of the two parameters, ΔC or D_c , can be considered an essential spatial cue, neither suffices on its own to specify chemotactic prowess. Instead, the experimental results agree with the model's suggestion that chemotactic prowess depends on the difference in receptor occupancy across the cell,

defined by the expression $\Delta B \approx \frac{K_d}{(C_s + K_d)^2} \Delta C = \frac{K_d C_s}{(C_s + K_d)^2} D_c$. It is worth emphasizing

that we know very little about the precise dependence of CI on ΔB , except that it is expected to be an increasing function, cannot be linear, and must reach saturation when CI is equal to unity (see Results). Moreover, we do not know whether or how the relation between CI and ΔB may depend on duration of exposure to the gradient and are at present too ignorant of the biochemical events involved to probe that relation with appropriate molecular perturbations. Clearly, further experiments will be required to explore the relation of CI to ΔB .

Several caveats are in order. First, we note that the experimental results have appreciable margins of error and do not exclude other models based on spatial gradient sensing by saturable receptors. In Supplementary Information we discuss an alternative spatial sensing model which incorporates contributions of the mean receptor occupancy and of molecular noise. In addition, conformation of CI curves to a model that relates ΔB to ΔC , C_s , and K_d does not by any means imply that the initial ΔB of a cell exposed to an fMLP gradient completely defines its subsequent chemotactic response. Certainly, cells must continue to assess ΔC and C as they migrate, and respond accordingly. In particular, the cell may augment its spatial gradient interpretation with a temporal strategy, in which the local ambient concentrations, C , are compared at different times during the migration of the cell. Combining temporal and spatial modes of gradient interpretation would be in keeping with nature's frequent tendency to solve important problems by employing redundant, overlapping mechanisms. Our experiments do not test this possibility.

Finally, we note that none of the major new findings of this work could have been made without using microfluidic devices, which expose cells to well-characterized, stable gradients of attractant (Materials and Methods and Supplementary Information). The

stable gradients were applied in very short time intervals (1-3 sec), setting a well-defined time point for initial exposure for all cells in each experiment. Moreover, the interval between the application of fMLP to the front and back of individual cells was less than 0.05 sec (see Supplementary Information), thus reducing to a minimum the initial temporal component of the stimulus applied to the cells. Another key element of these experiments was the use of exponential concentration profiles, produced by specially designed microfluidic networks. Our experiments, the first to apply exponential profiles to analysis of chemotaxis, show that such profiles make it much easier to distinguish separate roles of the gradient and the ambient concentration. In addition, the exponential profiles made it possible to validate an important prediction of the proposed model, that chemotactic prowess at constant D_c should be maximal at ambient concentrations near the K_d , the dissociation constant for fMLP binding to its receptor.

As compared to linear concentration profiles, exponential concentration profiles furnish a number of practical advantages for studying chemotaxis. As we have shown, chemotaxis in linear profiles is most efficient at the low concentration edge of the gradient stream, where the actual profile shape is expected to differ from the designed shape because of diffusive smearing and other edge effects . Because such edge effects perturb the profile at the low concentration edge, they set a lower limit on the range of reliably accessible ambient concentrations, C_s , at any given ΔC . In contrast, in exponential profiles, chemotaxis is most efficient in a region near $C = K_d$. This region can always be placed in the middle of the gradient-carrying stream by choosing appropriate concentrations at the edges, so that the range of accessible C_s is unlimited at any D_c . Finally, exponential profiles can furnish a rather wide region of concentrations, near $C_s =$

K_d , in which chemotactic prowess is mainly defined by D_c , the fractional concentration difference across the cell and is almost independent of ambient concentration.

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Materials and methods

Microfluidic devices

Microfluidic devices used in this study (Fig. 11 and Supporting Information Fig. S5-S7) comprise two main elements: the test channel, where the chemotaxis of dHL60 cells in gradients of chemoattractant (fMLP) is analyzed, and the gradient-making network. The gradient-making network is fed by a small number of source solutions with different concentrations of fMLP and generates a steady stream with a stable gradient of fMLP of desired shape across the stream. The gradient stream is either diverted into a designated outlet (d in Fig. 11A) or directed into the test channel (Fig. 11B, C). The injection of the gradient stream into the test channel sets the time point of exposure of the dHL60 cells to the gradient of fMLP (Supplementary Information Fig. S6) and begins a chemotaxis assay. In the test channel, the gradient stream is squeezed in a sheath flow between two streams of plain buffer (Fig. 11C) coming from two auxiliary inlets ($s1$ and $s2$ in Fig. 11A). The width of the gradient stream is usually substantially smaller than the

width of the test channel (Supplementary Information Fig. S6), and it is adjusted to vary the magnitude of the gradient in the stream. (The magnitude of the gradient is inversely proportional to the width of the gradient stream.) The microfluidic device has seven integrated membrane valves that can locally seal the microchannels beneath them. The valves enable fast switching of the flow, easy loading of dHL60 cells into the test channel (from a dedicated inlet, s1 in Fig. 11A), incubating cells in the test channel without flow, and preventing premature exposure of the cells to fMLP. These features provided by the valves were critical for conducting the extensive series of repeatable experiments and collecting data on the large number of cells chemotaxing in different gradients. This study used four different devices (Fig. 11A and Supplementary Information Fig. S5). They had gradient-making networks that generated a linear concentration profile and three types of exponential profiles, in which the ratios between concentrations at the high and low concentration edges, C_{high}/C_{low} , were 16:1, 81:1, and 256:1. Higher value of C_{high}/C_{low} at a given profile width, w , results in larger fractional difference of concentration across a cell, $D_c = \ln(C_{high}/C_{low}) \cdot L/w$, where $L = 10 \mu\text{m}$ is the cell diameter. By varying the actual values (but not the ratio) of C_{high} and C_{low} in each of the three exponential devices, we could expose cells in different experiments to exponential profiles with a particular D_c but different (and overlapping) ranges of fMLP concentration. In this way, we assessed chemotaxis over a total range of attractant concentration substantially wider than that produced by the C_{high}/C_{low} ratio in any individual experiment. Diffusion across boundaries between the gradient and sheath streams caused gradient profiles to degrade at the edges (especially at the high

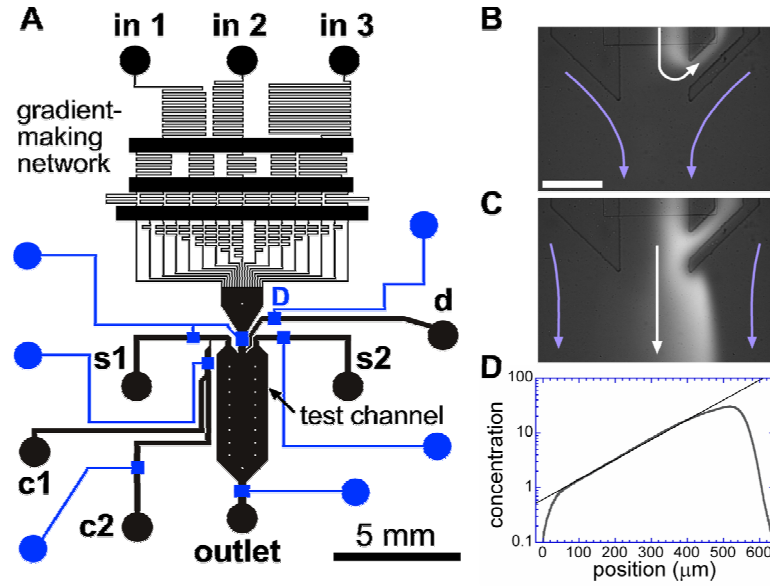


Figure 11. Design and operation of the microfluidic device producing an exponential profile with $C_{high}/C_{low} = 81$. (A) Layout of microchannels in the flow layer (black) and control layer (blue).

Rectangles at the ends of control channels are membrane valves. The ratio of concentrations of attractant (and fluorescein) fed into inlets in1, in2 and in3 is 1 : 9 : 81. (B) and (C) Micrographs of the central part of the beginning of the test channel (under mixed brightfield/fluorescence illumination) illustrating operation of the device with a 50 ppm fluorescein solution fed to in3. White arrows show the direction of the gradient-carrying stream coming from the gradient-making network. Blue arrows indicate the sheath flow from s1 and s2. In (B) valve *D* is open, and the gradient stream is diverted to port *d*. In (C) *D* is closed, and the gradient stream is injected into the test channel. Mean flow velocity in the test channel is 150 $\mu\text{m/s}$. Scale bar 300 μm . (D) Profile of concentration in the central part of the test channel ($\sim 300 \mu\text{m}$ from the beginning) in semi-logarithmic coordinates. The concentration is normalized to that of the solution injected into in1. The profile is close to an exponent (the straight line) in a region $\sim 400 \mu\text{m}$ in width.

concentration edge; Fig. 11C, D). Nonetheless, the desired exponential shape was preserved within easily definable limits (Fig. 11C, D). Here we report data only from those cells that remained within these limits along their entire migration trajectories.

Chapter 2.1.2.1, in full, is a reprint of the journal article *Bound attractant at the leading vs. the trailing edge determines chemotactic prowess* in Proceedings of the National Academy of Sciences, 2007. Herzmark, P.; Campbell, K.; Wang F.; Wong, K.; El-Samad, H.; Groisman, A.; Hourne, H.R., The National Academy of Sciences of the USA, 2007. Dissertation author was a participating investigator of this paper.

2.1.3 Yeast Chemotropism

In most of the previous experiments with gradient-sensing cells in microfluidic devices, including those presented in chapter 2.1.2, gradients were created across a stream of liquid and cell were to substantial hydrodynamic shear^{9, 10, 13}. This setup works with strongly adherent cells, but cells with no or poor adhesion will get washed away with the flow. Yeast cells are non-adherent and non-motile. Their gradient response is manifested in chemotropism rather than chemotaxis: when exposed to a gradient of a pheromone yeast cells grow mating projections (shmoo) in the direction of increasing pheromone concentration. Since chemotropism of yeast cells cannot be studied in the devices discussed in chapter 2.1.1 and 2.1.2, a device of a new type was needed that could create a stable chemical concentration gradient on long time scales (hours) with no active flow in the gradient region.

The microchannel network consists of two large (deep and wide), parallel channels with an array of shallow chambers connecting the channels. Solutions of two different concentrations (high and low) of the chemical in question flow through the large channels to provide a constant source and sink of the chemical. If the pressures in the two large channels at the opposite sides of a shallow chamber are equal, there is no active fluid flow across the chamber and a linear concentration profile of the chemical is created in the chamber by molecular diffusion between the two channels (source and sink). The fluid flow through the main channels has to be sufficient to ensure that the concentration of the chemical in the source channel does not get depleted and the concentration in the source channel does not rise because of the constant diffusive flux through the chambers.

Similar to the devices described in chapter 2.1.2, the microfluidic devices have two layers of microchannels and integrated valves, as well as dedicated inlet and outlet for cell loading. The valves make it easy to load cells into the chambers by closing off the chemical inlets and outlet and feeding a cell solution to the cell inlet. The cells then flow to the cell outlet through the main channels and the chambers. Once there are enough cells in the chamber, the flow of the cell suspension, and the cells left in the main channels are rinsed, once flow through them towards the main outlet is started.

These devices were designed, fabricated and characterized in Alex Groisman's lab at UCSD. Experiments with yeast cells using the microfluidic devices were conducted in Andre Levchenko's lab at Johns Hopkins University. My contribution to the project included device fabrication, upgrades of device design, and characterization.

Fabrication was similar to the neutrophil chemotaxis devices described previously. To fabricate the master mold for the flow layer of microchannels, a thin layer ($\sim 5\text{ }\mu\text{m}$) of SU8 photoresist is first spin-coated onto a wafer and patterned to make the chambers. A $\sim 25\text{ }\mu\text{m}$ thick layer of AZ100 is spin-coated on top of the SU8 pattern to create the relief for casting the main flow channels. This $\sim 25\text{ }\mu\text{m}$ thick relief is then rounded so control valves can be used with the device. The master mold for the control layer is fabricated with a single coating with an SU8 photoresist. The two-layer devices are assembled using the procedure described in chapter 2.1.2.

To ensure that the devices work correctly, I have done basic experiments using a fluorescent dye to check the shape of the chemical concentration gradients in the chambers. Slight differences in inlet pressures or in the resistances of the main channels can cause a flow through the chambers. This flow can not only wash away any cells

present, but also changes the shape of the concentration gradient. I tested the devices to determine the gravity of this problem. When the inlet pressures were appropriately adjusted, the flow through the chambers was very small and did not cause any negative effects. However, potentially dangerous flow across the chambers could be caused by improper adjustment of the inlet pressures or fabrication defects. A practical way to detect flow across the chambers, which I recommended to the collaborators in the Levchnko's lab, was by adding small ($< 2\mu\text{m}$) beads to the solutions.

Over the course of the project described in the paper we published and the continuing work in the area, I have made upgrades to the design of the microfluidic devices. Minor changes include reducing the resistances of the main flow channels to increase the rate of flow of the source and sink solutions. Our collaborator once informed us that it was difficult to get cells into the chambers due to the high flow resistance of those chambers in comparison to the main flow channels. To resolve this problem, I added pressure-actuated flexible membranes above the main flow channels, which reversibly constricted the cross-section of the channels. This constriction increases the resistance of the main channels and thus directing a larger part of flow of the cellular suspension through the chambers and facilitating the cell-loading. Another important modification of the microchannel network was a device that allows for 2 different strains of yeasts to be concurrently exposed to identical pheromone gradients.

2.1.3.1 MAPK-Mediated Bimodal Gene Expression and Adaptive Gradient Sensing in Yeast

The pheromone sensing MAPK pathway in yeast *S. cerevisiae* affects expression of an estimated 200 genes and directly participates in yeast chemotropism in pheromone gradients³². The pheromone response involves the formation of a diploid zygote from two haploid cells of opposite mating types through pheromone gradient sensing, directed localized cell growth (shmooing) and eventual cell fusion, and requires an integrated regulatory network. In spite of a long and productive research history, the control mechanisms involved in pheromone gradient sensing and chemotropism are still not completely understood. For example, it is not clear how the mean value and the gradient of the pheromone concentration are integrated by a chemotropic cell, whether the dose response in this pathway is graded, as suggested by a recent analysis or switch-like as observed in other MAPK cascades³⁴, and whether the response is affected by molecular-level noise. In addition, the respective roles of two MAP kinases activated by the pathway, Fus3 and Kss1, remain unclear.

Microfluidic chip-based experimental platform

To carry out repeated high throughput experiments on yeast pheromone response in precisely defined gradients, we developed a novel microfluidic device (Fig. 12a,b and Fig.S1 in Supplementary Information). Its functional area consists of an array of 5 μm deep parallel rectangular test chambers of various lengths (short horizontal channels in Fig. 10a,b) and two 25 μm deep mirror-symmetric flow-through channels adjacent to opposite edges of the test chambers (long vertical channels with kinks in Fig. 12a,b). Cells loaded into the test chambers are exposed to linear concentration profiles of

pheromone (Fig.S2). The pheromone gradient profiles are created by molecular diffusion between the left flow-through channel with a high concentration pheromone solution and the right flow-through channel with plain medium. In contrast to a previous microfluidic design for experiments on gradient response⁹, cells in the test chambers are not exposed to any active flow, which is critical for experiments with non-adherent yeast cells. The pheromone gradient profiles in the test chambers remain stable over at least 20 hours. A single microfluidic device contains multiple copies of test chambers of different lengths, allowing exposure of cells to a continuous range of pheromone concentrations presented in gradients of different steepness, as well as analysis of hundreds of cells per experiment. Therefore, long term pheromone dose and gradient responses of individual cells can be analyzed at high throughput. In addition, the cell phenotype and the expression level of specific fluorescently tagged genes can be monitored concurrently, an important capability unachievable with flow cytometry.

Phenotype co-existence and bimodal gene expression

We used the device to study the phenotypic dose response of individual cells to pheromone concentration, c_{ph} , gradually varying from 0-80 nM. Although cell response was tracked continuously, the final analysis was performed six hours after pheromone exposure to reduce the effect of cell cycle stage variability at the beginning of the experiment. Specifically, cells past the G1-stage upon pheromone exposure had sufficient time to complete their cell cycles and then attain their eventual phenotypic states. At six hrs, cells adopted a variety of pheromone response phenotypes. At relatively high c_{ph} , cells formed multiple mating projections or exhibited prolonged chemotropism ('shmooing' or SM phenotype), while at lower c_{ph} they underwent cell cycle arrest

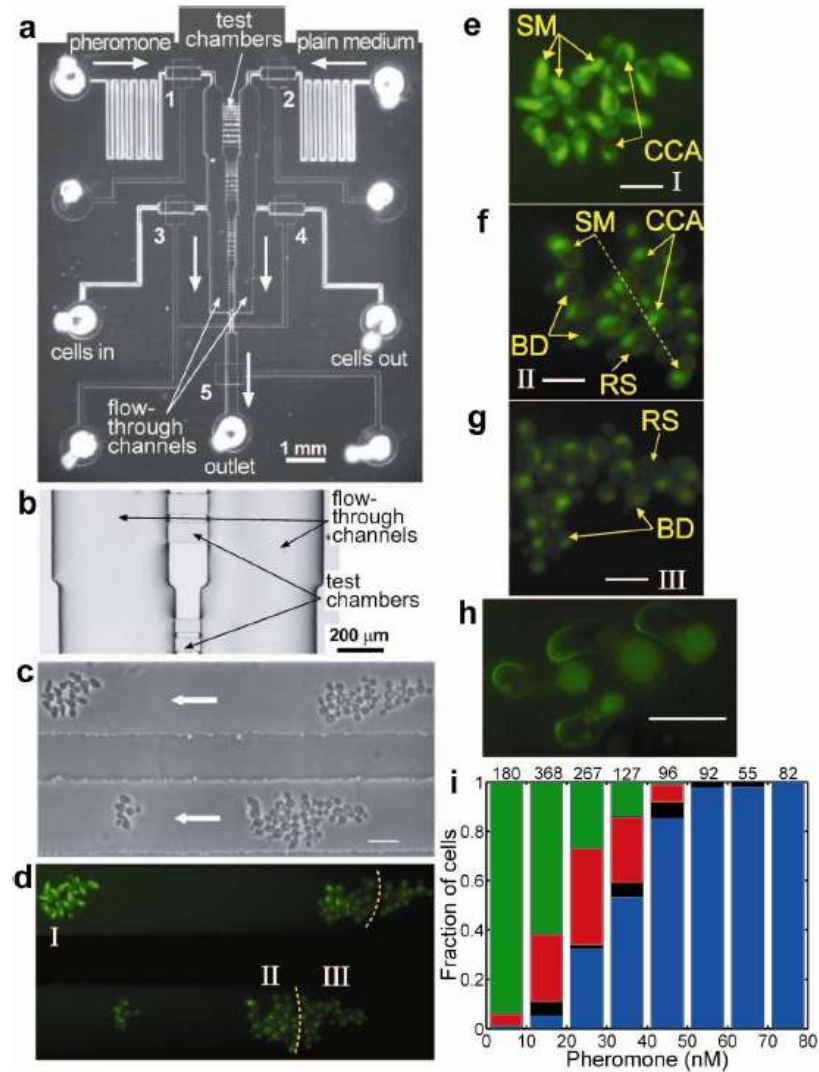


Figure 12. Pheromone dose response analysis using a microfluidic experimental setup. **(a)** Microfluidic device consists of two layers of microchannels: a flow layer (labeled inlets) of flow-through channels and test chambers and a control layer, with 5 membrane valves (numbered 1-5) actuated from 4 separate control inlets (not labeled). **(b)** Magnified view of an area with two 25 μm deep flow-through channels (wide vertical strips) and 5 μm deep test chambers between them (see Supplementary Information for details). **(c)** and **(d)** Phase contrast and fluorescence images of two adjacent chambers with Fus3-yEGFP cells six hours after pheromone exposure. (c_{ph} in the field of view increases from right to left, from 10 to 50 nM). Yellow dashed lines indicate boundaries between cell clusters merged due to cell division. Arrows in **(c)** denote the direction of increasing pheromone (see Fig. S2 for gradient image) **(e)-(g)** Close-up Fus3-yEGFP fluorescence images of cell clusters I, II and III displaying mixtures of different phenotypes. **(h)** Fus1-yEGFP expressing cells displaying chemotropism in a similar chamber. Scale bars are 20 μm (c)-(d), 10 μm (e-g) and 5 μm (h). **(i)** Fraction of different phenotypes of WT cells six hours after pheromone exposure. Phenotypes are color coded as: Budding: BD (green); Cell cycle arrested: CCA (red); Reverted from shmooing: RS (black) and Shmooing: SM (blue). Numbers on the top are the total numbers of cells in the bins.

without formation of a shmoo (CCA phenotype). At yet lower c_{ph} , they displayed no detectable response and continued dividing ('budding' or BD phenotype)³⁵. Additionally, some cells reverted from SM to BD phenotype ('reverted from shmooing' or RS phenotype; see Supplementary Methods for cell phenotype determination). Surprisingly, distinct phenotypes often co-existed in cell subpopulations exposed to the same c_{ph} (Fig. 12 e-g,i), indicating the existence of c_{ph} , where the decision to acquire a specific phenotype might depend on stochastic features of the underlying biochemical reactions.

To gain insight into the phenotype co-existence, we generated cells expressing Fus3-yEGFP and Fus1-yEGFP fusion proteins by genomic integration of *yEGFP* at the respective native loci (referred to as WT strains henceforth, see Supplementary Methods). These two genes, known targets of the pheromone pathway³², were selected as indicators of a more general gene expression response to pheromone. To exclude the effects of possible gradient variability between different chambers, we analyzed Fus1-yEGFP expression levels of cells in multicellular clusters (4-15 cells) with c_{ph} variation < 5 nM across a cluster (Fig. 13a). (The same c_{ph} was assigned to all cells within one cluster for all further analysis.) At $c_{ph} = 20-50$ nM, cells within one cluster frequently exhibited different phenotypic responses uncorrelated with their positions in the cluster. In the same c_{ph} range, there was clear bimodality in the expression of Fus1-yEGFP in individual cells (Figs. 13a, S4). The fluorescence intensity of membrane-localized Fus1-yEGFP measured with an epi-fluorescence microscope (as in Fig. 13a) correlated well with the total membrane-localized fluorescence estimated by confocal imaging (Supplementary Information and Fig. S4). We sub-divided cell clusters into groups of cells (4-11 cells per group) displaying one of the BD/CCA/SM/RS phenotypes and explored the correlation

between phenotype and gene expression. Strikingly, differences in Fus1-yEGFP expression levels between BD, CCA and RS cells were insignificant, whereas the difference between SM cells and cells of all other phenotypes was highly significant (Fig. 13a). The dose response for Fus3-yEGFP expression was qualitatively similar, but the bimodality was much less prominent (Fig.S5).

Mathematical model for transcriptional regulation

The phenotype co-existence and bimodality in Fus1-yEGFP and Fus3-yEGFP expression suggested that the underlying gene regulation network might exhibit at least two distinct stable states (bistability). Many natural and engineered biochemical networks can exhibit bistability or multi-stability, usually as a consequence of one or more positive feedbacks interactions³⁷⁻⁴⁴. Deterministic bistable systems eventually converge to one of the two distinct steady states available under a given constant input, where the chosen steady state is determined by the system's history (hysteresis). Such systems display histograms with unimodal distributions. In contrast, a combination of bistability with inherent stochastic noise might allow the system to switch between the two stable steady states, resulting in co-existence of two discrete response levels in cell populations exposed to the same level of stimulus, i.e. response bimodality^{34,46}. To investigate possible mechanisms of bistability in the pheromone pathway, we constructed a mathematical model incorporating the activity of the MAPKs Fus3 and Kss1, the pheromone-response specific transcription factor Ste12, and up-regulation in expression of *FUS1*, *FUS3* and *STE12* by Ste12 (Figs. 14a, S8 and S9-S15 of Supplementary Information). Unphosphorylated Kss1 exerts an inhibitory effect on the pathway by binding to Ste12 and potentiating Dig1,2 mediated repression of Ste12⁴⁷, whereas

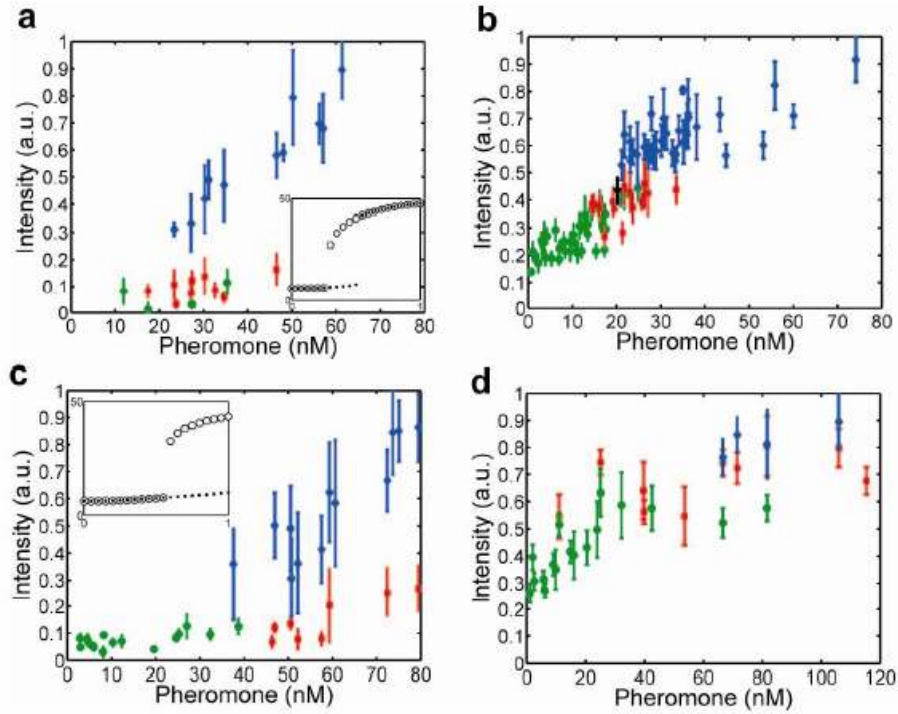


Figure 13. Quantification of Fus1 protein and gene expression. **(a)** Membrane localized Fus1-yEGFP in *WT* (strain AL4); **(b)** YFP driven by the *FUS1* promoter in *WT* (strain SP42); **(c)** Membrane localized Fus1-yEGFP in a *PSTE12₃PREmut* strain (strain ZH579); **(d)** YFP driven by the *FUS1* promoter in *fus3Δ* (strain SP44). The data (mean $\pm$ standard deviation) from different cell groups ($n = 4-11$ cells) are color-coded as in Fig. 1i: BD (green circles), CCA (red squares), RS (black triangles) and SM (blue diamonds). Fluorescence intensities are normalized so that the maximal (mean + S.D.) value in each experiment corresponds to unity. Insets in (a) and (c) are the corresponding predictions of the model (See also Fig.S11). Expression levels of SM and CCA cells in the region of co-existence are significantly different according to a two sample t-test (assuming a two-tailed distribution and unequal variances): $P = 2.6 \times 10^{-4}$ for **(a)**, $P = 6.3 \times 10^{-6}$ for **(b)** and $P = 1.1 \times 10^{-5}$ for **(c)**.

phosphorylated Fus3 and Kss1 can activate Ste12 thus enhancing the pathway activation⁴⁷⁻⁴⁹. Therefore, the model included activation of Ste12 by phosphorylated Fus3 and Kss1 and repression of Ste12 by unphosphorylated Kss1. The model generated *FUS1* and *FUS3* transcription dose response curves with a combination of monostable and bistable regions (Figs. 13b inset, 14b inset, S5d), thus reproducing the experimentally observed dose response curves.

The bimodal expression of Fus1-yEGFP and Fus3-yEGFP (Figs. 13a, S5a) suggested substantial stochastic variation in the concentrations of key pheromone pathway molecules across the cell population. Cell-cell differences in the signaling pathway components can be a significant source of variation in the pheromone response, especially at low pheromone concentrations. Stochastic variability is also likely to diminish the effect of hysteresis in a bistable system⁴⁵. Indeed, we did not observe any significant hysteretic effects in the response (Figs. S3, S5b)

MAPK mediated transcriptional regulation and *STE12* autoregulation

To verify that the bistability is regulated at the transcriptional level, as suggested by the model, we studied cells expressing YFP under the control of *FUS1* promoter (also referred to as a WT strain, see Supplementary Methods). These cells displayed a bimodal distribution of fluorescence intensity (Fig. 13b), which was qualitatively similar to but less pronounced than that of the Fus1-yEGFP cells. (See Supplementary Information for a discussion).

Analysis of the model further suggested that the non-linear transcriptional autoregulation of Ste12 is essential for the bistable response. To investigate this, we mutated the three consensus pheromone response element (PRE) sequences in the

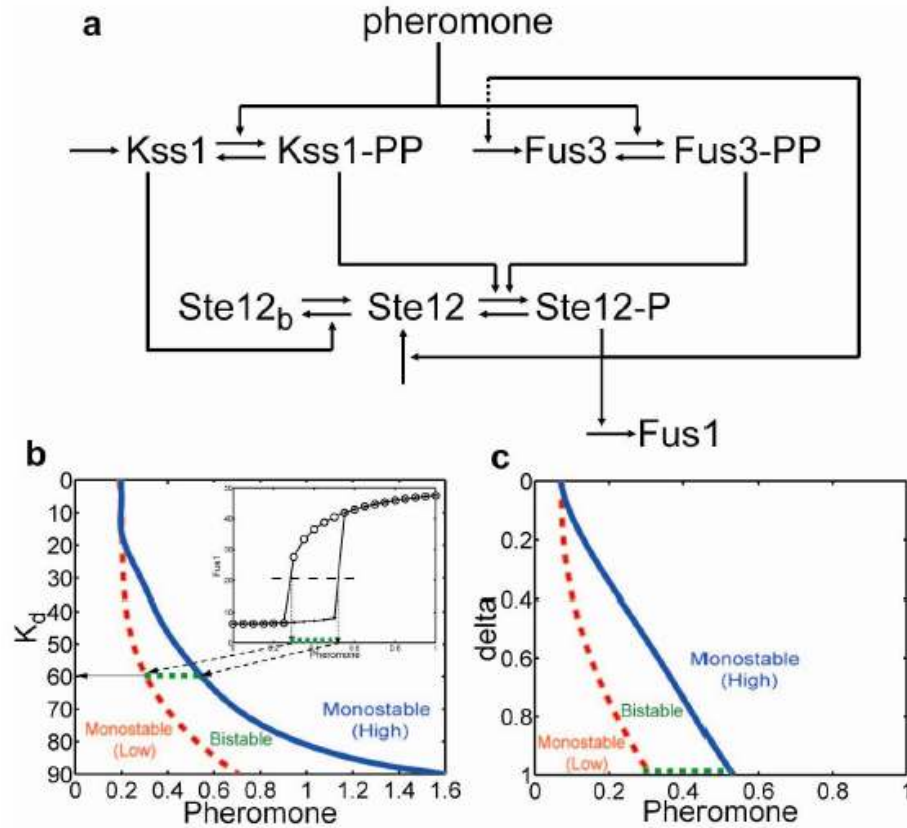


Figure 14. Computational modeling of transcriptional regulation in the pheromone response. **(a)** A simplified diagram of signaling and transcriptional regulation in the pheromone pathway. "P" denotes phosphorylated forms of the species. Ste12_b denotes the Kss1-bound repressed Ste12; **(b)** Ste12 autoregulation is a critical determinant of bistability in the model. For each binding constant K_D of Ste12-P to the *STE12* promoter, the bistability region is confined between the red and blue curves. The inset shows the hysteretic dependence of [Fus1] on pheromone for nominal K_D . Dashed green lines in the inset and (b), (c) indicate the bistability ranges for nominal K_D . Solid and open circles in the inset correspond to low and high initial concentrations of the relevant species, respectively (Supplementary Table 1 and 2). **(c)** Plot similar to (b) shows the region of bistability for various values of normalized Kss1 expression (δ). $\delta=1$ corresponds to the WT case and $\delta=0$ to *kss1* Δ case. (see Supplementary Information for model description)

promoter region upstream of *STE12* (*PSTE12_{3PREmut}*), leaving only non-consensus sites available for binding by activated Ste12 (Supplementary Information). We modeled this mutation by increasing K_d of Ste12 binding, which resulted in the bistability region moving to higher c_{ph} and becoming wider (Figs. 14b, S11d, S12i). Both effects were observed experimentally, with the CCA phenotype in mutated cells found at much higher c_{ph} than in WT cells (Fig. 13c).

To investigate the respective roles of Fus3 and Kss1, we first analyzed *fus3Δ* cells. Shmooing and polarized growth were severely hampered, occurring at much higher c_{ph} (Figs. 13d, S6, S7), in agreement with previous studies that have highlighted the importance of Fus3 for the mating response⁵⁰. Up to the highest c_{ph} tested ($c_{ph} > 100$ nM), these cells displayed a mixture of different phenotypes. We also observed hyper-invasive growth at low c_{ph} ⁴⁹, and a moderate increase in the *FUS1* promoter driven transcription over the entire pheromone range.

To study the role of Kss1, we evaluated Fus1-yEGFP expression (Fig. 15a) and *FUS1* promoter driven transcription (Fig. 15b) in *kss1Δ* cells as functions of c_{ph} . The model predicted substantial reduction of bistability in these cells, which was expected to lead to reduced bimodality in Fus1-yEGFP expression. Additionally, knocking out *KSS1* was expected to reduce c_{ph} at which *FUS1* expression starts to increase and saturate (Figs. 14c, S10, S11a). In close agreement with the model, bimodality in gene expression in *kss1Δ* cells was drastically reduced, and the pheromone sensitivity saturated at significantly lower c_{ph} . Strikingly, in contrast to WT cells the levels of gene expression in Kss1-deficient BD and CCA cells were highly sensitive to variation in c_{ph} , whereas in SM cells gene expression was essentially saturated (cf. Figs. 13 a,b and 15 a,b). The

increased activation of BD cells is consistent with previously observed Fus1 upregulation without concomitant cell cycle arrest in *kss1* Δ cells⁵⁰.

We hypothesized that the graded increase of Fus1-yEGFP expression in WT SM cells was closely connected to pheromone dependent phosphorylation of Kss1, leading to gradual reduction in Kss1-mediated repression of Ste12. To test this hypothesis, we transformed Fus1-yEGFP expressing *kss1* Δ cells either with the YCpU-*kss1*(AEF) or the YCpU-*kss1*(Y24F) plasmids. Kss1_{AEF} cannot be phosphorylated by the MAPKK Ste7, and hence lacks kinase activity. Kss1_{Y24F} can be phosphorylated by Ste7 to the same level as WT, but its phosphorylated form lacks kinase activity. Unlike in *kss1* Δ cells, in both mutants, CCA cells showed only a modest increase in gene transcription, which was likely a consequence of repression of Ste12 mediated gene expression by the unphosphorylated Kss1_{Y24F} and Kss1_{AEF}, which acted similarly to unphosphorylated Kss1 in WT cells. The bimodality and the growth of Fus1-yEGFP expression with c_{ph} in Kss1_{Y24F} cells were both similar to those in WT cells, indicating that the kinase activity of phosphorylated Kss1 is not essential for the observed behaviors (Fig. 15c). In contrast, the Kss1_{AEF} mutant displayed a stronger bimodality than WT (Fig. 15d), and unlike WT SM cells, Kss1_{AEF} SM cells showed no appreciable enhancement of Fus1-yEGFP expression with increasing c_{ph} . This behavior is predicted by the model: since Ste7 does not phosphorylate Kss1_{AEF}, the repression of Ste12 by Kss1_{AEF} is not likely to be weakened with increasing c_{ph} . All major trends in Kss1 mutants were consistent with mathematical model predictions (Fig. 15, S11).

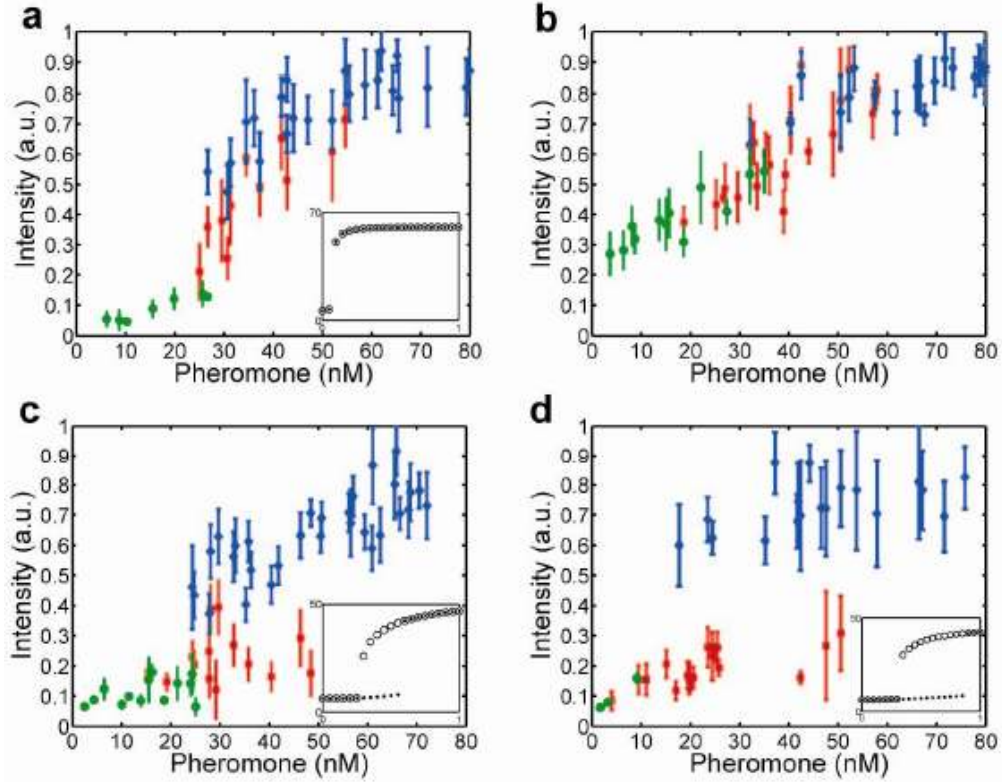


Figure 15. Role of Kss1 in regulating the pheromone response. *kss1Δ* cells expressing Fus1-yEGFP (ZH552) **(a)** and YFP driven by the *FUS1* promoter (SP43) **(b)** were exposed to the same conditions as in Fig. 2. Similar experiments were performed with a YCpU-*kssI*(Y24F) plasmid **(c)** and a YCpU-*kssI*(AEF) plasmid **(d)** introduced into the Fus1-yEGFP *kss1Δ* strain used in **(a)**. Fluorescence intensities are normalized as in Fig. 2. Insets **(a)**, **(c)**-**(d)** are the predictions of the mathematical model for the corresponding strains (see also Fig. S11). P-values (calculated as in Fig. 2) were 1.0×10^{-8} for **(c)** and 3.2×10^{-11} for **(d)** indicating significant difference in expression levels of SM and CCA cells. In contrast, this difference was relatively insignificant in **(a)** with a P-value of 0.017 and was

Gradient sensing characteristics of WT and *kss1Δ* cells

Our observations of WT SM cells indicated that, as expected, the mating projections were predominantly oriented in the direction of increasing c_{ph} . Gradient sensing was strongest at lower c_{ph} , whereas, at higher c_{ph} , the orientation of projections became increasingly random. Interestingly, the c_{ph} range of gradient sensitivity appeared to coincide with the range of sensitivity of *FUS1* expression to variation of c_{ph} . Pheromone dependence of *FUS1* expression may be representative of a large subset of genes, some of which are likely to be essential for gradient sensing. Hence, we hypothesized that the c_{ph} sensitivity of gene expression reflects sensitivity of the signaling apparatus necessary for precise gradient sensing. Accordingly, the saturated *FUS1* expression in SM *kss1Δ* cells might coincide with impaired gradient sensitivity. Therefore, we used the microfluidic device to further examine gradient sensing in WT and *kss1Δ* cells.

We found (Fig. 16a, d-e, h-i) that the directional bias in WT cells increased significantly with time, in general agreement with previous reports⁵¹. Moreover, the highest gradient sensitivity was consistently observed at high values of the gradient, $\gamma = \partial c_{ph} / \partial x$ and low c_{ph} . We quantified the precision of gradient sensing within a cluster of cells (4-11 SM cells) by a parameter σ , the root mean square of the angles between the observed projections in a cluster and the direction of the gradient (precision decreases with increasing σ). σ values were binned into two equal intervals, 0°–55° and 55°–110°, covering the whole experimental range, and were plotted on the c_{ph} - γ plane, with bin-specific symbols (Fig. 16f). The results suggested a linear relationship between the γ and

c_{ph} values for cell clusters displaying the same range of σ , i.e. $\gamma = \rho \cdot (c_{ph} - c_0) + \gamma_0$, where γ_0 and c_0 are constant and ρ is different for the two sets of cell clusters. We notice that $c_0 \approx 15\text{-}20$ nM, the value of c_{ph} where the two linear regression lines intersect, is also the lowest concentration at which SM cells are observed. σ remained constant at constant fractional gradient, $\rho = (\gamma - \gamma_0) / (c_{ph} - c_0)$, and more precise gradient sensing (lower σ) corresponded to larger ρ (Fig. 16f). These findings suggest that yeast cells respond to the fractional rather than absolute gradient of pheromone, and that gradient sensing in yeast is optimized for exponential concentration profiles (See Supplementary Information). The sensitivity to the fractional gradient of a chemical cue is in surprising agreement with the results reported for gradient sensing by amoebae *D. discoideum* and grasshopper neurons^{52, 53}.

Sensitivity of cells to fractional gradients further implies that gradient sensing can dramatically deteriorate at higher pheromone concentrations. We observed this deterioration in both WT and *kss1Δ* cells, but in *kss1Δ* cells it occurred at significantly lower c_{ph} than in WT (Fig. 16g). Therefore, deficiency in Kss1 leads to a decrease in the range of c_{ph} , in which gradient sensing is efficient, just as hypothesized above. Similarly, we observe that WT cells sense gradients substantially better than *kss1Δ* cells by comparing their values of σ for different ratios of γ to c_{ph} (Fig. 16i). Interestingly, WT cells performed only marginally better than *kss1Δ* cells in terms of the initial projection direction, but displayed substantially better alignment along the gradient over time as compared to *kss1Δ* cells (Fig. 16h-i). It thus appears that the progressive improvement in gradient sensing in WT SM cells is strongly correlated with the sensitivity of pheromone

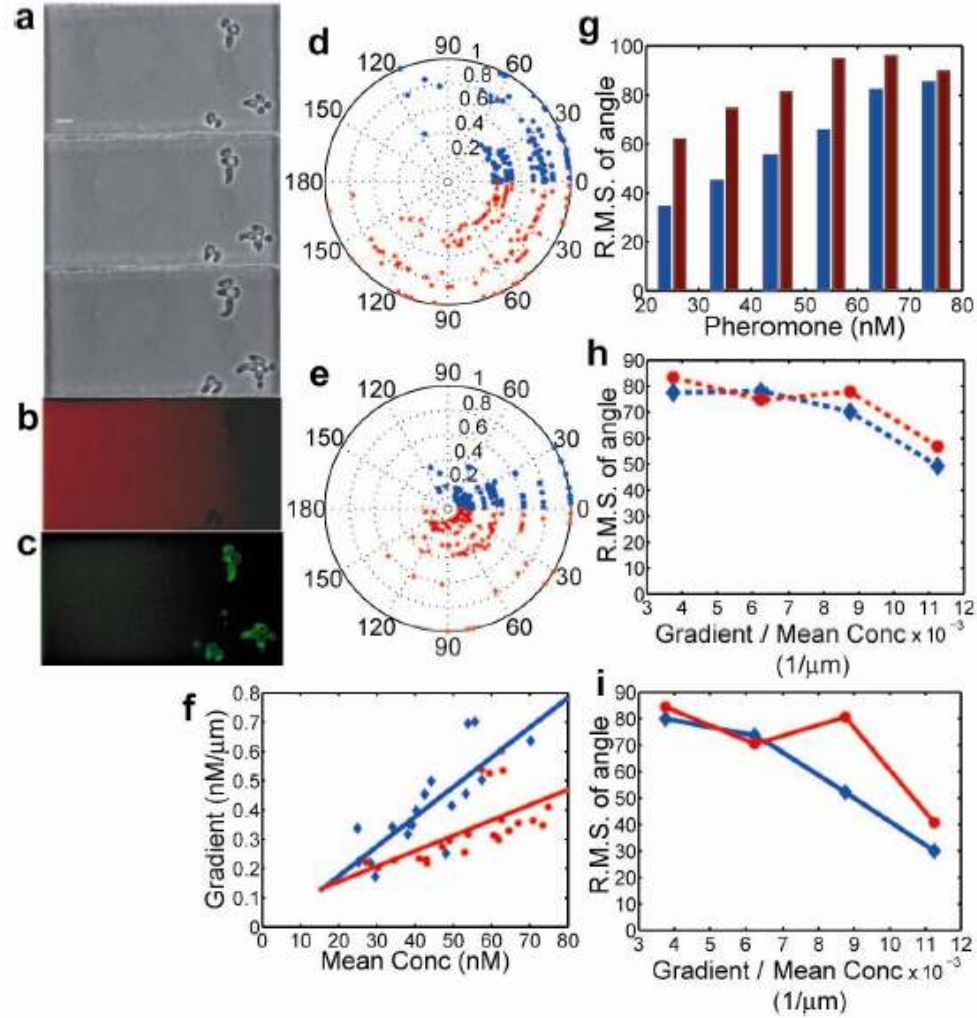


Figure 16. Quantification of pheromone gradient sensing. (a) Chemotropism at 4, 5 and 7 hrs. following exposure to a pheromone gradient (scale bar is 10 μm). (b)-(c) c_{ph} profile (visualized with Alexa Fluor 555) and GFP expression, respectively, at 5 hrs. (d)-(e) Polar plots representing the dependence of the absolute value of the angle between individual cell projections and the gradient, on c_{ph} , (radial dimension in (d)) and γ (radial dimension in (e)), normalized to the maximal values of c_{ph} and γ , respectively. Data are shown for the same cells at 4 hrs (red) and 20 hrs (blue) post-stimulation. (f) Cell clusters with $\sigma = 0-55^\circ$ and $\sigma = 55-110^\circ$ are plotted in c_{ph} - γ plane in blue and red, respectively (σ is the root mean square of the angle between the projection and the gradient direction); $n = 4-11$ SM cells per cluster. Straight lines are the corresponding linear regression lines $R^2 = 0.64$ (blue) and $R^2 = 0.38$ (red); (g)-(i) σ displayed by groups of SM cells in WT (blue) and $kss1\Delta$ (red) backgrounds at $t = 4$ hrs in (h) and $t = 9$ hrs in (g) and (i), as a function of the c_{ph} , (g) and γ / c_{ph} (h)-(i). Each bar (or point) represents at least 5 clusters of SM cells. Bars in (g) correspond to 10 nM widths in c_{ph} . Points in (h)-(i) represent bin widths of $0.0025 \mu m^{-1}$ in γ / c_{ph} .

induced gene expression to variation of c_{ph} . We also studied the gradient sensing of the strain containing the *STE12* promoter with 3 mutated consensus PRE sites (Fig. S16), as well as the two Kss1 mutant strains, Kss1_{Y24F} and Kss1_{AEF} (Fig. S17). In all cases, the existence of an extended dynamic range of gradient sensing was correlated with the c_{ph} sensitivity of the pheromone induced gene expression in SM cells.

Conclusions

This study yielded several important insights into the physiology of pheromone response in *S. cerevisiae*. The observed combination of switch-like and graded dose responses, combined with stochastic variability across a cell population, can provide significant benefits for mating yeast populations. First, bimodality in gene expression allows a cell population to diversify its transcriptional response at relatively low pheromone concentrations, reducing the cost of possible inappropriate engagement in expensive pheromone dependent gene amplification. Thus, when exposed to weak pheromone signals, some cells continue to suppress the pheromone response, whereas others display amplified gene expression correlated with the formation of mating projections. These shmooing cells, capable of sensing fractional pheromone gradients, benefit from the particularly high gradient sensitivity that we observe at low c_{ph} , (higher fractional gradient). Second, continued sensitivity of gene expression in shmooing cells to increasing c_{ph} appears to extend the c_{ph} range in which cells can sense gradients. Thus, as cells approach the mating partner, they can adjust to increasing pheromone concentrations with only partial compromise of gradient sensing. Strikingly, many features of the WT pheromone response, including bimodality in gene expression and high dynamic range of gradient sensing, are critically dependent on the presence of Kss1.

In particular, the switch-like increase of gene expression in shmooing cells is a result of the combined effect of Ste12 repression by unphosphorylated Kss1 and of Ste12 auto-regulation. The graded increase of gene expression with increasing c_{ph} , depends on pheromone regulated phosphorylation of Kss1 which results in graded reduction in Kss1 mediated repression of Ste12, and possibly, on the ability of phosphorylated MAPKs to activate the pathway. A reduction in the graded increase of gene expression in *kss1Δ* cells correlates with lower precision and reduced dynamic range of gradient sensing. Our results suggest that the role played by Kss1 is not redundant with that of Fus3, and provide functional significance for the strong negative regulation exerted by inactive Kss1 on Ste12, the transcriptional factor essential for the pheromone response.

Methods

Yeast and nucleic acid manipulations were performed as previously described. Details on strain construction are present in the Supplementary Information. Fabrication and operation of the microfluidic device is described in detail in Supplementary Information. Evaluation of the pheromone concentrations and gradients and of the levels of fluorescent reporter expression was performed using custom-written Matlab programs (See supplementary information for details). Mathematical model simulations were run in Matlab.

Chapter 2.1.3.1, in full, is a reprint of the journal article *MAPK-mediated bimodal gene expression and adaptive gradient sensing in yeast* in Nature, 2007. Paliqal, S.; Iglesias P.A.; Campbell, K.; Hilioti, Z.; Groisman, A.; Levchenko, A., Nature Publishing Group, 2007. Dissertation author was a participating investigator of this paper.

2.1.3.2 Exponential and two-dimensional gradients for non-adherent cells

Chapter 2.1.2.1 discussed a model of chemotactic prowess of live cells based on the two parameters, the fractional gradient $\frac{\Delta C}{C}$ and the ambient concentration C . In order to test this model, it was crucial to use the microfluidic gradient-making networks that generate exponential concentration profiles 2.1.1. Chapter 2.1.3.1 discussed the response of non-adherent yeast cells to a linear pheromone gradient. The use of a device similar to the one described in chapter 2.1.3.1, but with an exponential concentration gradient would broaden our understanding of the gradient response of yeast cells and other non-adherent cells.

The device for a generation of exponential concentration profiles that I built and tested is similar to the devices used in the yeast study⁵⁴ with two deep main flow channels and an array of shallow test chambers between them. The shallow chambers connect the main flow channels and allow for chemical diffusion, creating a gradient. The shape of the chemical concentration gradient can be adjusted by changing the shape of the chamber.

The shape of the chambers was optimized using the simulation software Comsol 3.2 (Comsol, Stockholm, Sweden) to obtain exponential gradients of the shape $C \propto \exp(\rho x)$ with the fractional gradient $\rho = \Delta C / C = 0.005, 0.0075, 0.01, \text{ and } 0.0125 \mu\text{m}^{-1}$. This range of the fractional gradient corresponded to the chemotropic response of yeast changing from weak to strong, based on the findings of Ref. ⁵⁴. A ratio 8:1 (high to low concentration) was fixed for all chambers.

The device was tested on a confocal microscope using FITC as the diffusive chemical. The shaped of the concentration profiles were found to be close to the shapes obtained from numerical simulations (Fig. 17). The slight increase in the fractional gradient ρ for all chambers is likely due to some sweeping of flow in the low concentration channel into the chamber at the curved boundary. All chemical concentration gradient schemes described thus far only allow for cells to be exposed to a concentration field of a single chemical. In order to test cellular response to non-parallel gradients of two different chemicals applied concurrently, a two-dimensional gradient device was designed and tested. This device can be used to test competing chemicals that elicit a specific response in cells, or to study a dose response of cells to different combinations of concentrations of two chemicals. The test area of the device consists of a shallow triangular chamber with corners connected to deep channels carrying three different solutions. With no fluid flow entering the chamber from the channels, chemicals diffuse into the chamber and establish a gradient. This creates two non-parallel chemical gradients within the same test chamber.

The device was tested on a confocal microscope. Two fluorescent dyes, FITC and sulphorhodamine, were used as the different diffusive chemicals, while the liquid in the third channel was a plain buffer. The dyes produced two symmetric concentration profiles in the test chamber (Fig. 18).

The design, fabrication, and characterization of exponential and two-dimensional microfluidic gradient devices were conducted by the dissertation author. These devices will be used for in-depth cell biology studies by collaborators in Andre Levchenko's group at Johns Hopkins University.

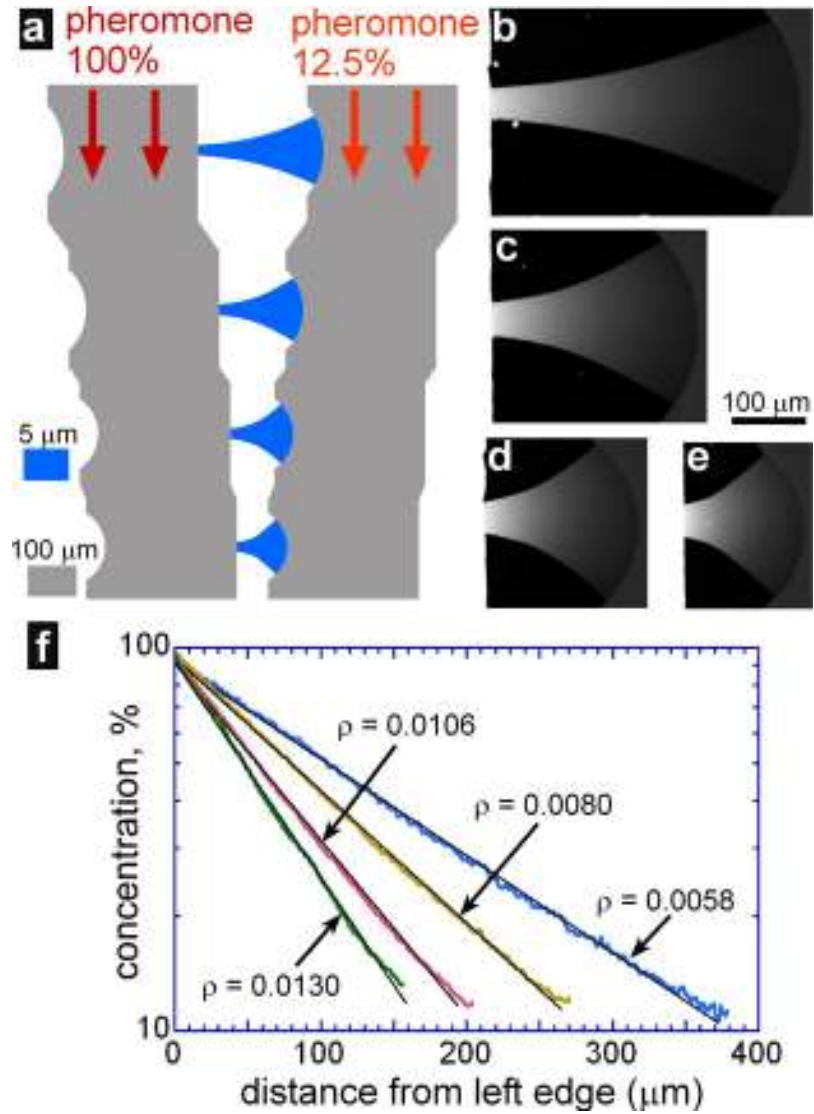


Figure 17. Microfluidic device with exponential concentration profiles. **(a)** Drawing of the functional area with 100 μm deep flow-through channels and 5 μm deep chambers (color coded). **(b)–(e)** Confocal images of different test chambers with 50 ppm (100%) and 6.25 ppm (12.5%) solutions of fluorescein in the flow-through channels. **(f)** Concentration profiles along the central axes of the chambers fitted with exponents, $c \propto \exp(-\rho x)$, where ρ is measured in μm^{-1} . Steeper profiles correspond to shorter chambers.

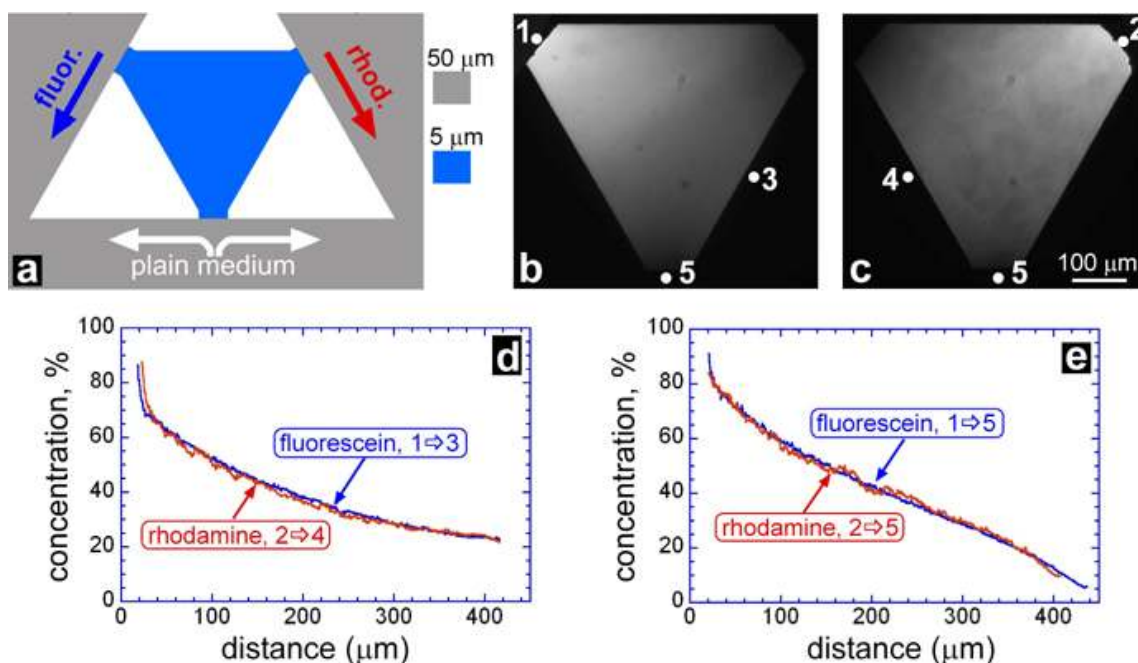


Figure 18. Microfluidic device generating a 2D concentration profile in a triangular test chamber, tested with solutions of fluorescein and sulphorhodamine. **(a)** Schematics of the functional area with color-coded channel depths and flow directions indicated by arrows. **(b)-(c)** Fluorescence micrographs of the test chamber with fluorescein and rhodamine filter sets, respectively. **(d)-(e)** Concentration profiles of fluorescein and rhodamine along lines between different points indicated in **b** and **c**;

2.2 CHEMOSTAT

The task of growing bacteria or yeast to high density colonies starting from a few cells is difficult with the standard laboratory *in vitro* methods. At high densities, cells quickly deplete the nutrients, while the metabolic waste builds up, making the medium unfavorable and causing an arrest of cellular growth. Large scale chemostatic perfusion devices have a constant flow of a nutrient solution over the cells providing continual nutrient supply and waste removal. However, cells with poor or no adhesion can get washed away and their colonies do not reach high densities in the perfusion devices. We designed a microfluidic device that allows cells to multiply over many generations under constant medium conditions without exposure to flow.

The microchannel network consists of flow-through channels and growth chambers in between the channels. Connecting the flow-through channels to the chambers are shallow capillary lines. The depth of these lines is designed to be smaller than a cell diameter so that once in the chamber, cells cannot get out. On the other hand, the capillary lines enable efficient diffusion of chemicals between the flow-through channels and chambers thus providing stable favorable medium conditions inside the chambers, where the bacterial or yeast cell colonies grow. The cells are loaded into the chambers by using the flexibility of PDMS the device is machined from. When the ambient pressure in the microchannel network is increased, the roofs of the capillary lines are lifted to the point where cells can pass through them.

Constant temperature on the microfluidic chip is achieved by flowing water from a temperature controlled water circulator through an O-shaped deep flow channel, which is situated above the chambers and separated from them by a thin ($\sim 200\ \mu\text{m}$) PDMS

membrane. The two-layer design of the device similar to the devices with membrane valves discussed in chapter 2.1.2 and 2.1.3.

The microfluidic chemostats were fabricated and physically characterized by me and used in a collaborative project with Andre Levchenko's group at Johns Hopkins University for quantitative cell biology experiments. Chemostats used for experiments with bacteria had $\sim 0.6 \mu\text{m}$ deep capillary channels in order to keep $\sim 1 \mu\text{m}$ diameter bacteria from escaping the chambers. The widths of these channels were $\sim 20 \mu\text{m}$. So the ratio of the width to depth was rather high, and the PDMS chips were prone to spontaneous collapse of the capillaries and sealing off the chambers, leaving no way to get the cells into the chambers. Collapse would often happen while bonding the device to the glass cover slide by baking in an oven, leaving no way to re-open the capillary channels without delaminating the entire device from the glass. Different types and mixing ratios of PDMS were tested in addition to different baking regimens in an attempt to minimize channel collapse and maximize their long-term stability. Eventually it was determined that the commercial PDMS Sylgard 182 (Dow Corning) had a higher Young's modulus than two other commercial PDMS products, Sylgard 184 (Dow Corning) and RTV615 (GE), and was better for preventing collapse. In addition, baking the device at $130 - 150^\circ\text{C}$ for 1 hour also increases the stiffness of the PDMS.

Once a procedure was found to keep the capillary channels from collapsing, I studied the dependence of the depth of the capillaries on the ambient pressure in the microchannel network. The chemostats were filled with a 0.05% FITC solution and the fluorescence of this dye in the capillary channels was measured under a microscope with a cooled digital camera (SPOT RT SE-18 by Diagnostic Instruments). As pressure was

increased, the roofs of the channels lifted creating a thicker layer of FITC dye solution and proportionally greater integral intensity of fluorescence. Three different widths of capillaries were measured: 14 μm , 18 μm , and 23 μm . As expected, the widest channels at 23 μm showed the greatest compliance. Capillary channels of all three widths were shown to reach 2 μm or greater depth at 8 psi of ambient pressure, indicating they were all appropriate to allow $\sim 1\mu\text{m}$ *E.coli* cells to enter the chambers.

Cell growth can be very sensitive to temperature. To set the temperature of the microfluidic chip, water from temperature-controlled circulator was pumped through a 0.3 mm deep by 1.4 mm wide O-shaped channel that surrounds the chambers of the chemostat. The distribution of temperature in the device was measured with a thermal imaging camera (far infrared, IR, camera Omega, by Indigo, Goleta, CA), while 37 °C water was pumped through the O-shaped channel. Glass is opaque in infrared. Therefore imaging of the coverglass provided direct information about its temperature. The coverglass was thin (~ 0.17 mm) and had relatively high thermal conductivity, and microfluidic chambers were in direct contact with its back side of the cover glass. Therefore, it was assumed that the IR radiation of the exposed side of the coverglass corresponds to the actual temperature of the cell growth chambers. Two rectangular hollowed aluminum blocks at 37 °C and 35 °C with glass slides attached to them were used as reference IR sources and were imaged simultaneously with the microfluidic device.

Using the known temperatures of the reference IR sources, the temperature of the growth chamber array of the chemostat was mapped and found to be in the range of 36.5 C and 37.0 C.

Lastly, I analyzed the distribution of particles in the chambers of the device, when particles were loaded from a mixed suspension using the standard loading procedure. The device I tested had 306 chambers with dimensions of 140 μm by 100 μm . Neutrally buoyant solutions of 1.5 μm diameter polystyrene beads (Cat. # 64040-15 by Polysciences, Warrington, PA) with concentrations of 1.7×10^6 , 1.7×10^7 , 6.8×10^7 and 3.4×10^8 particles/ml were used for the test. The pressure inside the device was increased to 7 psi and the beads were loaded into the chamber using the same method as loading live cells. After the pressure was reduced so that the beads could not escape the chambers, the number of beads in each chamber was counted. As expected, the number of beads in a chamber follows a Poissonian distribution $n_k = N e^{-\mu} \frac{\mu^k}{k!}$ where $N = 306$ is the total number of the chambers, n_k is the number of chambers having k particles in them and μ is the mean of the distribution. When applied to loading of cells into the chambers and growing cells colonies in the chamber, this finding implies that it is possible to have a number of chambers with colonies originating from a single cell, but the majority of the chambers will be unfilled.

Biological tests carried out in Andre Levchenko's group at Johns Hopkins University demonstrated the growth of *E. coli* colonies in the chambers of the devices to be chemostatic up to very high densities of cells. The results of this study were published in Nature Methods and republished with permission in this dissertation.

2.2.1 A Microfluidic Chemostat for Experiments with Bacteria and Yeast Cells

Although commonly thought of as self-sufficient single cell organisms, bacteria and yeast can establish communities of high morphological and functional complexity⁵⁵⁻⁶². The density of growing bacterial and yeast colonies can reach extremely high values (e.g., 10^{10} to 10^{11} *Vibrio fischeri* cells/ml in the light organs of *Euprymna scolopes*⁶³) that are hard to achieve in laboratory using common *in vitro* techniques due to rapid depletion of nutrients and accumulation of metabolic waste. Batch culture chemostats are also inappropriate as the convective flow used to refresh the medium leads to extensive mixing and loss of cells, preventing formation of naturally occurring morphologically complex multi-cellular superstructures. Flow chambers used to study biofilm formation or chemotaxis in adherent cells^{64, 65} are not suitable for cells with absent or weak surface adhesion, including the common lab strains of bacterium *Escherichia coli* (*E. coli*) and yeast *Saccharomyces cerevisiae* (*S. cerevisiae*). Therefore, at this point, there is no device to perform experiments with non-adherent cells growing to high densities in chemostatic conditions.

Custom made microfluidic devices have been increasingly used in cell biology^{36, 66, 67}. The bacterial applications include studies of chemotaxis⁴, phenotypic heterogeneity⁶⁸ and “social” interaction between motile *E.coli*⁶⁹. Here we propose a microfluidic device made of a silicone elastomer polydimethylsiloxane (PDMS) that allows growing cells in chemostatic and thermostatic conditions in an array of shallow microscopic chambers. The medium in the chambers can be quickly redefined to transiently expose the cells to an exogenous signal. The device is used to grow cell

colonies to high density starting from as few as one cell, and monitor them for extended periods of time at a single-cell resolution.

Results

Device design and operation

The micro-chemostat device (Fig. 19) was designed to confine growth of non-adherent cells inside small chambers of different sizes while keeping chemical contents of the medium inside the chambers consistently close to that of the medium fed from an outside reservoir. The main functional area of the chemostat is an array of parallel channels with continuous flow-through of fresh medium and parallel rows of chambers between the channels (Fig. 19a-c). The channels and chambers have a depth $h_1 \approx 6 \mu\text{m}$. The channels are $150 \mu\text{m}$ wide; the chambers have width, w , around $100 \mu\text{m}$ and lengths $70\text{-}200 \mu\text{m}$ (Fig. 19 and Supplementary Fig. 1 online). The channels and chambers are connected by capillaries, which are $\sim 20 \mu\text{m}$ wide, $\sim 40 \mu\text{m}$ apart from each other and have depth $h_2 \approx 0.6 \mu\text{m}$ and length $l \approx 25 \mu\text{m}$. The low depth of the capillaries makes them impenetrable to *E. coli* cells (about $1 \mu\text{m}$ in diameter and $2\text{-}3 \mu\text{m}$ in length) and to other bacterial and yeast species (Supplementary Fig. 2 online). The characteristic time of the device is designed to prevent any active flow through the chambers and possible perturbation of cell position, motion or intercellular interaction that may result. On both the inlet and outlet sides (Fig. 19a), branching from a single channel into the array of 16 parallel channels is binary and symmetric. This arrangement leads to equal flow rates through the channels, highly balanced pressures at capillaries on opposite sides of the chambers and thus very small flow through the chambers. The chamber flow is normally

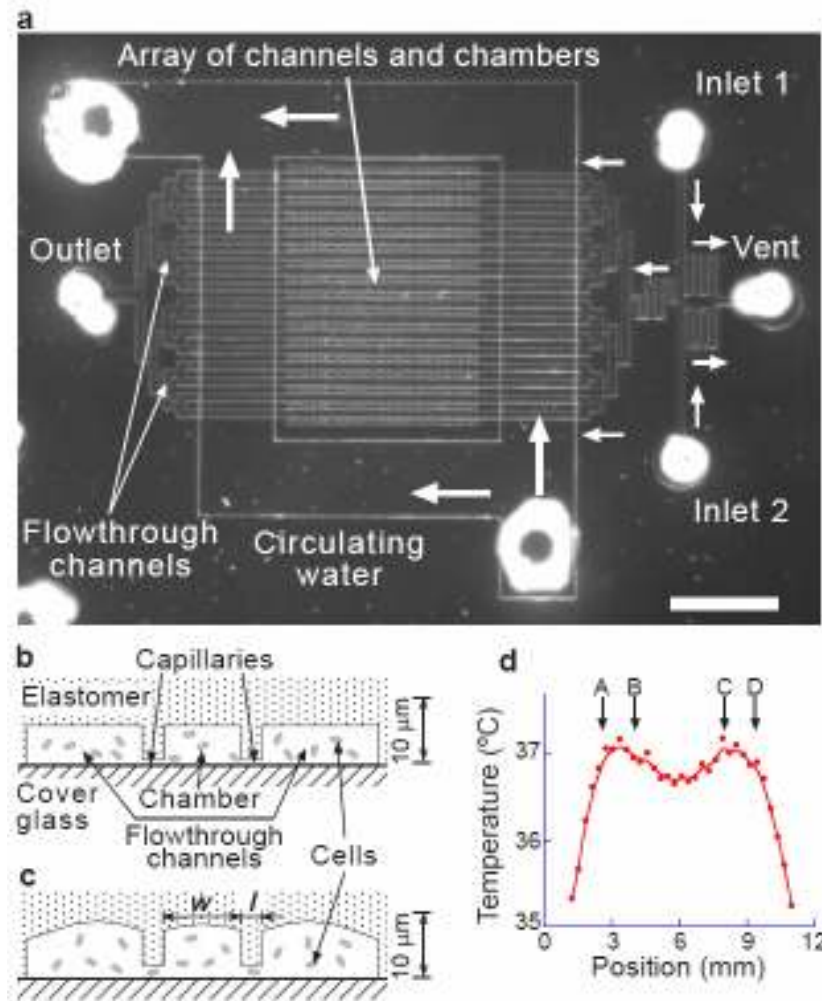


Figure 19. Design and operation of the two-layer microfluidic device. **(a)** Micrograph of the PDMS device. Scale bar, 2 mm. Horizontal and vertical arrows show direction of flow. The chip has an array of 16 flowthrough channels and 340 chambers. Inlets 1 and 2 are symmetric and are connected to the array by a T-junction. Each inlet is also connected to the vent a short distance upstream of the T-junction. The pressures applied to the inlets are adjusted so that medium from only one of the inlets is fed to the chamber array at any time, but there is always some flow from both inlets to the vent. Therefore, the streams from both inlets are always close to the T-junction, and the medium in the array is quickly replaced once the driving pressures are flipped. **(b)-(c)** Schematic drawing of a cross-section of the array showing two channels, a chamber of width w and two capillaries of length l connecting the channels and the chamber. At low gauge pressure inside the device **(b)** the capillaries are impermeable for the cells, but they become permeable at high gauge pressure **(c)**. **(d)** Temperature profile along a line going through the center of the device (a horizontal line at half-height in panel **(a)**) measured with an infrared camera. Points A and D, and B and C mark the outer and inner boundaries of the water circulation channel, respectively. Thermostatic water circulator was set at 37.0 °C. The room temperature was ~22 °C.

at least 1000 times weaker than the flow through the channels themselves, i.e., about 0.1 $\mu\text{m/s}$ chamber flow at 100 $\mu\text{m/s}$ channel flow.

A key issue in operation of the proposed chemostat - loading of the cells into the chambers - is resolved by taking advantage of high flexibility of PDMS (**Supplementary Methods** online). When the gauge pressure inside the device increases, the microchannels inflate and the “roofs” of the capillaries bulge up, increasing capillary depth and allowing passage of cells (Fig. 19c). We found that the depth of the capillaries increased linearly with the gauge pressure, reaching $\sim 2.5 \mu\text{m}$ at 8 PSI (Supplementary Fig. 4 online). When the gauge pressure is dropped to zero, the channels deflate, and the capillaries become inaccessible to cells again. (Compliance of PDMS under pressure was used previously for construction of membrane valves in two-layer devices¹.)

Device characterization

As a test we loaded into the chambers 1.5 μm monodisperse polystyrene beads. We used bead suspensions of 10^6 - 10^8 particles per ml resulting in 0.18 - 14 of beads per chamber in average (Supplementary Fig. 5 online). As expected, the distributions of beads in chambers followed the Poisson law. At concentrations $\ll 1$ beads per chamber, when most of the chambers are empty, the majority of the chambers with beads have only one bead inside. This distribution suggests a possibility of having most colonies originate from single cells. Although chambers are predominantly empty in this case, the number of colonies can be increased by augmenting the functional area of the device and the number of chambers. We could indeed capture single cells in chambers and observed colonies developing from them in numerous experiments. In a representative experiment shown in Fig. 20, a single cell introduced into a chamber divided multiple times, initially

forming a characteristic branched network that then broke as the cells reinitiated locomotion prior to filling the chamber completely. Cell densities approaching complete (dense) chamber packing (estimated at $\sim 2.5 \times 10^{11}$ cells/ml for *E.coli*) were also consistently achieved with several other bacterial and yeast strains (Supplementary Fig. 2 online).

Next we used a truncated quorum sensing *lux* operon from *V. fischeri* expressed in *E. coli*⁷⁰⁻⁷² to study the response of a growing colony to a fast diffusing substance and to track the rate of colony growth. In quorum sensing, bacteria at high densities exchange a membrane permeable compound termed autoinducer (AI) and overexpress genes contained in the *lux* operon, including *luxR*. We took advantage of this inducible up-regulation by fusing LuxR (the AI-receptor), to a low stability Gfp(LVA) mutant⁷³. We estimated the steady state (Supplementary Fig. 6 online) and dynamic (Fig. 21a) responses to exogenously added *V. fischeri* specific AI, N-3-oxo-hexanoyl homoserine lactone. In a dynamic response experiment, when regular medium was replaced with the medium containing 10 nM of AI, following a latency period of approximately 20 min, there was a fast exponential increase of fluorescence over approximately 3-4 orders of magnitude (fitted with $\exp(28.7 \text{ hr}^{-1}t)$) and a slower exponential growth ($\exp(0.8 \text{ hr}^{-1}t)$) afterwards (Fig. 21a). (Concentration of 10 nM of AI was below saturation value, but caused uniform response across cell population, Supplementary Fig. 6 online.) The initial increase was likely due to transcriptional *luxR* and *gfp* upregulation (cf. $\sim 1,000$ -fold transcriptional upregulation of the *lux* operon reported in⁷³). The later, slower exponential rise was probably due to cell proliferation (cf. Fig. 21b,c). Following AI removal, a transient biphasic behavior for approximately 45 min was followed by a rapid

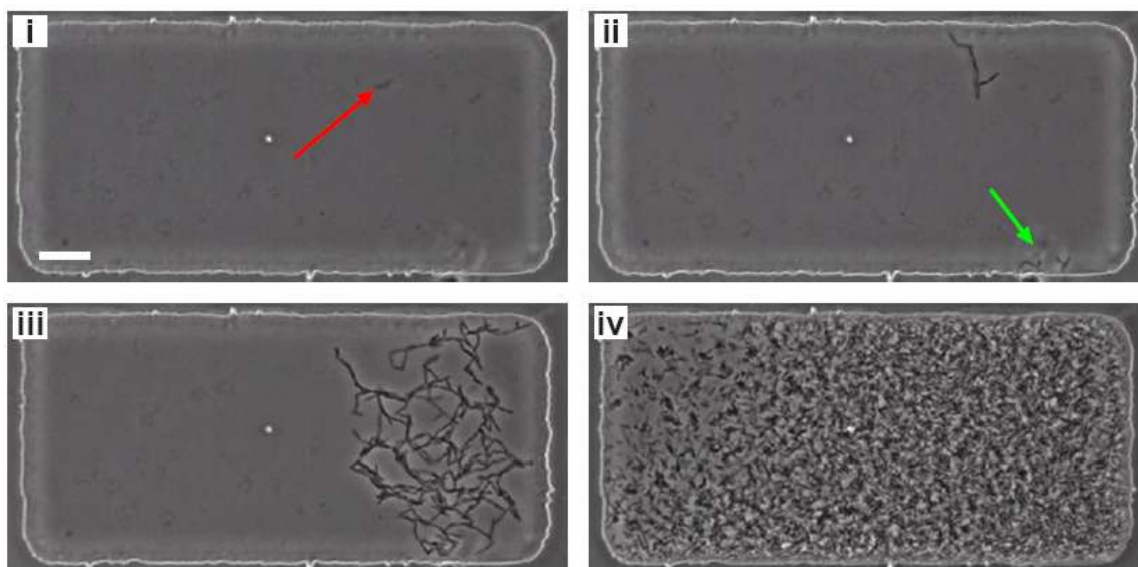


Figure 20. A representative experiment showing development of a JM109 *E. coli* colony inside a chamber at 35 °C from a single cell captured at the beginning of the experiment. Scale bar, 20 μm . The time frames are taken in sequence shown: 0 h (i), 3 h (ii), 5 h (iii) and 8 h (iv). The originating cell (red arrow) grows into a cell cluster that breaks into two (one indicated by a green arrow) and then forms a complex cell network that ultimately grows to fill the whole chamber.

decay characterized by two exponential rates. The slower rate ($-1.07 \pm 0.2 \text{ hr}^{-1}$) observed first was equal to that reported for the degradation rate of the Gfp(LVA) (cf. 0.018 min^{-1} reported in ⁷³). The subsequent faster rate may characterize an as yet unknown mechanism of Gfp(LVA) degradation. The chemostat design thus allows the analysis of the dynamic responses of growing colonies to exogenous signals and recording of the growth curves of the colonies over extended periods of time.

Testing chemostatic conditions

The exponential increase of Gfp(LVA) fluorescence in growing colonies at constant AI (Fig. 21a and Supplementary Fig. 7 online) suggests uniform rates of cell proliferation throughout the chambers. In an extreme non-uniform case of proliferation at a colony periphery only, fluorescence intensity would increase linearly. To further assess proliferation uniformity, accessibility of nutrients and AI to the cells, and experimental variability, we recorded colony growth curves at a constant $[\text{AI}] = 10 \text{ nM}$ in chambers of three different widths ($w = 70, 105 \text{ and } 140 \text{ }\mu\text{m}$) at two different temperatures (20 and 35 °C), using three different chips at each temperature (Fig. 21b-c and Supplementary Fig. 6 online). The growth curves were exponential over at least two orders of magnitude in all cases. The rates were close to those observed in exponential batch culture: $0.72 \pm 0.07 \text{ hr}^{-1}$ vs. 0.8 hr^{-1} at 20 °C and $1.19 \pm 0.02 \text{ hr}^{-1}$ vs. 1.3 hr^{-1} at 35 °C, on the chip and in the batch culture respectively. Deviations from the exponential growth became significant at very high cell concentrations, typically only 3-10 times (depending on temperature and chambers size) below the plateau corresponding to a dense packing of the cells in the chambers (cf. Fig. 20). The range of exponential growth reached 3-4 orders of magnitude in cell concentration in colonies starting with 1-3 cells (Supplementary Fig. 7 online).

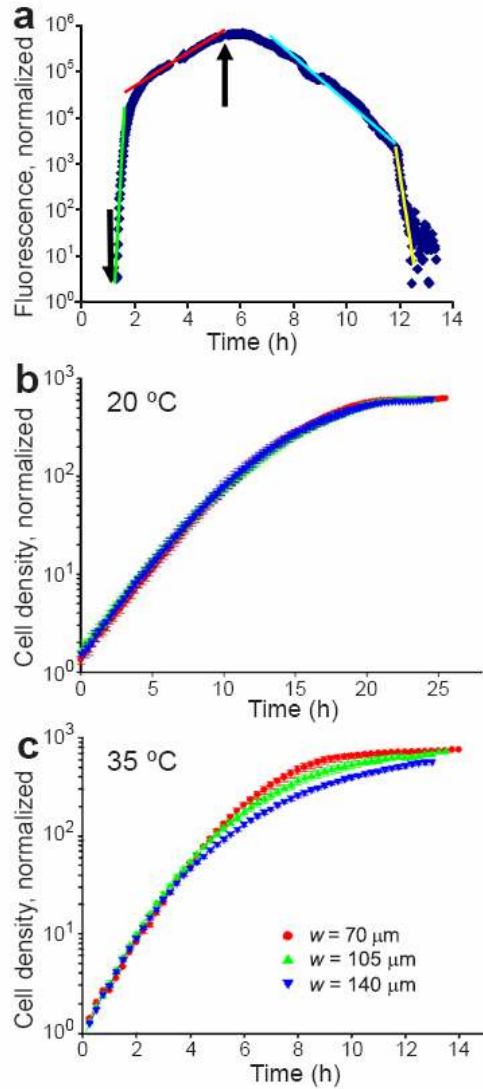


Figure 21. Response of cells to addition and removal of exogenous signal, and growth curves of colonies under different conditions. **(a)** A representative time dependence of the whole colony fluorescence intensity (in a $100 \times 200 \times 6 \mu\text{m}$ chamber) due to autoinducer-dependent Gfp(LVA) expression in response to addition (down arrow) and removal (up arrow) of 10 nM homo-serine-lactone to the medium fed to the channels. The straight lines indicate exponential curves fitted to the data set. The quantitative parameters determined from fitting are given in the text. **(b)-(c)** Study of the random and systematic variations in colony growth in chambers of different sizes at 20 °C and 35 °C. The chambers had dimensions $w \times 100 \times 5 \mu\text{m}$. To plot the curves, recorded fluorescence intensities were normalized to represent cell density by division by chamber volume to compare chambers of different size. The cell density of approximately 1 was taken to be the lowest initial cell density. To account for different initial number of cells in the chambers and thus compare proliferation rates at the same cell density, the curves were shifted along the time axis to the point where their early time segments overlapped. The initial numbers of cells in the chambers varied from 15 to 30. The averaging was done over 12 chambers on three chips for all sizes at 20 °C, and over 7, 6 and 6 chambers on three chips for $w = 70, 105$ and $140 \mu\text{m}$, respectively, at 35 °C. Error bars are s.e.m.

Fluorescence in the plateau regions was highly uniform throughout the chambers, suggesting uniform AI access even at highest cell densities.

Variability in growth rates (and, ultimately, colony sizes) between different equally-sized chambers on the same chip and different chips was similar: 5-10% at 20 °C and 25-30% at 35 °C (Supplementary Fig. 6 online). At 20 °C, the growth curves were also highly consistent for chambers of all three widths (Fig. 21b). The exchange of chemicals between the chambers and channels occurs through capillaries by diffusion and its efficiency decreases with w . Therefore, compromised medium conditions anywhere in the 70 and 105 μm chambers would imply even less favorable cell environments in significant portions of the 140 μm chambers, resulting in slower colony growth for the 140 μm chambers. Hence the overlapping curves in Fig. 21b present a strong evidence for essentially chemostatic conditions throughout the 70 and 105 μm wide chambers at 20 °C at all cell densities. The shape of the growth curve for the 70 μm chambers at 35 °C (Fig. 21c) is the same as at 20 °C (up to rescaling of the growth rates, Fig. 21b), suggesting that the conditions remain largely chemostatic there as well. As the chamber width increases, the colony growth rates at 35 °C systematically decrease, as colonies' sizes approach the capacities of the chambers (Fig. 21c). This result suggests that due to high intrinsic rates of metabolism and, possibly, reduced diffusion rates in dense colonies growing at 35 °C, chemostasis may be somewhat perturbed in chambers with $w > 70 \mu\text{m}$. Nevertheless, chemostasis is quite robust in narrow chambers, at room temperature and at lower cell densities.

Discussion

Functionality of the proposed chemostat is somewhat similar to that of the device described in Ref.⁶⁸, assembled of two PDMS parts with a sandwiched porous membrane and a cover glass. Nevertheless, the proposed chemostat has a few important advantages. The whole functional area (Fig. 19a-c) is made of a single cast of PDMS sealed with a cover glass (no assembly required) and can be readily integrated with other microfluidic elements. The chemostat permits growing high density bacterial and yeast colonies with large numbers of cells in chambers matching the field of view and working distance of the standard high resolution objective lenses, and loading cells into the chambers is simple and reliable.

The architecture of the micro-chemostat described here resembles complex structures of bacterial and yeast biofilms, where cells often grow in clumps separated by ‘channels’ supplying nutrients and removing waste. The dimensions of the chambers are similar to the size of the microcolonies found in some biofilms. This device therefore allows analysis of development of bacterial and yeast species in the environment resembling naturally occurring high density colonies. Additionally, the single cell resolution achieved on the chip can allow analysis of the effects of noise in gene expression^{46, 74, 75}, the onset of ageing⁷⁶, appearance of spontaneous mutations or the segregation of a population into particular subpopulations (e.g. sporulation⁷⁷). The flexible design allows control of multiple parameters regulating colony growth and development, including the chambers size (and thus the maximal size of the colonies) and temperature. Devices with similar design can be used for experiments with other non-adherent cell types, including mammalian cells, such as cells of hematopoietic origin.

Methods

Device fabrication. The microchannel device was made of two-layers of polydimethylsiloxane (PDMS) (Sylgard 184 by Dow Corning) using soft lithography⁷⁸ and sealed to a #1.5 microscope cover glass (Fig. 19). The master mold for the first layer (for cells and medium) was a silicon wafer with a two-level relief with heights of 0.65 and 6 μm (Supplementary Fig. 1 online). The mold was spin-coated with about 150 μm thick layer of PDMS prepolymer (Fig. 19d), which was then cured at 80°. The master mold for the second, water circulation layer had 300 μm thick relief. It was used to make a ~5 mm thick PDMS cast that was cut into individual chips. The chips were aligned and placed on top of the 150 μm thick PDMS layer on the first wafer, which was pre-coated by ~15 μm thick layer of partially cured PDMS for bonding. After curing, monolith two-layer PDMS chips were formed¹. (See **Supplementary Methods** online for more details). After use the PDMS chips were separated from the glass, washed in a mild detergent and recycled.

Flow driving. Working liquids (the media and cell suspensions) were kept in 30 ml plastic syringes connected to the microfluidic devices by Ø0.5 mm tygon tubing. The flow was driven by pressure differences between the inlets and outlets. The syringes were held upright attached to platforms sliding on vertical rails to generate the hydrostatic pressure²⁹. The syringes could be either opened to the atmosphere or filled with compressed air with pressure in a range from 0 to 12 psi.

Microscopy and Image Analysis. Fluorescence, bright-field, and phase-contrast micrographs were captured at 20×, 40× and 60× magnification with a Nikon Eclipse TE2000 epi-fluorescence microscope equipped with electronic shutters and a Spot RT camera. The rate of colony growth was studied taking series of fluorescence images of

the same chamber at fixed time intervals (usually about 3 min) and with a constant exposure. We assumed that the number of cells is proportional to the total fluorescence of the area. The latter was calculated by summing the intensity values for all the pixels in the image of the chamber and subtracting the background level. See **Supplementary Methods** online for description of bacterial and yeast species used and the construction of the plasmid.

Chapter 2.2.1, in full, is a reprint of the journal article *A microfluidic chemostat for experiments with bacteria and yeast* in Nature Methods, 2005. Groisman, A.; Lobo, C.; Cho, H.; Campbell J.K.; Dufour, Y.S.; Stevens, A.M.; Levchenko, A., Nature Publishing Group, 2005. Dissertation author was a participating investigator of this paper.

3. OPTOFLUIDICS

Optofluidics refers to the merger of optical systems with microfluidics. These optical systems can include lasers, as well as optical detection, plasmonic and optoelectronics setups. Using fluids with these systems gives a higher degree of flexibility and adaptivity compared with solid materials. Optical properties of the medium, such as refractive index and absorption, can be changed by replacing one fluid with another. Also, in traditional microfluidics, most optical support elements are located off-chip, whereas optofluidics helps integrate those elements to have a more compact stand-alone device. Another type of optofluidics are pneumatically and hydraulically driven optical components, such as sensors, scanners, actuators, lenses and other light-manipulators. By creating new techniques with the use of optofluidics, we can increase the functionality of existing machines by utilizing the available adaptation of the optofluidic devices. Three projects presented in this thesis are motivated by this general idea.

The first project is a microfluidic 2x2 optical switch. The device makes use of total internal reflection (TIR) and refraction index matching to switch between a transmission state and reflection state. The difference of indices between PDMS ($n=1.41$) and water (1.33) allow for TIR at angles greater than 70 degrees to the normal. By filling a wide flat-parallel PDMS channel with water, a laser beam can be reflected off the PDMS-water interface for a reflection state. The transmission state is accomplished by filling the channel with a concentrated aqueous salt solution that matches the index of PDMS. PDMS valves allowed us to control which liquid is in the channel and switch between the two states by replacing the liquids within about 20 ms.

The second project uses high flexibility, durability, and good optical properties of PDMS elastomer for two adaptive optical applications. We created a new type of composite material, consisting of a PDMS membrane with hard epoxy pieces grafted in it. By lithographically defining the shape and orientation of these pieces, we can condition and adjust the bending and stretching modes of the membrane when pressure is applied to it. One device focuses stretching to a specific region of the membrane. Another device causes rotation of the central region.

The third project is an adaptive lens made from a thin (1.5mm – 2mm) PDMS membrane affixed to a circular (doughnut shaped) mount. Application of vacuum to the mount causes the membrane to bend inwards and act as a negative meniscus lens. This lens is adjustable from 0 to -4 diopters and has a response time in the millisecond range.

3.1 A Microfluidic 2x2 Optical Switch

Optical switching devices play an important role in modern telecommunication networks providing unique functionalities such as optical add/drop, optical cross connect, and ring protection. A variety of switching devices based on electro-optic, thermo-optic, mechanical, acousto-optic and magneto-optic modulation techniques have been developed during past years⁷⁹⁻⁸³. A few designs used the effect of total internal reflection (TIR) and had crossing regions between coupled waveguides filled with index matching liquids^{84, 85}. A gas bubble with the liquid-air interface acting as a mirror was either driven into the crossing region by a thermo-capillary force⁸⁴ or created in it using the inkjet technology⁸⁵.

In this manuscript we demonstrate a 2×2 all optical switch based on control of TIR by flowing two different mixable liquids through microscopic channels made of a transparent silicon elastomer. With the advent of soft lithography and rapid prototyping⁷⁸, the silicon elastomer Polydimethylsiloxane (PDMS) has become a material of choice for many microfluidic R&D and lab-on-a-chip applications. The devices made of PDMS are inexpensive and their fabrication does not require high-level facilities. PDMS has quite low Young's modulus that allows construction of adaptive lenses with thin flexible membranes^{86, 87}. The flexible elastomer membranes are also a key element in pressure-actuated micro-valves that can be integrated in the microfluidic devices^{1, 88}. A specific advantage of PDMS for a fluidic optical switch is its low index of refraction, $n_e = 1.41$, which can be easily matched by low viscosity, non-toxic aqueous salt solutions.

The device, shown schematically in Fig. 22 and 23, has two distinct layers of microchannels separated by thin flexible membranes in the regions where they overlap. The first, flow layer contains the main functional element of the device – a 10 mm long, 2 mm wide and 75 μm thick flat-parallel mirror channel. It can be filled with either pure water from inlet 1 or an index matching salt solution from inlet 2 (see Fig. 22). The channels connecting the inlets and the outlet with the mirror are 100 μm thick and 800 μm wide and have rounded shapes. They are completely sealed by the flexible membranes when a pressure of 15 psi is applied to the membranes through channels (control lines) in the second, control layer of the chip¹. The membranes serve as pressure-actuated integrated “push-up” valves⁸⁸. Three of the control lines are connected to the valves on the two inlet channels and the outlet channel (valves 1, 2 and 5). The fourth control line (simultaneously switching two membrane valves, 3 and 4) and the vent in the

flow layer serve the purpose of purging the dead volumes between the two inlet valves (1 and 2) and the T-junction connecting the inlets with the mirror channel (Fig. 22).

The liquids were held in 60 cc plastic syringes, mounted vertically and connected to the elastomer chip by flexible tygon tubing with an inner diameter of 1 mm. The outlet and vent syringes were open to the atmosphere, while the syringes feeding the inlets were sealed and pressurized to 6 psi by compressed air. The pressure in tubing connected to the control lines was switched from zero to 15 psi using a manifold of solenoid valves with a response time of about 4 ms. The valves were driven by a home made controller interfaced with a PC through a National Instruments DAQ card.

All the membrane valves were normally pressurized and closed, and there was no flow in the channels. The optical switching was performed by filling the mirror channel with a liquid having a different index of refraction. If, for example, the water had to be exchanged by the salt solution, valves 2-5 were opened simultaneously (by switching pressure in the corresponding control lines to zero) to start flow from inlet 2 toward the outlet and the vent. The valves 2, 3 and 4 were closed after 100 ms, a time interval corresponding to a liquid discharge of about 4 mirror channel volumes (7 μ l) in the flow from inlet 2 to the outlet. Closing valve 5 was delayed by 20 ms to allow pressure relief in the mirror channel.

The condition of TIR at the interface between the PDMS elastomer with a refraction index $n_e \approx 1.41$ and deionized water with $n_w \approx 1.33$ is met at angles of incidence larger than 68.3° . Therefore the microfluidic chips were made with optically flat, clear facets at angles of 75° to the plane of the channels on both sides (see Fig. 23), and the incident beams were directed normally to the facets, Fig. 23c. The index

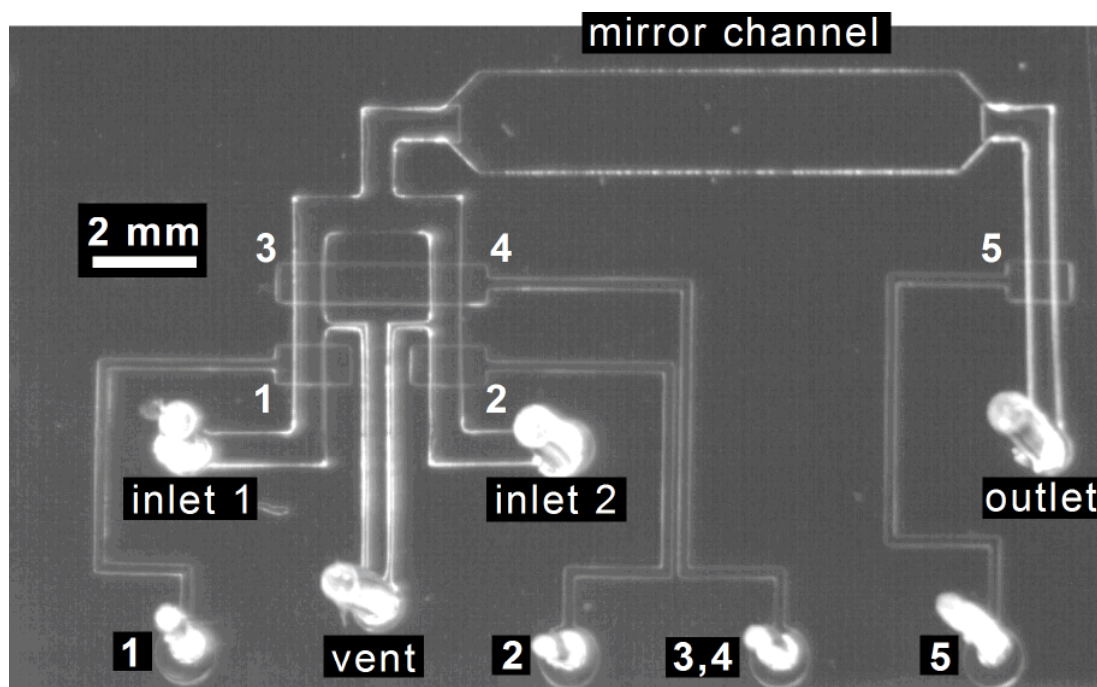


Figure 22. A microphotograph of the switch device. Control layer inlets are labeled by numbers of the membrane valves they actuate. Rounded channels can be clearly distinguished from rectangular ones (the mirror channel and channels in the control layer).

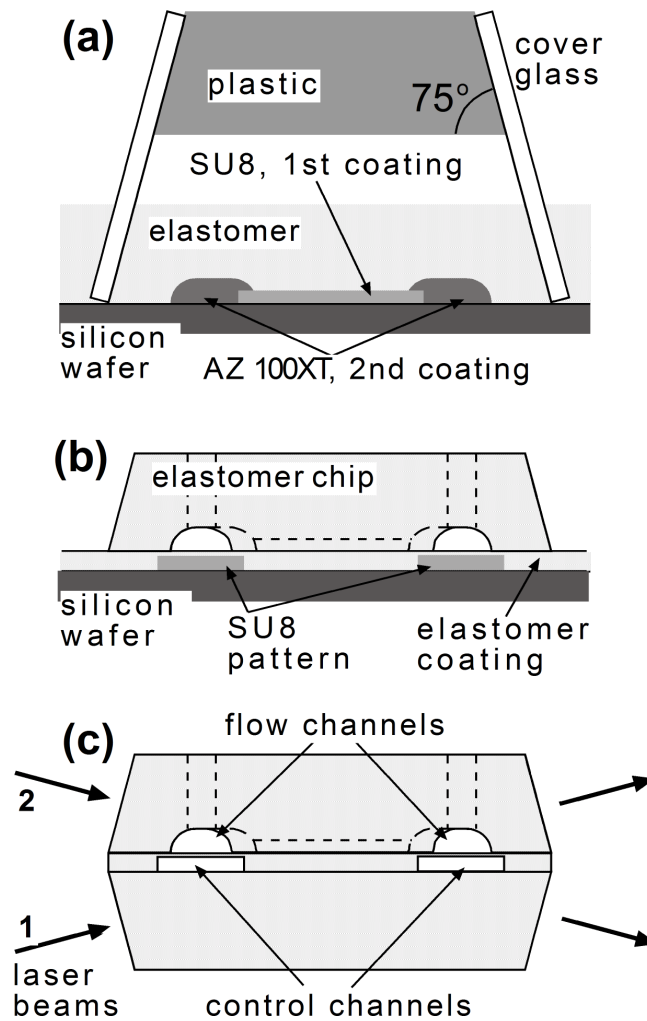


Figure 23. Schematic drawings showing construction and consecutive stages of fabrication of the switch. (a) The flow layer master mold with the PDMS cast and facet forming structure on top of it. (b) PDMS chip with engraved flow layer channels aligned on top of the control layer mold coated with a thin layer of the elastomer. (c) The complete 3-layer elastomer chip with 4 optical quality facets. The arrows on the left show directions of incident laser beams 1 and 2, and the arrows on the right show transmitted and reflected beams.

matching liquid was a solution of about 35% KI and 15% NaBr (by weight) in water. Its viscosity was nearly as low as the viscosity of water (10^{-3} Pa·s) allowing high flow rate and fast switching at moderate driving pressures.

The device was made using the technique of soft lithography and was assembled from 3 layers of PDMS (see Fig. 23). Two of the layers had microchannel relief engraved on their surfaces (flow and control channels), and they were cast using two different master molds. The molds were fabricated by the regular near-UV contact photolithography using 8,000 dpi resolution masks designed to implement the desired geometry of the molds. To make the master mold for the flow layer, a 4" silicon wafer was first spin coated with 75 μm thick layer of a negative photoresist (SU8-2050 by MicroChem) and patterned through a photomask. That produced flat parallel relief to cast the mirror channels. Next, the wafer was coated with about 100 μm layer of a positive resist, AZ100 XT by Clariant. It was patterned through another photomask (aligned with respect to the 75 μm SU8 layer), and baked on a 140° hot plate for 30 min after development to round the AZ 100XT relief^{1, 88} (see Fig. 23a). The mold for the control channels was made by spin coating another 4" wafer with a 100 μm thick layer of SU8-2050 resist and patterning it through a third photomask.

The resin and catalyst parts of the silicon elastomer (Sylgard 184 by Dow Corning) were mixed in a proportion of 5:1 and poured onto the first mold to a depth of 5 mm. In order to make the flat 75° facets and assure optical quality of the surfaces we used trapezoidal plastic bars with silanized cover glasses glued to their sides (see Fig. 23a). The cover glasses were immersed into the liquid elastomer and the construct was baked for 25 minutes in an 80 °C oven to partially cure the elastomer. The elastomer was then

separated from the glasses and the wafer, cut into individual chips, and holes were punched in the chips with a gauge 16 luer stub to make ports for the flow layer.

In parallel the second mold was spin coated with a ~ 160 μm thick layer of elastomer (20:1 mixture of the resin and catalyst). The elastomer was partially cured in the oven, and the chips were placed on top of it aligned with the photoresist relief on the mold^{1, 88} (see Fig. 23b). After 2 hours of baking in the oven a monolith of completely cured elastomer with integrated valves was formed. The elastomer was cut into individual chips and holes were punched in the chips with a gauge 20 luer stub to make ports for the control layer. The control channels on the surface of the chips were sealed by ~ 5 mm thick pieces of the elastomer with flat surfaces and the same trapezoidal profile with 75° facets (see Fig. 23c), which were made using a procedure similar to that described above. (Both surfaces were treated with Oxygen plasma for improved bonding.) The complete 3-layer device had a hexagonal profile with the microchannels near the mid-plane. It was optically clear and the interfaces between different layers were not detectable by eye.

Characterization of the device was performed using a 2 mm collimated beam derived from a single mode HeNe laser source ($\lambda=632.8$ nm). The laser beam was directed to the mirror channel (see Fig. 22) either from above (beam 1, Fig. 23c) or below (beam 2, Fig. 23c) normally to one of the facets. Thus, the angle of incidence on the surface of the mirror channel was 75° for both beams. The channel acted as a mirror (exchange mode) and a transparent window (bypass mode) when it was filled with water and with the index matching solution, respectively.

Images of the transmitted (bypass) and the reflected (exchange) beams (captured with a Cohu CCD camera) are shown in Fig. 24. The mode of the beam in the bypass

state of the switch is characterized by a high quality Gaussian shape (see Fig. 24a). In the exchange state the mode is somewhat elliptical, which is probably due to a slight curvature of the PDMS-microchannel boundaries. The beam quality could be improved by using a more elaborate fabrication procedure and PDMS with a higher Young's modulus⁸⁹. Power of transmitted and reflected beams was measured with a Newport 818-SL power meter. A linear polarizer was placed in front of the device to explore its performance for two orthogonal states of polarization. Results of the measurements for the beams 1 and 2 coming from below and above (see Fig. 23c), the two orthogonal linear polarization states and the two switching states are summarized in Table 2 as values of insertion loss and extinction ratio in dB. The latter was defined as the ratio between the powers of the reflected (transmitted) and the incident beam in the bypass (exchange) mode. The TE polarization corresponds to the electric field orthogonal to the plane of incidence.

The cross talk of the device in the bypass state strongly depends on exact matching of refractive indices of PDMS and the salt solution. At the incidence angle of 75° the measured extinction ratio of 20 dB (see Table 2) in the bypass state for TE polarization corresponds to an index mismatch of about 0.001. An extinction ratio of ~45 dB, which is a standard requirement for modern telecommunication switches, should be reached by reducing the index mismatch down to 0.0001. The extinction ratio in the exchange state is mainly limited by the small width of the mirror channel (2 mm) and can be further improved by better collimation of the beam.

Part of the insertion loss, about 0.3 dB, is due to reflections from the two PDMS-air interfaces and can be eliminated by AR coating the facets. The insertion loss inside

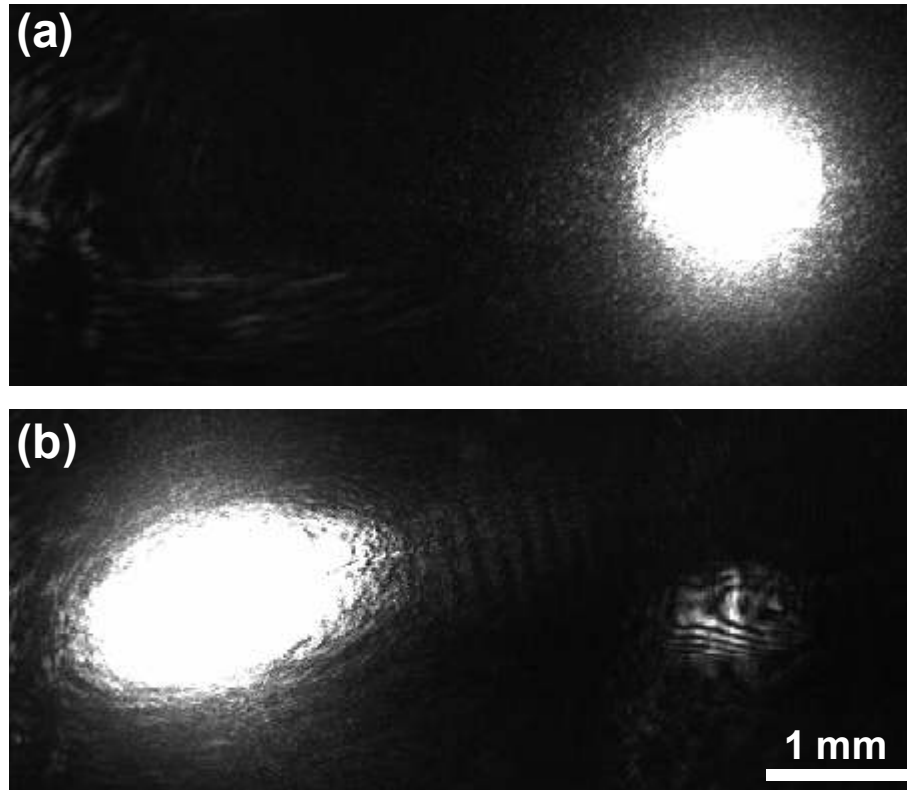


Figure 24. Images of reflected (left) and transmitted (right) beams (after passing the switch) formed on a CCD array in the bypass mode (a) and exchange mode (b).

the device is $\sim 0.4\text{-}0.8$ dB, which is comparable with state of the art optical switching components. The polarization dependent loss (PDL) is on the order of $0.1\text{-}0.3$ dB. Those numbers could be reduced by improving the index matching, quality of the mirror channel surface and by increasing the width of the mirror.

We tested dynamic performance of the device by switching it periodically and measuring power of the beams with fast Silicon photodetectors (Thorlabs 201/579-7227) connected to a digitizing Tektronix oscilloscope. A representative time series is shown in Fig. 25. The transition from bypass to exchange state occurred within about 20 ms (based on 10%-90% criterion), while the transition from exchange to bypass took less than 10 ms. This speed meets current standards for most switching applications (e.g. protection and routing). The switching speed is mainly limited by the rate of flow in the microchannels and can be further increased by applying higher pressures and increasing channel depths.

The demonstrated optical switch is a free space device with the width of the mirror (2 mm) sufficient for even non-collimated laser beams. Since it is based on one-phase flow of two mixable liquids, its size can be varied without subtle effects on its performance. Those are two main advantages of this device compared with the bubble photonic switches implemented in waveguides⁸⁵. In addition, the device fabrication is based on the soft lithography replication process, which is expected to reduce cost per unit, making this switching technology appealing for low-end applications. The switch can also be integrated with other microfluidic devices for lab-on-a-chip applications.

This research has been partially supported by DARPA, NSF, and AFOSR.

Table 2. Insertion loss and extinction ratio (in dB) for transmitted and reflected beams in the two states of the switch and for two inputs at TE and TM.

	Bypass TE, dB		Exchange TE, dB		Bypass TM, dB		Exchange TM, dB	
Laser beam	1	2	1	2	1	2	1	2
Insertion Loss	0.78	0.84	1.1	1.1	0.71	0.67	0.83	0.78
Extinction ratio	22.7	24.4	18.2	17.7	17.8	22.5	21.7	16.7

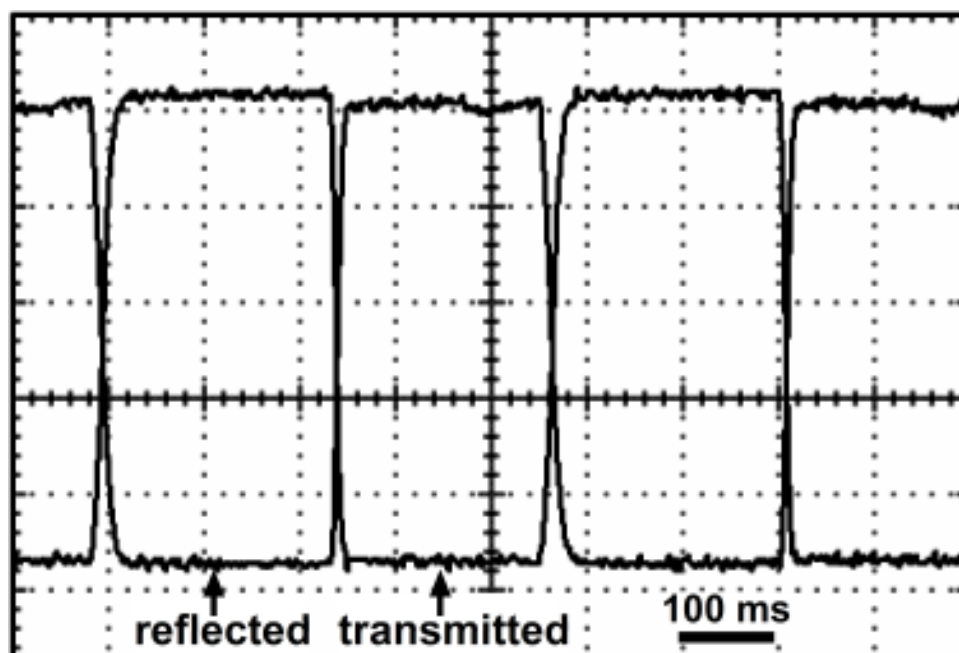


Figure 25. Power of transmitted and reflected beams as a function of time measured by fast photodetectors and visualized with an oscilloscope during a series of periodic switching events.

Chapter 3.1, in full, is a reprint of the journal article *A microfluidic 2x2 optical switch* in Applied Physics Letters, 2004. Campbell, K.; Groisman, A.; Levy, U.; Pang, L.; Mookherjea, S.; Psaltis, D.; Fainman, Y., American Institute of Physics, 2004. Dissertation author was the first author and a leading contributor of this paper.

3.2 Pressure-driven Devices with Lithographically Fabricated Composite Epoxy-Elastomer Membranes

Thin flexible membranes made of a silicon elastomer polydimethylsiloxane (PDMS) have been increasingly used in microfabricated devices to produce pressure actuated valves^{1, 88, 90}, check valves^{91, 92} and adaptive lenses⁸⁷. The manner of deformation of a plain membrane under pressure is defined by the shape of the frame it is attached to, allowing for very limited tunability. Here we describe a technique for fabrication of composite membranes with pieces of rigid UV-cured epoxy grafted inside PDMS. The dimensions and positions of the epoxy parts are defined with a high precision by UV-lithography. Patterning the soft membrane with the rigid epoxy makes its mode of deformation under pressure highly adjustable. To demonstrate applications of the proposed technique we designed, fabricated and tested two devices that we call stretcher and rotator (Fig. 26). In the stretcher, the grafted pieces of epoxy focus the pressure-induced extension of the membrane to a thin strip of PDMS. In the rotator, the epoxy patterning causes in-plane rotation of a central area of the membrane when pressure is applied.

Both the stretcher and the rotator consist of ~ 750 μm thick composite PDMS-epoxy membranes (with ~ 200 μm thick pieces of epoxy grafted in them) bonded to identical ~ 5 mm thick PDMS supports, which have rectangular openings in the middle and 1 mm thick glass windows glued to their back sides (Fig. 26). The side of a PDMS support bonded to the membrane has a ~ 100 μm deep lithographically defined indentation around the opening. The indentation has a shape of a rectangle with rounded corners. The epoxy elements in the two composite membranes consist of identical

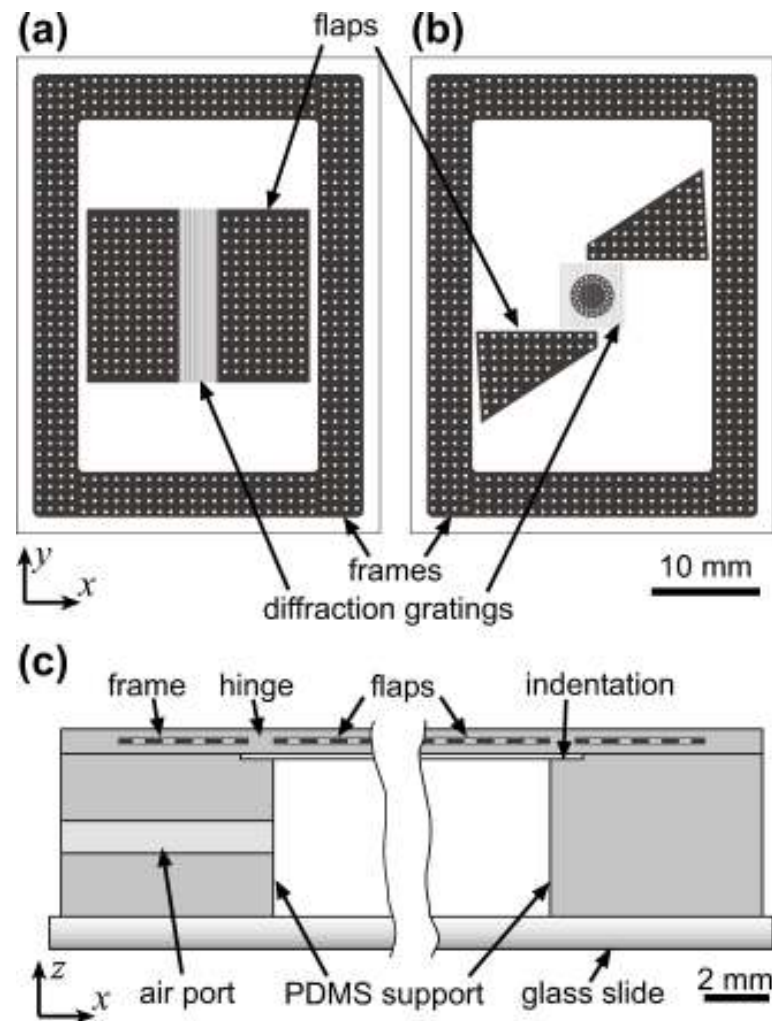


Figure 26. Composite membrane devices, the stretcher and rotator. (a) and (b) Schematic drawings of the composite membranes in the stretcher and rotator, respectively. Dark areas correspond to epoxy parts inside the membranes. (c) Schematic drawing showing a cross-section of a device.

rectangular frames with rounded corners and different parts inside the frames. Both inner dimensions of the frames, $a = 22$ mm and $b = 32.4$ mm, are 0.5 mm smaller than the corresponding dimensions of the indentations in the supports, and the frames are aligned concentrically with the indentations. Therefore, the edges of the contact area between a frame and a support are set by boundaries of the indentation, whereas the edges of the flexible membrane in the interior of the frame are set by the lithographically defined epoxy frame (Fig. 26c). All epoxy elements are perforated, and the holes are filled with PDMS to ensure grafting of epoxy with PDMS. The air pressure in the cavity between the membrane and the glass is adjusted through a via in a side wall of the support.

Fabrication of the composite membranes (Fig. 27) started from coating a silicon wafer with a 0.6 μm thick layer of UV-curable epoxy SU8 and patterning it through a photomask to produce a diffraction grating relief (Fig. 27 b,c). The wafer was coated by a ~ 200 μm layer of PDMS (GE RTV615; 25:1 mixture of parts A and B; Fig. 27c) that was cured and then spin-coated by a ~ 200 μm layer of SU8 epoxy (Fig. 27 d). The SU8 layer was exposed to UV-light through another photomask and developed to generate the rigid epoxy elements of the composite membranes with lithographically defined dimensions and positions (Fig. 27e). Next, the wafer was coated with another layer of PDMS that was cured generating a flat-parallel composite membrane with a total thickness of ~ 750 μm (Fig. 27f). To complete a device, the membrane was bonded to a PDMS support (Fig. 27g), separated from the wafer, and the support was bonded to a glass slide (Fig. 27h).

The stretcher (Fig. 26a) has two rectangular epoxy flaps separated from the frame by 750 μm wide strips of PDMS, which act as flexible hinges. When the interior of the device is pressurized and the membrane is inflated, the flaps are lifted by turning about

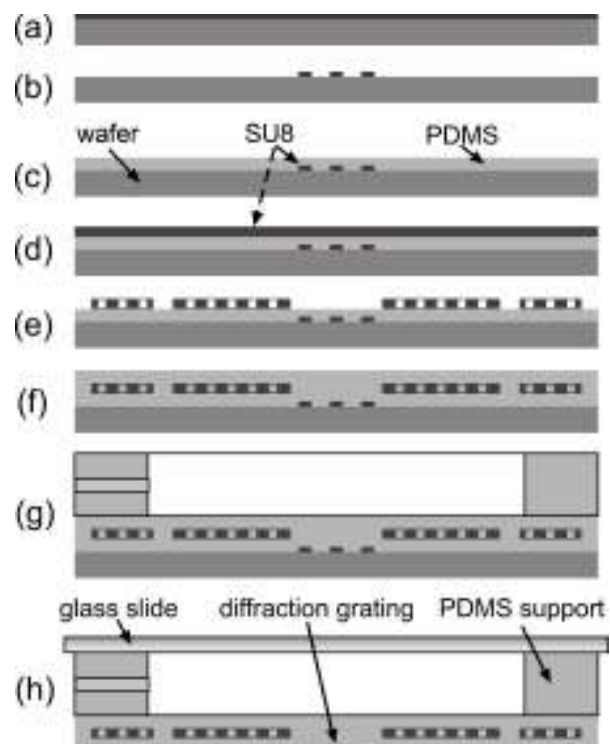


Figure 27. Fabrication of a composite membrane device is shown step-by-step from top to bottom.

the edges of the frame. The Young's modulus of the SU8 epoxy (4-5 GPa⁹³) is about four orders of magnitude higher than the Young's modulus of PDMS in the membrane (measured at 0.3 MPa). Thus, a substantial part of the membrane extension occurs in the narrow central strip of PDMS between the flaps with a diffraction grating engraved on its surface (Fig. 26a) that has a width $x_0 = 3.5$ mm when the device is not pressurized. The composite membrane in the rotator (Fig. 26b) has a circular piece of epoxy in the middle and two trapezoidal flaps adjacent to the frame. The epoxy patterning has a central but no axial symmetry. When the membrane is inflated and the flaps are lifted, the tips of the flaps apply a torque to the circle, making it turn in the plane of the membrane.

To evaluate the extension of the central PDMS strip in the stretcher and rotation of the central area of the membrane (with the epoxy circle in it) in the rotator, we looked at diffraction of light in the diffraction gratings engraved on the surface of PDMS (Fig. 26 a,b). Both gratings had ~ 12.5 μm wide 0.6 μm deep groves parallel to the longer side of the frame and a period of 25 μm . They were illuminated with a ~ 1 mm wide 632 nm laser beam (derived from a HeNe laser) through the glass windows on the back sides of the devices. Application of air pressure to the stretcher caused reduction of the distance between maxima of diffraction (Fig. 28a-c), whereas the pressure applied to the rotator caused rotation of the whole diffraction pattern without its appreciable deformation (Fig. 28d-f).

The period of the grating and the distance between diffraction maxima at a given pressure, d and X , respectively, are connected to the grating period and the distance between diffraction maxima at zero pressure, d_0 and X_0 , by the equation $d/d_0 = X_0/X$. We measured X at various air pressures, P , and calculated the relative

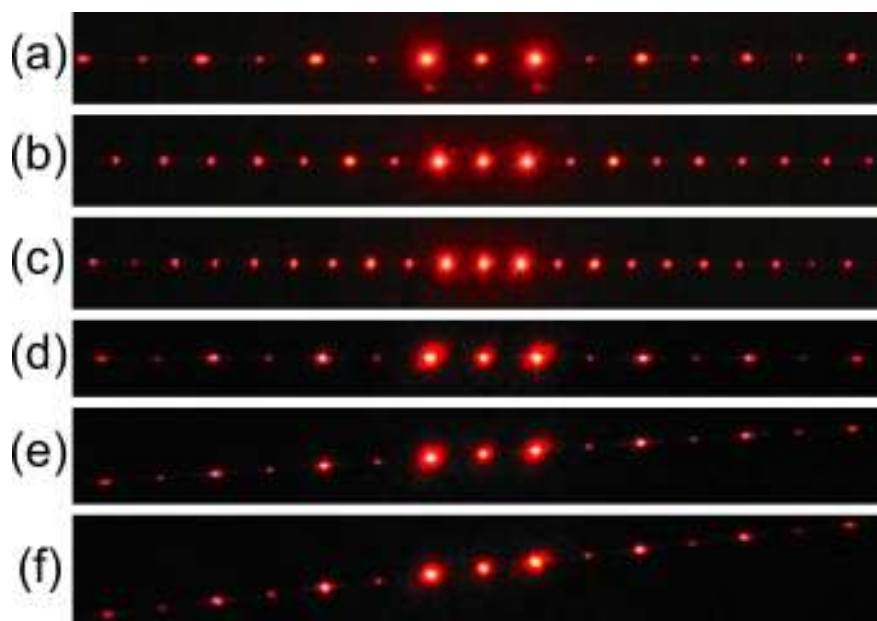


Figure 28. Photographs of diffraction patterns generated at different pressures, P , by the diffraction gratings engraved in the membranes of the stretcher, (a)-(c), and of the rotator, (d)-(f). The pressures are: (a) 0; (b) 0.75 psi; (c) 1.5 psi; (d) 0; (e) 0.75 psi; (f) 1.5 psi.

extension of the central PDMS strip in the stretcher as $\Delta x/x_0 = X_0/X - 1$. Elevation, h , of the center of the membrane as a function of P was measured in a separate test. The diffraction grating in the center of the membrane was inspected under a darkfield microscope that had a mechanical stage equipped with a precise gauge of its vertical (z -) position. Dependence of $\Delta x/x_0$ on h is shown in Fig. 29 along with the corresponding dependence for a plain ~ 750 μm thick PDMS membrane attached to the same support.

Because of the flexibility of the epoxy flaps (which are 8.5 mm wide and only 0.2 mm thick; Fig. 26), the xz -plane profile of the inflated composite membrane was close to an arc with a chord of a constant length, $l_0 = a = 22$ mm. The radius of the arc can be estimated from $r^2 = (l_0/2)^2 + (r-h)^2$ as $r = (l_0^2/4 + h^2)/(2h)$, and the contour length of the inflated membrane in the xz -plane can be estimated as $l = 2r \sin^{-1}(l_0/2r)$. The ratio between Δx and $\Delta l = l - l_0$ found at different P was consistently at 0.44 ± 0.01 , suggesting that the 3.5 mm wide central strip of PDMS contributed $\sim 44\%$ of the total extension of the membrane. The rest of the membrane extension originated from elongation of the strips connecting the flaps with the frame and from slippage of PDMS along the flaps and the frame. The ratio of $\Delta x/x_0$ to the estimated relative extension of the whole membrane, $\Delta l/l_0$, at different P was consistently at 2.7 ± 0.1 . It was the same as the ratio between $\Delta x/x_0$ for the composite membrane and for the plain PDMS membrane at equal h (Fig. 29). The high values of the two ratios indicate that the introduction of the epoxy flaps leads to substantial enhancement of the membrane extension in the narrow central strip of PDMS.

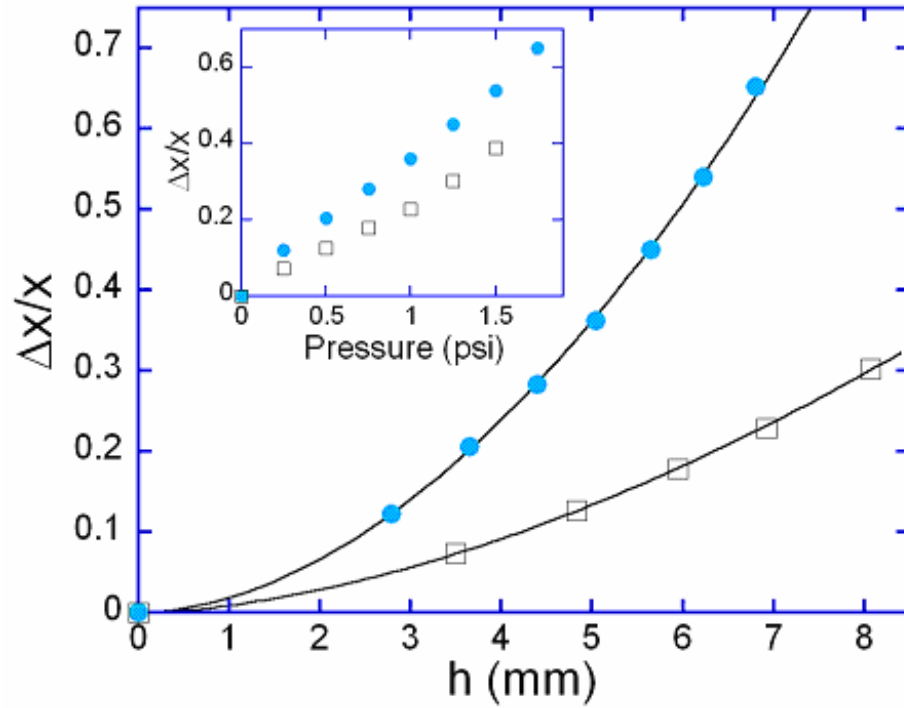


Figure 29. Relative extension of the central strips, $\Delta x / x_0$, as a function of elevation of the center of the membrane, h , for the stretcher (circles) and for the plain membrane device (squares). Error bars are smaller than symbols and are not shown. *Inset:* $\Delta x / x_0$ as a function of pressure, P , for the stretcher (circles) and for the plain membrane device (squares). Error bars are smaller than symbols and are not shown.

Dependence of $\Delta x/x_0$ on P in the composite membrane device was close to linear (inset in Fig. 29). In low pressure range ($P \leq 1$ psi), the ratio between $\Delta x/x_0$ measured at a given P for the composite and plain membranes was ~ 1.6 , indicating that the stretching of the central strip of PDMS in the composite membrane device was significantly more sensitive to pressure. To measure the uniformity of stretching of the central strip of PDMS, we analyzed patterns of diffraction from different regions at the central axis of the strip (the axis of symmetry of the composite membrane along the y -direction, Fig. 26a). The variations of $\Delta x/x_0$ along the middle 8 mm of the 14 mm strip were within 1% at 0.5 psi and within 2% at 1 psi ($\Delta x/x_0$ was always a maximum at the center). For the plain membrane, the corresponding variations of $\Delta x/x_0$ were $\sim 10\%$ at both pressures. We also examined the central strip of PDMS under the microscope to evaluate its curvature in the yz -plane (Fig. 26). At $P = 0.5$ psi the radius of curvature of the middle 8 mm of the strip was ~ 150 mm for the composite membrane and ~ 30 mm for the plain membrane. These last two results show that the epoxy flaps substantially improve the uniformity of extension the central strip of PDMS and render the extension substantially more unilateral.

The turning of the diffraction pattern generated by the rotator (Fig. 28 d-f) is a manifestation of in-plane rotation of the central area of the composite membrane with the epoxy circle (Fig. 26b). The pressure induced rotation in the plane of the membrane is not found in the case of the plain PDMS membrane device. Therefore, it is an essentially new effect introduced by the patterning of the membrane with the rigid epoxy parts. The dependence of the rotation angle, ϕ , on P was close to a power law (Fig. 30). The angle

reached 8° at $P = 2$ psi (and $h = 8$ mm). At $P = 1$ psi and $h = 5.6$ mm, φ was 5° , whereas the out-of-plane tilt of the central circle (measured under a microscope) was within $\sim 0.7^\circ$. The circle thus remained practically parallel to itself as it elevated and turned in the plane of the membrane. This type of motion of the circle was a consequence of a highly symmetric layout of the composite membrane with respect to its center (the center of the circle). The symmetry in the layout was enabled by the lithographic definition of the dimensions and positions of the frame, flaps and circle, as well as by high uniformity of the membrane thickness.

The response time of the devices to variations of P was measured at ~ 100 ms for the stretcher and at ~ 200 ms for the rotator. The durability of the devices was tested by putting them through $\sim 2 \times 10^5$ cycles of inflation and deflation at $P = 0.5$ psi and a frequency of 1 Hz, and measuring $\Delta x/x_0$ and φ as functions of P (for the stretcher and rotator, respectively) after the cycling. The dependences of $\Delta x/x_0$ and φ on P were found unchanged within the experimental error.

The UV-curable epoxy SU8 has been used before to produce micromechanical elements⁹⁴ as well as complex pressure-actuated devices with moving parts on flexible springs^{95, 96}. The proposed fabrication technique is different in that it permits lithographic definition not only of individual parts but also of their mutual arrangement in the final functional device, thus allowing building complex multi-part micromechanical systems without the need of assembly. The two devices constructed to demonstrate the usage of the composite membranes, the stretcher and rotator, have been shown to operate as

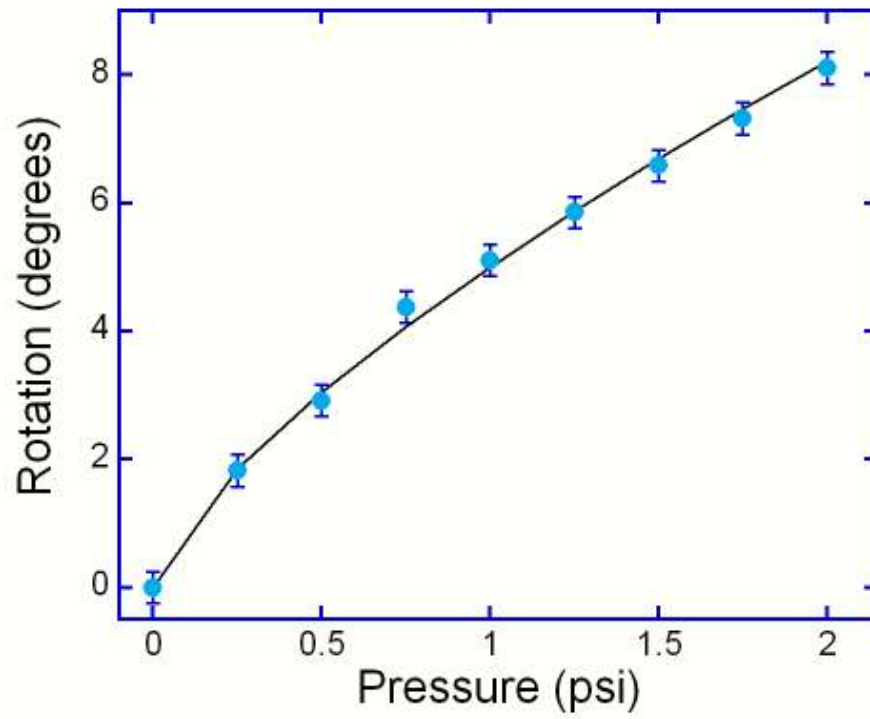


Figure 30. Angle of the in-plane rotation, φ , of the central circle of the composite membrane in the rotator as a function of pressure, P , applied to the device.

pressure-driven adaptive optical systems, modifying diffraction patterns of light. The performance of the devices can be enhanced by improving the adhesion between epoxy and PDMS, and the devices can be miniaturized by proportional reduction of dimensions of all their components. A prospective area of applications for the composite membranes is pressure-driven actuators.

Experimental

Fabrication of the supports. To produce the PDMS supports, first, a 5 in. silicon wafer was spin-coated with a 100 μm layer of a UV-curable epoxy (SU8-2050, Micro-Chem, Newton, MA) and exposed to UV-light through a photomask with transparent areas corresponding to the indentations in the supports (Fig. 26c). After development of the wafer, a $\sim 5\text{mm}$ layer of PDMS pre-polymer (GE RTV 615; 5:1 mixture of parts A and B) was poured onto it and partially cured by 45 min baking in an 80 $^{\circ}\text{C}$ oven. The PDMS cast was peeled off the wafer, cut into individual chips $\sim 34 \times 44$ mm in size, and rectangular openings were made within the indented areas of the chips (Fig. 26c). A support was completed by punching a ~ 1.5 mm via in its side wall.

Fabrication of the composite membranes. Fabrication of both composite membranes was started by spin-coating a 5 in. wafer with a 0.6 μm layer of the SU8 epoxy (SU8-2015 diluted to 18% resin content) and exposing it to UV-light through a photomask to produce a relief for engraving a diffraction grating on the surface of the membranes (Fig. 27a, b). PDMS in the membranes was a 25:1 mixture of parts A and B of RTV 615. The wafer was spin-coated with a ~ 200 μm layer of PDMS pre-polymer and was left to cure overnight at room temperature on a horizontal support to produce an even PDMS film (Fig. 27c). The curing was finished by 2 hrs baking in a 60 $^{\circ}\text{C}$ oven. The

cured PDMS was spin-coated with a $\sim 200\ \mu\text{m}$ layer of SU8-2100, exposed to UV-light through a photomask and developed that produced the epoxy patterning of the composite membranes (Fig. 26 and Fig. 27d, e). A measured amount of the PDMS pre-polymer corresponding to the total membrane thickness of $\sim 750\ \mu\text{m}$ was added onto the wafer and spread over its surface by spin-coating at 200 rpm. The wafer was put through several cycles of degassing and opening to the atmosphere to remove air bubbles trapped in the holes of the perforated epoxy parts and to ensure filling of the holes with PDMS (Fig. 27f). Next, the wafer was left overnight at room temperature on a horizontal support, to let the pre-polymer layer level off as it slowly cured and to produce a composite membrane with an even thickness and flat surface. The curing of PDMS was completed by 2 hrs baking in a $60\ ^\circ\text{C}$ oven. The adhesion between the UV-cured SU8 and the PDMS substrate was sufficiently strong to prevent any displacement of the epoxy parts with respect to the substrate during the SU8 development and the subsequent PDMS coating, degassing and curing. Therefore, the SU8 patterning of the composite membrane was an accurate replica of the pattern on the photomask.

Bonding a supports to a membrane. To bond the composite membrane to the supports, the wafer with the membrane was spin-coated with a $\sim 15\ \text{mm}$ layer of the PDMS pre-polymer that was partially cured by 45 min baking in an $80\ ^\circ\text{C}$ oven. The supports were placed on the wafer, aligned with respect to the epoxy frames (there were four supports and four frames on a wafer), and the wafer was baked for 2 hrs in an $80\ ^\circ\text{C}$ oven to completely cure PDMS (Fig. 27g). The membrane was cut along the perimeter of the supports, and the supports with the bonded membranes were peeled off from the wafer. Finally, back sides of the supports were dipped into a $20\ \mu\text{m}$ layer of PDMS pre-

polymer, placed on clean 1 mm thick glass slides cut to the size of the supports (Fig. 27h) and bonded to the slides by 2 hrs baking in an 80 °C oven.

Measuring device response times. To measure the response times of the stretcher and rotator, each of the devices was connected to a source of air pressure, $P = 1$ psi, through a solenoid valve with a switching time of ~ 4 ms. Extension of the central strip in the stretcher was assessed by measuring the intensity of the 0th maximum of diffraction with a photodetector. The intensity varied with the extension because of changing depth of the grooves in the diffraction grating. To record the rotation of the diffraction pattern generated by the rotator with a photodetector, the diffracted laser beams emerging from the device were expanded in the direction of rotation with a cylindrical lens, and a narrow slit perpendicular to the direction of rotation was put in front of the photodetector.

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Chapter 3.2, in full, is a reprint of the journal article *Pressure-driven devices with lithographically fabricated composite epoxy-elastomer membranes* in Applied Physics Letters, 2006. Campbell, K.; Levy, U.; Fainman, Y.; Groisman, A., American Institute of Physics, 2006. Dissertation author was the first author and a leading contributor of this paper.

3.3 Pneumatically Actuated Adaptive Lenses with Millisecond Response Time

Adjustment of focus is one of the most basic functionalities of imaging and scanning systems, which usually requires mechanical motion of some of the lenses or mirrors in the system. An alternative is a motionless lens with variable refractive power that can potentially enable more compact imaging systems with fewer parts and faster response. Functional adaptive lenses have been built using several different technologies, including liquid crystals (LC)^{97, 98}, flexible liquid-filled shells^{86, 87, 99}, and electrowetting^{100, 101}. The amplitude of the voltage dependent modulation of the wave front of light in LC is typically a few wavelengths. Therefore, adaptive LC lenses with large apertures are usually diffractive Fresnel lenses⁹⁷ with discrete sets of refractive powers and substantial chromatic aberrations.⁹⁸ The old technology of liquid-filled flexible shells⁹⁹ has been recently revived due to the wide use of elastomer materials.^{86, 87} Nevertheless, the liquid filling of the lenses could be a problem for their durability and thermal stability, and their readjustment time is usually relatively long. A recently introduced adaptive lens based on electrowetting¹⁰¹ has a wide range of refractive power and a response time of ~ 7 ms. However, its aperture is 1.6 mm only, and its response time is expected to increase as a power 1.5 of the aperture.¹⁰¹

Here we describe the design and operation of adaptive lenses with millisecond response time that are based on transparent flexible elastomer membranes affixed to circular mounts. For the purposes of this study, the membrane lenses were integrated in compound camera lenses that contained two more elements attached to the same mount: a plano-convex glass lens and a diaphragm between the membrane and the lens (Fig. 28a).

The mount was sealed by the membrane and the lens, and the pressure of air in it was adjusted through a connector on a side.

We built and tested two adaptive compound lenses. Lens 1 had a membrane radius $R_0 = 9$ mm, membrane thickness $h_0 = 2.06$ mm, diaphragm aperture $d = 5$ mm, and the glass lens focal length $f_0 = 24.5$ mm. Lens 2 had $R_0 = 6.0$ mm, $h_0 = 1.66$ mm, $d = 3.3$ mm, and $f_0 = 19$ mm. The flat-parallel membranes were fabricated out of polydimethylsiloxane (PDMS). Their optical flatness was <0.003 % rise/run, *i.e.*, <0.3 μm over 10 mm. The mount was made of acrylic and had concentric groves filled with PDMS to enhance the bonding with the membrane (Fig. 28a, b; see Supplemental Materials for more details).

Application of vacuum to the interior of the mount pulls the membrane inwards (Fig. 28b, c). The central area of the membrane acquires the shape of a meniscus lens (Fig. 28b, c) that can be approximated as a spherical shell with a radius, R , thickness, h_0 , and refractive index n ($n = 1.41$ for PDMS). At radius r (Fig. 28b), the extent of the bent membrane along the optical axis is $h(r) = h_0 R / \sqrt{R^2 - r^2}$. For $R \gg r$, it is $h(r) \approx h_0 / [1 - r^2 / (2R^2)] \approx h_0 [1 + r^2 / (2R^2)]$. As compared with the optical path along the central axis, the optical path at a small r is increased by $\Delta z \approx [h_0(n-1)/(2R^2)] \cdot r^2$, and for paraxial rays, the spherical shell acts as a negative lens with focal length $f_m \approx -[h_0(n-1)/(2R^2)]^{-1}$.

The shape of a deformed membrane (Fig. 28c) is well described by a model where the membrane is represented by a thin circular plate with clamped edges,¹⁰² and its z -axis

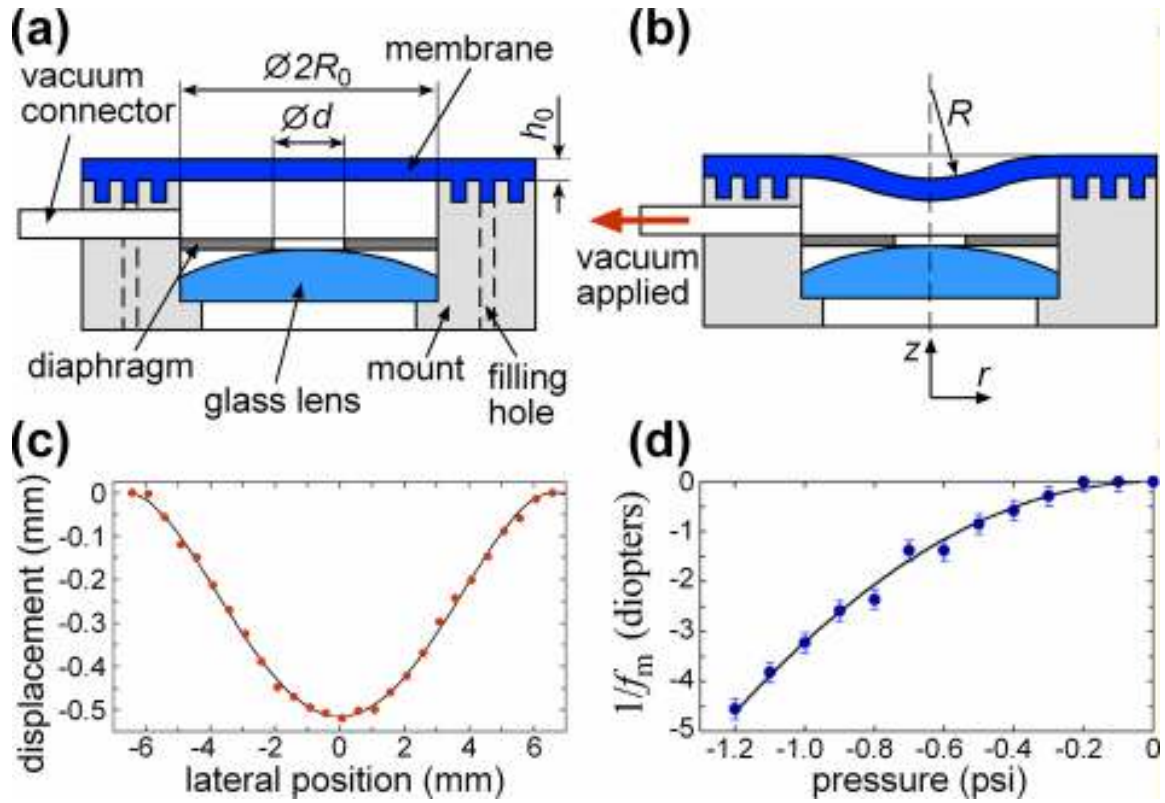


Figure 31. (a) Schematics of an adaptive compound camera lens with a flexible membrane. (b) The same camera lens with the membrane pulled inwards, when vacuum is applied. (c) Displacement of the membrane, ζ , in lens 2 at the gauge pressure of vacuum applied to the lens $P = -0.5$ psi as a function of lateral position with respect to the membrane center, fitted by equation $\zeta = -a(R_0^2 - r^2)^2$ with $R_0 = 6.6$ mm. (d) Refractive power, $1/f_m$, of the meniscus lens formed by the membrane in lens 2 as a function of P .

displacement, ζ , is given by a fourth order polynomial equation, $\zeta(r) = [(R_0^2 - r^2)^2 / (64D)] \cdot P$, where P is the gauge pressure inside the mount and D is the bending modulus of the membrane. In this model, the central area of the membrane has a curvature $1/R = [R_0^2 / (16D)] \cdot P$ and a refractive power $1/f_m = -[h_0(n-1)R_0^4 / (512D^2)] \cdot P^2$. The deviations of Δz from the parabolic dependence, $\Delta z \propto r^2$, become appreciable at $r > R_0/4$ (see Supplemental Materials). Therefore, to reduce spherical aberrations, it was important to use the diaphragms.

We measured the dependences of the refractive power of the compound lenses, $1/f$, on P by imaging an array of narrow strips onto a CCD camera, and calculated the refractive power of the membranes as $1/f_m(P) = 1/f(P) - 1/f_0$. The dependence of $1/f_m$ on P measured for lens 2 (Fig. 28d) was well fitted by a parabolic curve $1/f_m \propto P^2$, as predicted by the model. A refractive power of -4 diopter (dpt) was reached at $P = -1.0$ and -1.1 psi for lens 1 and 2, respectively. The resolution of the lenses was measured by attaching them to a CCD camera and imaging arrays of bright and dark strips of equal width with different periods. The linear resolution was defined as the width of the strips, at which the array visibility (peak-to-peak divided by twice the mean) was reduced to 50%. The resolutions of lenses 1 and 2 corresponded to 2 and 3 arc min, respectively, and remained unchanged in the entire tested range of $1/f_m$ from 0 to -4 dpt for both lenses. Therefore, the variation of the refractive power of the membrane lenses did not cause deterioration of resolution of either of the compound lenses.

To test the response of the lenses to rapid variations of pressure, we used 3-way solenoid valves with their two inputs connected to different sources of vacuum (or pressure) and their common ports connected to the lenses. Lens 1 was tested with a low flow resistance valve (MHE3, by Festo AG), and lens 2 was usually tested with a relatively high flow resistance valve that had a shorter response time (two LHDA valves connected in parallel driven with a Spike&Hold circuit by Lee Company, Westbrook, CT).

In the first set of tests (Fig. 29a), a HeNe laser beam was directed onto an edge of a semiconductor photo-detector through a lens placed at an angle to the beam. Variation of the refractive power of the lens changed the direction of the beam and its footprint on the detector, thus changing the detector output. When lens 1 was connected to the valve through a wide tube with a low flow resistance (Fig. 29b), the valve switching resulted in fast transitions that were followed by decaying oscillations in the detector signal (ringing). The shortest transition time with suppression of the oscillations was achieved by tuning the tube flow resistance to near-critical damping and was ~ 5 ms based on 10%-90% criterion (Fig. 29c). The valve used with lens 2 produced a slight overdamping with rise/fall times of $\sim 4.5/2.5$ ms (Fig. 29d). With a lower resistance valve, the lens 2 transition times were as low as ~ 2 ms (not shown). From the frequencies of the decaying oscillations upon the valve switching (cf. Fig. 29b) and responses of the lenses to varying frequencies of switching the valves, the natural frequencies of the membranes in lens 1 and 2 were estimated as ~ 80 and ~ 375 Hz, respectively.

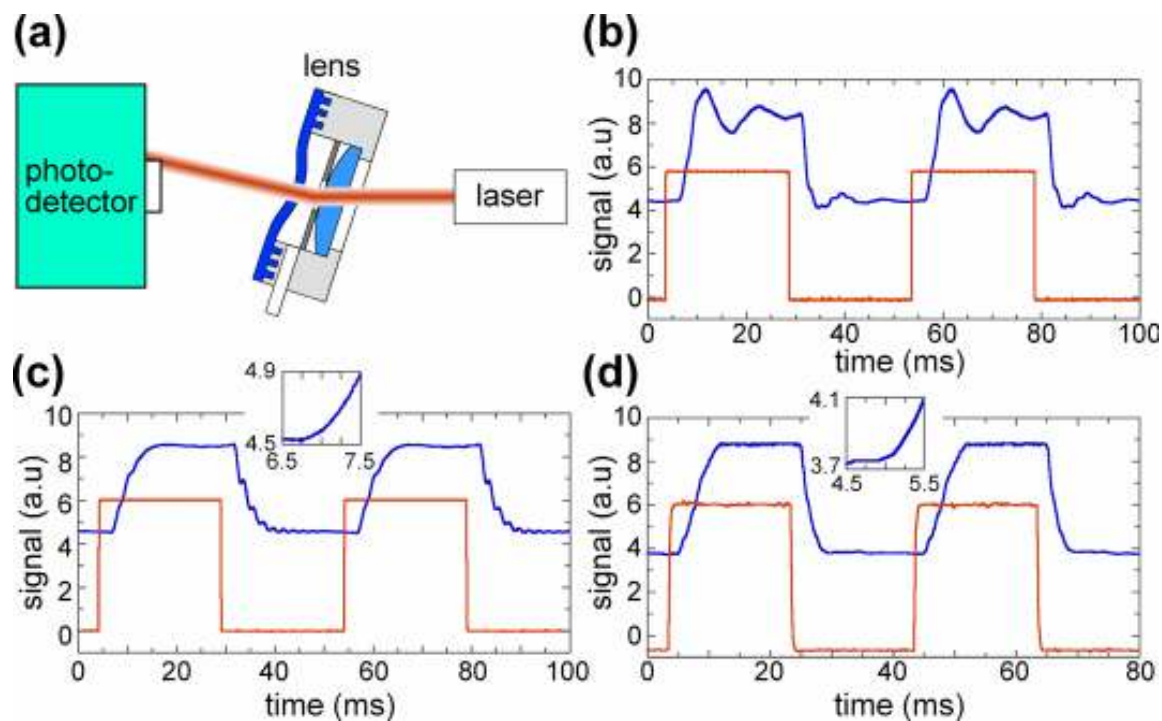


Figure 32. Temporal response of the lenses, as measured by a photo-detector, to switching of pressure. **(a)** Schematics of the optical setup. **(b)-(d)** Square-wave signal driving the solenoid valve and readout of the photo-detector, both in arbitrary units (a.u.). **(a)** Lens 1 with a low flow resistance between the valve and the lens, driven at 25 Hz between 0 and -0.3 psi. **(b)** Lens 1 with the flow resistance tuned to near-critical damping, driven at 25 Hz between 0 and -0.5 psi. **(c)** Lens 2 driven at 20 Hz between 0 and -0.8 psi. *Insets* in **(c)** and **(d)** are close-up views of the onsets of the first rise transitions.

The latencies between the rises and falls in the signal driving the valve and the beginning of the lens response, ~ 3.0 and ~ 1.5 ms for lens 1 and 2 (Fig. 29c, d), respectively, were close to the nominal response times of the valves. For both lenses, both the rise and fall transitions had very short onset times (insets in Fig. 29c, d): switches between flat segments and steep rise and fall segments occurred within < 0.5 ms and < 0.3 ms for lens 1 and lens 2, respectively, suggesting that the lenses can be driven at frequencies up to ~ 1000 Hz and ~ 1600 Hz, respectively.

In the second set of tests, the lenses were attached to a CMOS camera (Basler 602fc) with an acquisition rate of up to 8000 frames per second and were used to image an array of strips placed at an angle to the optical axis (Fig. 30a and Supplemental Materials). Time series of the distribution of light along a selected line of CMOS pixels perpendicular to the strips were recorded and plotted (Fig. 30b). Changes in the pressure supplied to the lens caused changes in the z -axis and lateral position of the in-focus region of the array (Fig. 30a) and thus in the location of the highest contrast point of the pixel line. This point was obtained for each frame by numerically differentiating the pixel values along the line and finding the maximum of the absolute values of the local derivatives. The location of the highest contrast point was converted into the z -axis position of the in-focus region of the array that was used to calculate $1/f$ and $1/f_m$.

For both lenses, the time dependences of $1/f_m$ (Fig. 30c, d) were generally similar to the corresponding photo-detector signals. The transitions accompanied by ~ 4 dpt variations in $1/f_m$ occurred within ~ 5 ms and $\sim 2/5$ ms rise/fall for lens 1 and lens 2, respectively. Both lenses were responsive up to the highest frequencies at which the

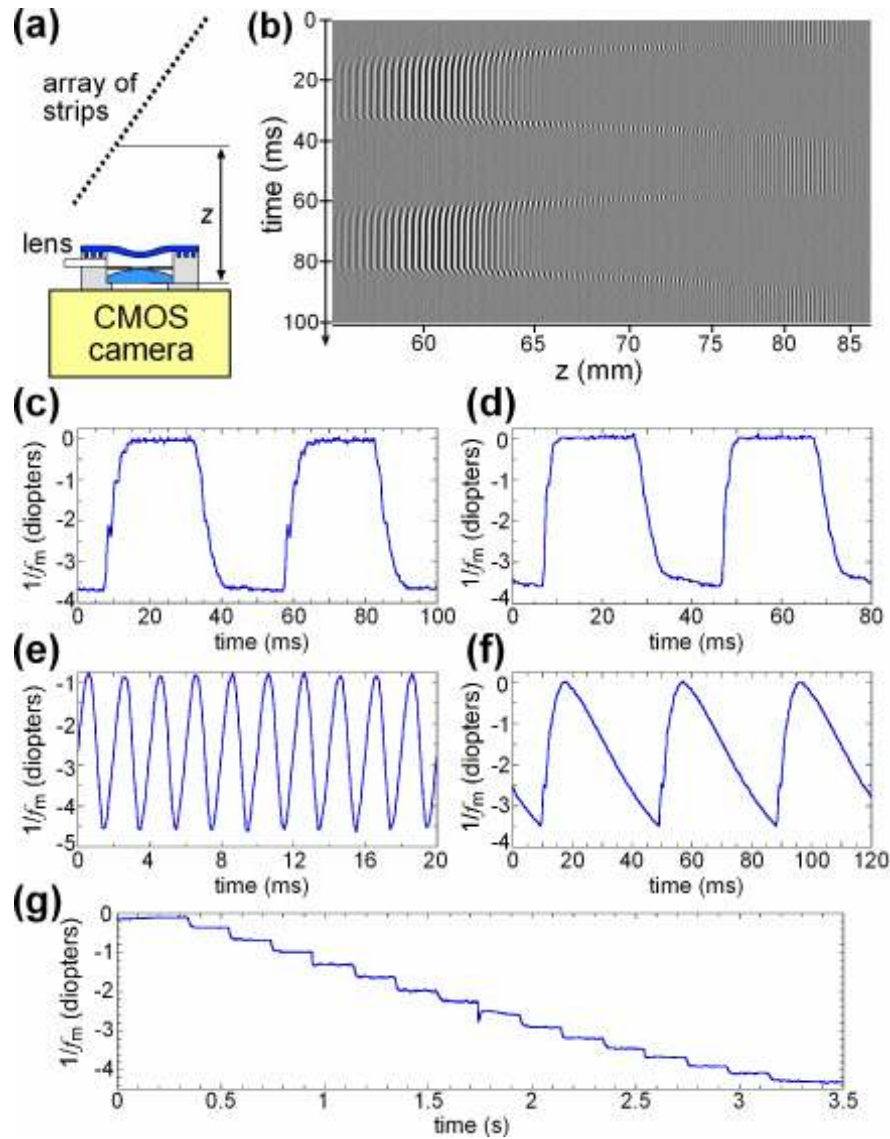


Figure 33. Variation of the refractive power, $1/f_m$, of the membrane lenses with time in different regimes. **(a)** Schematics of the imaging setup. **(b)** Space-time diagram obtained from imaging of a strip array with lens 1 attached to a CMOS camera operating at 4000 fps. CMOS pixel readouts along a selected line in consecutive frames are plotted from top to bottom. The pressure supplied to the lens is switched between 0 and -1 psi at 20 Hz. Two periods are shown. Labels at the horizontal axis indicate the distance, z , between the lens and the in-focus region of the array of strips, when the highest contrast region of the diagram is found at the corresponding position along the horizontal axis. **(c)-(g)** Refractive power of the membrane lens, $1/f_m$, as a function of time in different tests: (c) the test shown in panel (b); (d) lens 2 is switched between 0 and -1 psi at 25 Hz; (e) lens 1 is switched between 0.5 and -2.0 psi at 500 Hz. (f) lens 2 is driven at 25 Hz, with each 40 ms period comprised of 4 ms pulse connecting the lens to $P = 2.5$ psi and 36 ms when the lens is connected to $P = -4$ psi, both through high flow resistance lines; (g) the pressure fed to lens 1 is switched from -0.2 to -1.1 psi in 15 steps at a rate of 5 steps/s.

corresponding valves could be driven, ~ 530 and ~ 740 Hz, respectively. The amplitudes of variation of $1/f_m$ generally decreased at high frequencies, but the high-frequency response was enhanced by applying a larger difference of pressures to the valve inlets. We achieved ~ 4 dpt variation of refractive power at a frequency of 500 Hz for both lenses (Fig. 30e and Supplemental Materials). Importantly, there was no detectable change in the visibility of the strips compared with images taken at various fixed pressures (see Supplemental Materials). Therefore, the high frequency of oscillations of the refractive power did not compromise the resolution of the system. A sawtooth pattern of variation of $1/f_m$ appropriate for z -axis scanning was realized by customizing the pressures at the two valve inlets, the resistances of the inlet lines, and the duty cycle of the rectangular wave driving the valves (Fig. 30f).

To switch a lens between a large number of states with a small step in refractive power, we used 4 solenoid valves connected in series through tubing segments with different flow resistances to build a pressure divider (see Supplemental Materials). The divider was fed by air flow between a source of positive pressure and a vacuum source, and its output pressure varied between 16 discrete values defined by a 4-bit control signal sent to the valves. When lens 1 was connected to the pressure divider, its refractive power was switched in the range from -0.1 to -4.3 dpt with steps of ≤ 0.33 dpt (Fig. 30g). The transition times were typically ~ 20 ms. An exception was an ~ 100 ms transition at the eighth step, when all 4 valves were switched simultaneously.

The pneumatically driven adaptive membrane lenses described in this study have a continuous range of >4 dpt in refractive power and are liquid-free, thus avoiding the thermal stability and durability problems typical for the liquid-filled lenses. The transition

times of the membrane lenses are about an order of magnitude shorter compared to the electrowetting lenses with similar apertures. An important potential application of the proposed lenses is the z -axis scanning for 3D imaging, in particular in confocal and two-photon microscopes, and for 3D particle image velocimetry. The modulation of refractive power by 4 dpt achieved at 500 Hz (Fig. 30e) corresponds to a scanning depth of $\sim 80\text{ }\mu\text{m}$ with a $40\times$, 180 mm tube lens microscope objective. The variation of refractive power in discrete steps with short transition times (Fig. 30g) could be used for fast focusing in imaging and adaptive vision devices.

Chapter 3.3, in full, is a reprint of the journal article Pneumatically actuated adaptive lenses with millisecond response time in *Applied Physics Letters*, 2007. Campbell, K.; Fainman, Y.; Groisman, A., American Institute of Physics, 2007. Dissertation author was the first author and a leading contributor of this paper.

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