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Enabling Systems Biology Approaches Through Microfabricated Systems

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Abstract

With the experimental tools and knowledge that have accrued from a long history of reductionist biology, we can now start to put the pieces together and begin to understand how biological systems function as an integrated whole. Here, we describe how microfabricated tools have demonstrated promise in addressing experimental challenges in throughput, resolution and sensitivity to support systems-based approaches to biological understanding.

Keywords

Systems biology; microfluidics; microfabrication; MEMS

INTRODUCTION

A reductionist approach has long enabled researchers to gain insights into the structure and function of individual components of complex biological systems (including molecular networks, cells, tissues, organisms, and ecological systems). This approach provided a convenient, conceptually tractable approach to systems about which little was known. However, the operation of any system as a whole often transcends what the function of these individual units may suggest. For example, the functional capabilities of a microprocessor are not readily apparent from the function of an individual transistor, just as the properties of the brain are not readily apparent from the function of a single neuron. It is increasingly clear that to gain a comprehensive understanding of these complex systems and their functions, there is need to study the systems as a whole to incorporate an understanding of component interactions and dynamics within the native context of the system[1]. Thankfully, at the same time, the historically reductionist approach to biology has now amassed a wealth of knowledge about individual biological components, giving biologists a meaningful starting point from which to understand component relationships, system function and emergent properties[2,3].

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Early breakthroughs in molecular and structural biology resulted in the identification of key molecular players in the biological networks including nucleic acids and proteins. The resulting knowledge of biomolecule structure and function enabled the understanding of physical associations between these biomolecules and the identification of small interaction networks. This process of puzzling together knowledge of individual components into the context of a whole system is the fundamental aim of systems biology. Early attempts at this system integration focused on an integrative understanding the information stored in certain classes of biomolecules within an organism. Trying to fully understand the information stored in DNA marked the early genomic era of the systems level approach in biology and resulted in the sequencing of the whole genome of a number of species including human, mice, fly, nematode and yeast[4]. Genomics laid the groundwork for an expansive approach of discovery science that exhaustively probed individual layers of information within a system. The subsequent emergence of other 'omics' tools (e.g. transcriptomics, proteomics, metabolomics, and interactomics) then sought to probe other layers of information, moving down the central dogma and beyond[3,4]. Armed with these 'omics' toolsets, biological studies are heading into exciting new territory where whole systems can be studied with both breadth (large number of readouts that indicate system states and responses) and depth (readouts at multiple layers of information, from genetic to phenotypic).

The acquisition of 'omic' data was facilitated by the development of high-throughput platforms such as automated DNA sequencing, mass spectrometry, protein chips and microarrays. System-wide experimental approaches require innovative high-throughput experimental platforms, and computational approaches that facilitate acquisition and processing of large data sets. Thus far, the history of biology can be told as an intimate coupling of discoveries with their enabling technologies: basic structural biology with the advent of light microscopy, structural molecular biology with x-ray crystallography, molecular biology, genetics and genomics with the fundamental ability to replicate nucleic acids via polymerase chain reaction. The enabling tools for systems-level investigation are still emerging. At present, many microfabricated tools, such as microarrays, have demonstrated their enormous potential in the massive parallelization and throughput required for systems approaches. With the potential for high-throughput handling, stimulation and sensing of individual cells, tissue samples and microorganisms at a higher spatial and temporal resolution than conventional approaches, microfabricated tools seem poised to become one important enabling toolset in systems biology.

The foundational technologies for manufacturing on the micron scale stemmed from the development of transistor technology in the 1950s and the subsequent microelectronics boom[5]. The current microfabrication toolset is largely based on the lithographic, deposition and etching technologies developed in the microelectronics industry. Applying this set of technologies to a variety of materials have permitted the construction of micron-scale structures, sensors and actuators that leverage material properties and small-scale physical phenomena to perform experimental tasks. The resulting products, broadly categorized as "microsystems" have permeated the technologies in daily life and research laboratories. The printhead of inkjet printers, for example, are microfluidic systems that enable high-resolution ink deposition. The pressure and inertial sensors that are commonplace in our vehicles and consumer electronics operate based on the sensitivity of microfabricated structures to these physical changes.

In the context of biological research, the powers of microfabrication lie primarily in its abilities to match functional sensors and actuators with the size scale of cells and molecular components, and miniaturize and automate assays to facilitate massive parallelization and throughput of experiments. Many common examples of the power of microfabrication are evident in labs today. For instance, microfabricated multi-electrode arrays are now in

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common use for electrophysiological measurements of *in vitro* cell and tissue cultures with high spatial resolution, and *in vivo* as implantable electrode probes. Another example is atomic force microscopy, which applies the precision and cell-compatible mechanical properties of microstructures to enable probing the structural details of cellular components. In this case, microfabricated probes grant access to structural information far below the resolution limits of light microscopy and direct investigation of the mechanical properties of biological specimens. Yet another example of miniaturization and parallelization is commercial bio-microarrays, which permit simultaneous measurement of large panels of biomolecules; in the last couple of decades, microarrays have been an important part of the "omics" toolset in characterizing system behaviors based on biomolecular identification and quantification[6].

Many biological micro devices have leveraged the controlled, predictable flow of fluid at small scales to perform functional tasks. This microfluidic miniaturization of fluid handling has permitted the integration of several laboratory functions on discrete microchips, resulting in the growing field of "lab-on-a-chip" systems [7]. While the attraction of miniaturization in terms of reduced reagent consumption is one obvious advantage, operating at small scales also permits the use of highly controllable laminar flow to perform useful functions. Specifically, flow at the micro scale permits the creation of defined boundaries of two liquids (Figure 1a), the ability to produce robust, repeatable gradients (Figure 1b) and can be leveraged to generate repeatable droplets in systems with two immiscible fluids (Figure 1c). This review will illustrate how each of these functional flow phenomena has been employed to enable biological investigation.

While the technological advantages in the microelectronics industry have mostly focused on the creation of micro-scaled features on hard substrates such as silicon, biological microdevices have increasingly moved towards the use of optically transparent materials such as glass and a large range of polymers with different physical properties and chemical compatibilities[10]. Particularly, a softer, gas permeable and optically transparent polymer, polydimethylsiloxane (PDMS) , has gained popularity for devices that house biological specimens for manipulation and observation[11]. In practice, PDMS microstructures can be easily molded from structures already fabricated on conventional silicon substrates. The resulting devices have been demonstrated to be compatible with long-term cell, tissue and microorganism culture. Another dominant advantage of PDMS is that it exhibits elastic properties that are orders of magnitude less stiff than that of conventional hard substrates. In practice, this has meant that microstructures in PDMS can be made with mechanical properties that are compatible with most soft tissues. Importantly, this has also meant that PDMS structures can be deformed under relatively mild pressures, resulting in the creation of a host of pneumatically driven actuators, such as valves and pumps (Figure 1d) [12]. Yet, despite these advantages, PDMS has fundamental limitations in solvent compatibility that has made other polymers and glass the materials of choice in some applications [10,13,14]. In these cases, on-chip automation and active fluid control have been demonstrated using the movement or "slipping" of chip compartments on multiple layers of the device[15,16] or integrating elastomeric membranes into a glass device[14,17]. In combination with the advantages of small-scale flow, the microfluidic toolbox of biological investigators now comprises both passive control mechanisms that depend on geometry and active control mechanisms that can permit direct, and controllable physical manipulation of the microfluidic world.

This article is intended to illustrate how biologists and engineers have leveraged these tools to enable precise, high-throughput systems-level experimentation. In particular, we highlight some of the important experimental functions that microfabricated tools serve including sample handling and positioning, defining and dynamically altering environmental

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conditions, sample detection and analysis and even the construction of systems for mimicking whole tissues and *in vivo* experimental conditions. These micro-scale systems offer intrinsic advantages in terms of matching the scale of the experimental platform to the scale of molecular reactions, cells and small organisms. Moreover, we will show that micro-scale experimental platforms are often associated with a higher level of throughput and automation since miniaturization is often used as a means for parallelization and active on-chip components such as valves can enable automation of complex processes. Furthermore, to remove potential data processing bottlenecks downstream of high throughput data acquisition, the coupling of microfluidic control with microscopy and computer vision techniques has been used to facilitate both fully automated data acquisition and analysis. After a quick tour through the range of functionality currently available through microfabricated experimental systems, we will then explore two in-depth examples that highlight how microfluidics has both been commercialized to perform complex, automated functions and utilized to perform facilitate conceptually simple experimental designs. While other reviews of the capabilities of microsystems to enable systems-level biological investigations exist[18], this review aims to offer some historical perspective, an updated, broad and easily understandable overview of the field and insights on how microtechnology is being made accessible for general use for labs with little microfabrication experience.

SPATIAL POSITIONING OF SINGLE CELLS AND WHOLE ORGANISMS

One fundamental challenge in integrative studies of intact cells and multicellular models is that these biological specimens exist at a size scale that is not easily amenable to human manipulation. At the same time, the ability to precisely control the spatial positioning of biological specimens is crucial in controlling and understanding interactions between these cells or organisms and with their microenvironment. Even when the control of experimental conditions is not the critical concern, careful spatial positioning is often required to assess the state of the specimen using common techniques such as fluorescence microscopy. Yet, despite its fundamental importance, biological sample handling is often laborious, time-intensive and a source of error in conventional techniques. In contrast, microfabricated systems enable ease of sample manipulation and precise spatial positioning via the ability to match the size-scale of the experimental platform with the size-scale of the biological specimen. Specifically, manipulation of fluid flows on a micron scale can provide precise, passive mechanisms for orienting and positioning micron-scale specimens. These passive control techniques can also be integrated with active components such as fluidic valves that permit active control of the microenvironment Here, we will highlight microfabricated systems that have been developed to remove conventional experimental barriers in cell and small animal handling.

Cell trapping

Understanding environmental response and interactions on a single-cell level is key to understanding potential emergent behaviors of populations of cells in the body and intracellular mechanisms that may not be readily apparent from looking at population-averaged data. To address this need, several devices have been developed that utilized hydrodynamic flow and physical obstacles[19,20], dielectrophoresis[21,22], microwells[23,24], micropatterning[25], acoustic waves[26] and droplet encapsulation[22,27–29] to precisely place individual cells in defined compartments and allow single cell measurements. Specifically, many groups have used a combination of carefully controlled flow and physically defined cell traps to offer an experimentally simple, passive and generalizable method of placing cells in defined physical locations[19,20,23]. As an example, Chung *et al.* developed a densely packed cell trap array for the capture of cells into individual compartments at high efficiency (Figure 2a)[20]. The device, which consists of microfabricated structures in PDMS bonded

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directly onto a coverslip, is fully compatible with conventional microscopy and cellular techniques for analyzing cell surface marker expression and probing intracellular signaling. These traps and the other spatial positioning methodologies previously mentioned permit single-cell resolution assessments of cell population heterogeneity that would otherwise be masked by conventional flow cytometry approaches, and allow time-resolved tracking of individual cells. Moreover, by keeping cells in a defined location, temporally dynamic data can be acquired to investigate mechanisms in signaling.

Another microfluidic approach that has proven useful in handling biological specimens is of the use of two immiscible fluids (such as aqueous media and an oil) to form discrete, monodisperse droplets. These droplets can be used to encapsulate and isolate cells, small organisms and biomolecules into physically and chemically isolated compartments for analysis. Droplets can then be merged or split to facilitate stimulus addition or removal and easily transported, stored and analyzed within microfluidic devices. Droplet microfluidics leverages the ability to carefully control flow along with the surface properties of the device and properties of the immiscible fluids to form discrete, stable experimental chambers of a highly defined and uniform size. To control droplet formation based on these variables, many standard microfluidic droplet generators that facilitate the controlled dispersal of droplets into the carrier liquid have emerged (Figure 1c) [30]. Using these designs, cell encapsulation and droplet based experimentation has been demonstrated for yeast cells[31], bacteria[22,32], and mammalian cells[27–29]. For example, Brouzes *et al.* developed a platform for the encapsulation of single cells in droplets and used controlled merging of these cell droplets with a droplet-based drug library for cytotoxicity tests (Figure 2b)[28]. Furthermore, strategies have been developed to allow encapsulation of cells in matrices by using crosslinkable hydrogels[33]. This enables the encapsulation of cells in their native matrix, and is particularly crucial for adherent cells. Finally, above the single-cell level, droplet encapsulation has also been employed as a strategy for small multicellular model organisms[27,34,35], which we will discuss in the following section. In general, droplets represent a general strategy for partitioning single biological specimens and manipulating and assaying them as independent units.

Organism culture and experimentation

In addition to facilitating single-cell experimental techniques, microfluidics has advanced in the realm of spatial control of whole organisms like *Drosophila melanogaster*[36], zebra fish[35,37] and *Caenorhabditis elegans*[38,39,40,41,42,43]. Many platforms have been developed to enable the positioning, orientation, and manipulation of small multicellular model organisms for both imaging and stimulation. Due to its broad use as a biological model organism and its micron scale body size throughout its lifespan, the small, transparent nematode (roundworm) *C. elegans* has received the majority of the attention from microfluidic developers. Moreover, due to its optical transparency, short lifespan, sequenced genome, fully mapped neuronal network and genetic homology to humans, *C. elegans* provides an excellent model for an integrative understanding of how genetics and environmental inputs influence phenotypic outputs from development to behavior to longevity. Taking advantage of the optical transparency of the worm, many studies use high resolution optical imaging to derive both structural and functional information from genetically encoded fluorescent reporters or stains. However, these studies are also hindered by the difficulty in positioning, orienting and immobilizing the freely moving worm for imaging. To address this issue, many groups have used actively controlled valves in PDMS-based devices to enable the loading and immobilization of *C. elegans* in a controlled imaging region for high resolution imaging, manipulation and sorting[38,39,43]. Chung *et al*. showed that microfluidic devices could be used to introduce *C. elegans* to a fixed imaging region, immobilize them by the application of valves and cooling and perform high-resolution imaging to identify and

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isolate mutants for subsequent phenotyping and study (Figure 2c)[38]. Moreover, the ability of *C. elegans* to be cultured in liquid media and its small size makes it easy to handle in microfluidic devices throughout its life cycle and several platforms have been developed for short- and long-term on-chip culture of worms for both developmental and behavioral studies[40,42].

In addition to the worm, the fruit fly *Drosophila melanogaster* and the zebra fish *Danio rerio* are popular model organisms for developmental biology and are micron-scaled and amenable to microfluidic manipulation for part of their early development. Recently, a microfluidic platform was developed to precisely array and orient *Drosophila melanogaster* embryos with its anterior-posterior long axis aligned vertically (Figure 2d)[36]. The device consisted of a wide serpentine channel arrayed with cylindrical embryo traps along one side (Figure 2d). As embryos flow along the serpentine channel, small fluid flows across the traps through a resistance channel guide embryos towards to traps, where they are pushed upward into the vertical position by continual fluid flow. By orienting the embryos with its dorsal-ventral axis parallel to the imaging plane, patterning events along this axis, including the formation of morphogen gradients that guide differentiation and development, can be observed on a single imaging plane and without three dimensional image reconstruction that may introduce errors and aberrations. Similar to the single-cell trap array we discussed previously, this embryo trap array enables high-throughput and high-quality imaging of developmental events with single-embryo resolution, permitting the study of developmental variability and robustness in large populations. One could envision that this platform can be extended to other organisms of similar geometry from the more diminutive embryos of *C. elegans* to the larger embryos of the popular vertebrate developmental models of fish. Finally, for developmental model organisms such as the zebra fish, several other microfluidic platforms have already been developed to permit culture and observation of fish embryos under development[35,37].

SYSTEM PERTURBATION AND CHEMICAL STIMULATION

Organisms are subjected to dynamic environments and chemical, physical or genetic perturbations, both at a cellular and whole-organism level; these perturbations often affect system behavior and functions, and give rise to emergent properties. For example, chemical gradients play a significant role in a number of physiological processes such as homing of leukocytes to sites of inflammation, wound healing, cancer metastasis, and development. Of interest in systems biology studies is the ability to apply precise perturbations and observe the biological consequences. Conventional techniques for applying chemical gradients include the Boyden chamber [44], Zigmond chamber [45], Dunn chamber [46], and the micropipette approach [47], which have been used in cell migration and bacterial chemotaxis studies. However, these techniques suffer from lack of stability, have difficulty in producing complex gradients, and are not able to create temporally varying gradients. Beyond the spatial positioning functions that we have already discussed, small-scale fluid flow in microfabricated systems can be used to overcome these limitations in spatial and temporal control and help understand cellular and organismal signal processing by systematically perturbing environmental signals. The microfluidic length scales, which are comparable to the length scales of cells and microorganisms (thus making flow characteristically laminar), enable precise application of spatial and temporal chemical gradients. For example, by manipulating the laminar flow behavior in microfluidic systems, Takayama *et al.* demonstrated the ability to apply spatial soluble chemical gradients with sub-cellular resolution[48], and several papers have demonstrated strategies for temporal control using flow switching and flow focusing [49]. Here, we will discuss the common strategies that have been utilized to perform controlled stimulation of biological specimens in microfabricated platforms.

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Controlled convective and diffusive transport of biomolecules is utilized in forming spatial and temporal biochemical gradients on microfluidic devices. One of the prominent gradient generators, coined the “Christmas tree,” consists of input channels for several discrete chemical inputs and a network of bifurcating channels that mix and split to get solutions with differing proportions of the input solutions (Figure 1b). The channels then converge into a wider channel, and the laminar flow behavior of microfluidic fluid flow enable stable interfaces between adjacent solutions with mixing only through diffusion [50]. By modulating the fluidic resistances in the channels in this scheme, platforms with linear, logarithmic and polynomial concentration gradient profiles have been generated [8,50,51,52]. In addition to using the mixing and splitting “Christmas tree” approach to create spatial gradients, Amarie *et al.* also implemented flow switching and reduced the size of the device channels to add the ability to temporally control on-chip spatial gradients [52]. Alternatively, biochemical gradients can be created by diffusion of molecules from a reservoirs (sources and sinks) using porous membranes [53,54], hydrogels [55] or high resistance microcapillaries [56], where diffusive transport dominates over convective transport. While these schemes mainly rely upon diffusive transport for controlled mixing, several other devices have been fabricated that make use of a combination of convective transport for maintenance of bulk boundary concentration conditions and diffusive transport to deliver the biomolecules to cells under low shear stress [57,58]. The use of convective transport to rapidly change boundary conditions also permits an added level of temporal control. For example, utilizing a combination of convective and diffusive transport, Atencia *et al.* applied spatially and temporally varying gradients of growth media and glucose to observe bacterium migration under a two-dimensional glucose gradient (Figure 3a) [57]. Similarly, VanDersarl and colleagues fabricated a device capable of delivering a chemical gradient with both spatial and temporal gradient by diffusion of molecules through a porous membrane (Figure 3b) [54].

In addition to soluble gradients, cells also experience gradients of molecules anchored on a matrix. Techniques such as microcontact printing, selective surface adsorption of biomolecules from solution, and cross-linkage of biomolecules onto surfaces have been utilized in combination with a solution gradient generator to create surface-immobilized protein gradients [59,60,61]. For example, Dertinger *et al.* used a “Christmas tree” gradient generator to deposit gradients of immobilized laminin on to a surface. Using this scheme, they demonstrated that laminin surface gradients influenced the orientation of axons during neuronal development in rat hippocampal neurons [60]. Importantly, biomolecules can also be immobilized on biomimetic substrates commonly used in conventional cell-based assays such as polyethylene glycol (PEG) hydrogels, allowing for coupling between microfluidic and conventional techniques. Selective biomolecule attachment to various substrates can be accomplished using the same gradient generation approaches described above coupled with a covalent cross-linkage reaction to attach biomolecules to the material [61].

Moreover, spatiotemporal stimulation platforms for handling small organisms like *C. elegans* [40,43,62,63] and *Drosophila* embryos [64,65] have been developed to gain insights on whole organism response to environmental perturbations. Specifically, in the freely moving *C. elegans*, a key focus has been to understand the processing and integration of environmental cues to produce neural and behavioral responses. In order to understand how a freely moving animal integrates chemosensory inputs into behavioral outputs, both spatially and temporally variant gradients of odorant has been demonstrated in a microfluidic arena capable of accommodating freely moving animals (Figure 3c) [62]. Furthermore, to specifically investigate temporal responses in a high throughput fashion, Chung *et al.* designed a chemical screening device to place individual *C. elegans* in chambers and perform fast chemical exchanges to probe environmental cues in mating behavior (Figure 3d) [40]. Overall, for both cellular specimens and whole organisms, the microfluidic approaches that we have discussed here represent an important toolset that

helps biologists probe the input/output behavior of biological systems with a degrees of both spatial and temporal precision that would be impossible with conventional tools.

DETECTION, MEASUREMENT AND SORTING ON CHIP

Biomolecule detection and analysis

As we have discussed previously, early approaches to biology from a systems perspective focused on understanding the layers of information along the central dogma and beyond, with interests in genomics, proteomics, transcriptomics and metabolomics. At the core of these system level approaches are tools that enable quantification of biomolecules. Here, sensitivity, the ability to handle minute volumes, and integration with preparation steps are paramount in developing robust analytical tools to quickly quantify a large range of biomolecules. For example, in the realm of proteomics, techniques such as Western blotting, ELISA, mass spectrometry and electrophoresis are fundamental analytical tools in protein identification and immunoaffinity determination. However, these assays often involve multiple steps that require human intervention, have long incubation times due to mass transport limitations, and require significant volumes of reagents and protein samples. In addition, the cellular expression levels of different proteins vary widely, posing a challenge in the detection of concentration levels that may differ by orders of magnitude. Microfluidics has enabled the recapitulation of these conventional techniques on a micron scale to achieve fast diffusion times, reduce reagent and sample use and increase sensitivity. Furthermore, integration of preparation, separation and detection steps via the simple fluidic coupling of multiple microfluidic modules minimize human intervention, reduce reagent use, and increase sensitivity. There has been a plethora of microfluidic devices developed for translating conventional proteomic approaches on-chip, including devices for western blotting [66], ELISA [67], and electrophoresis [68]. Moreover, new on-chip functionality and microfluidic integration has been employed to develop more integrated and automated analysis platforms [69]. More detailed overviews of microfluidic approaches to "omics" are explained elsewhere [70].

Most importantly, microfluidics can be leveraged in performing single cell analysis owing to the minute volumes that allow amplification of biomolecules present in low quantities. There has been a growing interest in single cell mRNA sequencing, with specific applications in studying diseased cells and also basic science [71,72]. Recently, Ramsköld and colleagues developed Smart-Seq, an mRNA sequencing protocol that enables sequencing from either end of the mRNA sequence, improving the transcriptome coverage with high sensitivity, and demonstrated applications in studying circulating tumor cells (CTCs) [71]. One can envision that microfluidics can be leveraged in single-cell mRNA by employing cell-separation modules (discussed below) for identifying and separating cells of interest such as CTCs from blood cells, coupled with the Smart-Seq for rapid transcriptome quantification on chip.

Mass-based sensors

In addition to recapitulating conventional techniques on a micron scale, highly sensitive mass-based devices have emerged for single-cell and single-molecule detection. Single-molecule detection is crucial in allowing for quantification of precise protein expression levels on a single-cell level. Mass spectrometry is currently the technique of choice in molecule profiling, but the challenge is on achieving the sensitivity required when conducting single-cell studies. To improve sensitivity, Roukes *et al.* developed a nanomechanical mass spectrometry-based sensor for the detection of single molecules [73]. Whereas the low sensitivity in conventional mass spectrometry limits molecular analysis to samples from populations of cells, this device will allow for the measurement of proteins

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such as transcription factors on a single cell level. Similarly, a microchannel resonator was recently demonstrated to measure the mass, density and volume of yeast cells during the different stages in the cell cycle [74]. In this work, a microchannel that could accommodate the flow of single cells was embedded inside a cantilever. By measuring the resonance frequency changes of the cantilever as single cells travelled through the microchannel, single cell growth dynamics could be precisely captured. These two examples illustrate how miniaturization can fundamentally increase sensitivity in detection and facilitate single-cell studies where previously only population based studies were possible [75]. Single-cell resolution is currently an experimental challenge, and will ultimately be necessary to elucidate cellular signal processing events that may be masked by population averages and the complexity in cell-cell interactions.

Cell separation and sorting

In addition to performing measurements with single-cell resolution, the ability to isolate specific cells and probe for functionality can be insightful in understanding diseased cell phenotypes and the functional causes and consequences of cell population heterogeneity. Using cell properties such as size [76,77], shape [78], expression of surface markers [79,80], magnetic properties [81], and a combination of size and mechanical rigidity [77,82,83], microfluidic platforms have been developed for the detection and isolation of cells with specific attributes from heterogeneous populations such as whole blood. Several comprehensive reviews have been published on cell separation techniques [84]. For example, Kotz and colleagues developed a simple microfluidic channel for the neutrophil capture based on surface marker affinity. Subsequent genomic and proteomic analysis of neutrophils isolated from whole blood allowed for the identification of differences in expression in neutrophils at varying activation states. This device provided a tool that overcomes the challenge in handling sensitive neutrophils, reduced the sample preparation time, and demonstrates promise as a simple tool for use in a clinical setting [80]. Such tools also demonstrate promise in helping understand systemic disease progression and phenotypical changes in patient populations by quickly isolating relevant cell types.

Another crucial property of cells that has been used as a basis for separation is their deformability. Deformability can be indicative of cell's disease state, as evidenced by changes in mechanical properties of cells in sickle cell disease and cancer [85]. While tools such as micropipette aspiration, optical tweezers, magnetic forces, and atomic force microscopy have been established for measuring cell mechanical properties, they are limited in throughput and the ability to enrich specific cells with similar mechanical properties from a cell population for downstream analysis. The length scales in microfluidic systems lends itself to a unique ability to probe cell mechanical properties using constricted channels. Hur *et.al.* developed a platform that enabled separation and enrichment of cancer cells from peripheral blood using a combination of cell size and deformability [82]. These separated cells can then be used to analyze expression of specific genes and proteins that can give insight on disease progression and cell function using the analytical tools mentioned above. Ultimately, through the coupling of sample reaction schemes with separation and analytical techniques, microfluidics enables the creation of integrated, multifunctional platforms for systems level study.

RECAPITULATING ORGAN AND TISSUE FUNCTION ON CHIP

The sections above have illustrated how microfluidic technology can be used for high-throughput positioning, stimulation and detection of individual cells and whole organisms. However, being able to probe cellular response in the spatial context of other cells and the mechanical context of biological tissues will provide more information on the overall system behavior in tissue and organs. Conventional approaches often involve *in vivo* experiments on

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animal models such as mice, making the experiments laborious and expensive, and limit the extent of the type of system perturbations that can be applied to specific sites in a live animal. Organ-on-chip is an emerging field in microfluidic technology that attempts to provide a sophisticated mimicry of the physiological environment by creating cell-cell and cell-matrix interfaces that enable interaction dynamics to be elucidated. While these systems do not represent the organs in their entirety, they represent advancement to cell-culture based *in vitro* models and may offer cheaper alternatives to drug screening. Recreating organ architecture on-chip enables researchers to engineer a physiological microenvironment, where they can take advantage of microfluidic characteristic length scales to precisely apply chemical and mechanical stimulation.

Microfabricated platforms have enabled the emulation of brain [86], gut [87], blood vessel [88–90], liver [91], kidney [92], eye [93], and lung [94] models in microfluidic devices. Several published reviews provide an in depth overview of the application of organ models in microfluidic devices [89,95]. Recently, Huh *et al.* recapitulated the alveolar-capillary interface in a PDMS-based device by using a porous, flexible PDMS membrane coated with extracellular matrix material. Human alveolar cells and endothelial cells were seeded on either side of the membrane, and the membrane was sandwiched between two compartments to mimic the alveolar and vascular compartments (Figure 4a). The system was used to demonstrate pulmonary inflammation, mimic the mechanical stimulation dynamics in the alveolar-epithelial interface during normal breathing, and the effect of aerosols on endothelial inflammation that could have significant implications in toxicology studies [94]. Apart from its interfaces with other organ systems, the vascular system itself has been especially amenable to mimicry on chip and has garnered significant attention from microfluidicists [89]. For example, using a collagen matrix, Zheng *et al.* fabricated endothelialized three dimensional networks to mimic blood vessels in healthy and diseased states for angiogenesis and thrombosis studies (Figure 4b) [88]. They were able to mimic the matrix and cellular content of blood vessel wall, the geometry, branching and bifurcation of blood vessels observed in the physiological environment. Using this model, the researchers recapitulated remodeling events observed during angiogenesis, endothelial cell sprouting and vessel leakiness in diseased states. The platform could be used to elucidate the functional impact of cellular, chemical and physical stimuli. We envision that this blood vessel platform could be instrumental in complementing studies on thrombosis that have previously been done in mice in a cost effective manner. Furthermore, these tissue and organ mimicry models are not limited to functioning as a cost- and time-effective way to facilitate drug screening and toxin screening.

The ability to recreate systems on-chip fundamentally allow systems biologists to build systems from the ground up and establish necessary conditions for observed systems behaviors. Ultimately, organ-on-chip could facilitate an understanding of biological systems by providing an effective testbed for systems-based hypotheses.

ACCESSIBLE MICROFLUIDICS

Thus far, we have reviewed several emerging classes of microfabricated tools with the potential to eliminate experimental barriers to a systems-level approach to biology. While many of these micro-scale technologies still emerging from the developmental pipeline, some tools have already started to contributed to new biological knowledge and have demonstrated utility to the point of commercialization. Here, we will provide two in-depth examples of how microfabrication can be utilized to simplify, enhance and automate biological techniques of widespread, commercializable utility and how the application of even very simple, accessible, do-it-yourself fundamental microfluidic techniques can be used to elucidate novel biology.

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Digital PCR

Early microfluidic forays into nucleic acid detection focused on the fundamental ability of microfluidics to massively parallelize low -volume amplification reactions that could be rapidly thermally cycled[99]. However, it was the conceptual leap to digital detection schemes ultimately made the massive parallelization that could be achieved with microfabrication an indispensable part of the detection toolset. Digital polymerase chain reaction (digital PCR) applies the nucleic acid amplification techniques of PCR to single molecules of DNA or RNA in a highly parallelized manner. Via the dilution of samples such that only one DNA or RNA molecule initiates each PCR reaction, digital PCR allows the precise quantification of nucleic acid molecules despite the exponential nature and imprecision of the PCR reaction itself (Figure 5a and b). In its original conception, individual microliter PCR reactions were performed in parallel on a multi-well plate [100,101]. The throughput and therefore utility of the technology was therefore fundamentally limited by the requirement for tedious manual fluid handling, which increased the risk of contamination as well as decreasing throughput. Moreover, single molecule isolation and reaction conditions are both more easily established in smaller volumes of fluid than in conventional macro-scale PCR reactions. Finally, the sensitivity of digital PCR to quantify rare molecular species is dependent on the number of reactions that can be performed in parallel. Thus, while the conceptual basis of digital PCR has been established since the early 1990s, the true power of digital PCR was not unlocked until microfluidic technologies enabled massively parallelized, nano- to picoliter PCR reactions with automated fluid handling[102]. Over the last few years, many microfluidic realizations of digital PCR have emerged[16,103,104]. Some of these technologies, including the well-established micro-valve array based microfluidic platform from Quake and colleagues (Figure 5c) [105] and droplet-based implementations from RainDance Technologies and Bio-Rad, have been commercialized[106], making the technology available and accessible to non-microfluidics labs.

With the increasing sensitivity provided by microfluidic realizations with ever increasing reaction numbers, digital PCR technology is poised to render precise quantification of nucleic acids both accessible and precise. One dominant application of digital PCR has been in the area of molecular diagnostics, where the detections of rare mutations or allelic imbalances can provide early signals of disease[98,100,107] or detections of fetal nucleic acids in maternal blood can result in minimally invasive prenatal diagnosis of genetic abnormalities[97,108]. Digital PCR also has the potential to provide new insights into the inner workings of cells, tissues and ecological communities. Already, digital PCR technology has been applied to the transcriptional profiling of single cells, permitting an understanding of heterogeneities in cell populations and how individuals contribute to the behavior and robustness and behavior of the collective[104]. The technology has also shown promise in helping to dissect the microbial composition of environmental niches[109], making it a promising tool to help understand ecological architecture and symbiosis in the human microbiome[110]. Finally, digital PCR has been applied to the amplification of small samples and simplifying the analysis pipeline for next generation DNA sequencing technologies, making it a critical tool for enabling and expanding the power of high-throughput sequencing technologies[111]. This technological advance now enables highly sensitive nucleic acid detection and amplification that is capable of quantifying the rare but crucial elements that can inform us about organismal health, disease progression and microbial diversity.

Experimental Power from Simple Designs

While one advantage of microfluidic technology is its power to multiplex and handle complication in an automated manner (Figure 5c), even very simple applications of microfluidic concepts have yielded gains in biological knowledge. Fundamentally,

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reductions in the scale of cell-, tissue-, or microorganism-culture or experimentation results in better spatial and temporal control over culture conditions. In some cases, miniaturization helps mimic the natural microenvironment of the cultured body, allowing autocrine or paracrine signals to accumulate [112] or enabling confinement or micron-scale deformations as a mechanical stimulus [94,113]. In others cases where it is necessary to impose highly defined environments, miniaturization permits rapid fluid exchange by lowering the volume to be replaced, allowing for better temporal control of conditions [20,40,43,62]. Similarly, lowering culture volume also permits continuous media exchange while keeping overall media consumption low. In this case, continuous laminar flow can facilitate the generation of defined spatial boundaries and gradients (Figure 1a and b). Importantly, these applications of microfluidics are both conceptually simple and easy to implement in practice. With the availability of custom chip manufacturing from foundries such as the Stanford Microfluidic Foundry or companies such as Little Things Factory or Dolomite, these tools are accessible to laboratories without the intervention of a full-scale commercial developer or the purchase of dedicated toolsets.

An example of a very simple, elegant use of microfluidics to enable the investigation of the mechanisms of developmental robustness was presented in the work of Lucchetta *et al* [64,65]. Chemical cues, called morphogens, choreograph the formation of structure in embryonic development[114]. In the fruit fly, *Drosophila melanogaster*, a gradient of the *Bicoid* protein established by the selective deposition of maternal mRNAs governs anterior-posterior polarity in the embryo[115]. The maternally established Bicoid gradient then controls downstream expression of morphogens to drive the developmental process. Yet, it was noted that despite natural variations in the Bicoid gradient, the downstream expression of the Hunchback morphogen was robust, indicating compensatory or secondary patterning mechanisms that can overcome noise in the Bicoid gradient[116]. Using a simple Y-channel device to define a sharp boundary of two fluid flows of different temperature around a developing *Drosophila melanogaster* embryo (Figure 6a), the authors were able to investigate the nature of the compensatory mechanism by both spatially and temporally disrupting the Bicoid gradient (Figure 6b). Ultimately, the ability to control Bicoid gradient disruptions both spatially and temporally by reversing the temperature steps allowed the systematic investigation in the limits of developmental robustness and the timing of the compensatory mechanism. Thus, even very conceptually and practical simple microfluidics can address experimental needs lacking in conventional methods and address novel biological questions.

CONCLUSION

In this work, we have briefly introduced some of the experimental functions that can be accomplished in microfabricated systems in order to realize more carefully controlled, higher-throughput, higher-sensitivity biological studies. The experiments enabled by the platforms enumerated in this paper are often impossible to perform without the degree of fluidic and mechanical control offered by microfabrication. The range of work that we have discussed also demonstrates that microfluidics can be extremely useful at different levels of complexity. The superior spatiotemporal environmental control offered by conceptually simple, easy to implement microfluidic solutions can bring us new insights on environmental response and biological system robustness. At the same time, more advanced microfabrication techniques can be used to emulate organ and tissue functions on chip to provide more physiologically relevant disease and drug models and create fully automated systems for specimen handling, imaging and sorting. Finally, complex but robust microfluidic integration can be developed and commercialized to perform critical experiments by labs with no microfluidics expertise. Ultimately, we believe that the understanding and adoption of microfabrication and microfluidics at these diverse levels of

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expertise can bring about critical enabling technologies that bring us closer to an integrative understanding of the interactions between genotype, environment and phenotype.

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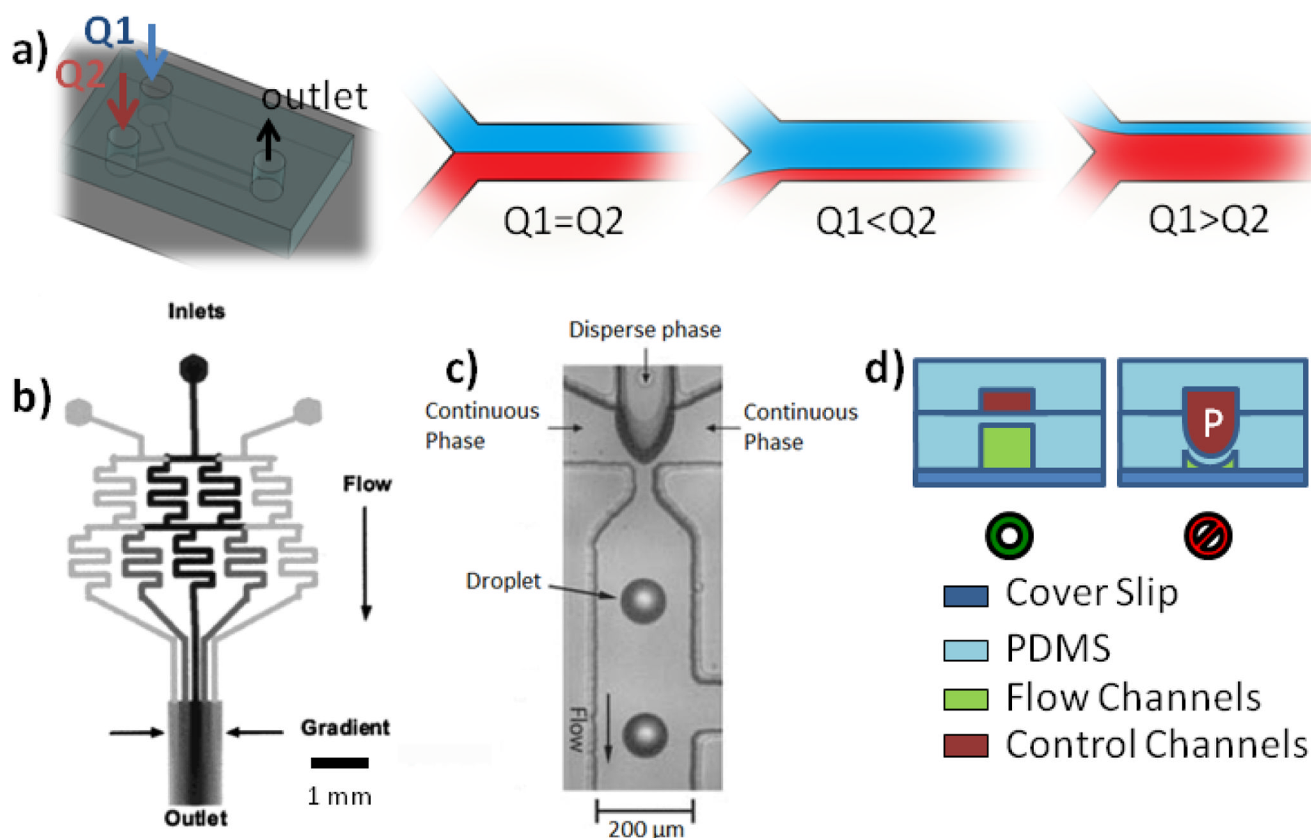

Figure 1.

Overview of basic microfluidic functions. a) Laminar flow at small length scales in microfluidic channels follow predictable, controllable trajectories that can be utilized to form controlled fluid boundaries. Two miscible liquids (blue and red) injected at various flow rates (Q1 and Q2) into a Y-shaped microfluidic channel can form stable boundaries that are disrupted only by diffusive mixing. b) Controlled flow boundaries and diffusive mixing can be employed to generate stable gradients. In this case, input concentrations at a few inlets can be passively mixed sequentially into multiple intermediate output concentrations[8]. Reprinted with permission. Copyright 2000 American Chemical Society. c) The flow of two immiscible fluids (usually an aqueous disperse phase and an oil-based continuous phase) can be used to form droplets of uniform size[9]. Applications include encapsulation and culture of cells and microorganisms, high-throughput parallel chemical reactions and generation chemical compound libraries for screens and subsequent processing. Reprinted with permission. Copyright 2009 American Chemical Society. d) Microfluidic fabrication with soft polymers such as polydimethylsiloxane (PDMS) can include active structures based on the deflection of thin membranes using pneumatic pressure. Applications include flow, cell and microorganism control, mechanical stimulation, fluid displacement, and pumping.

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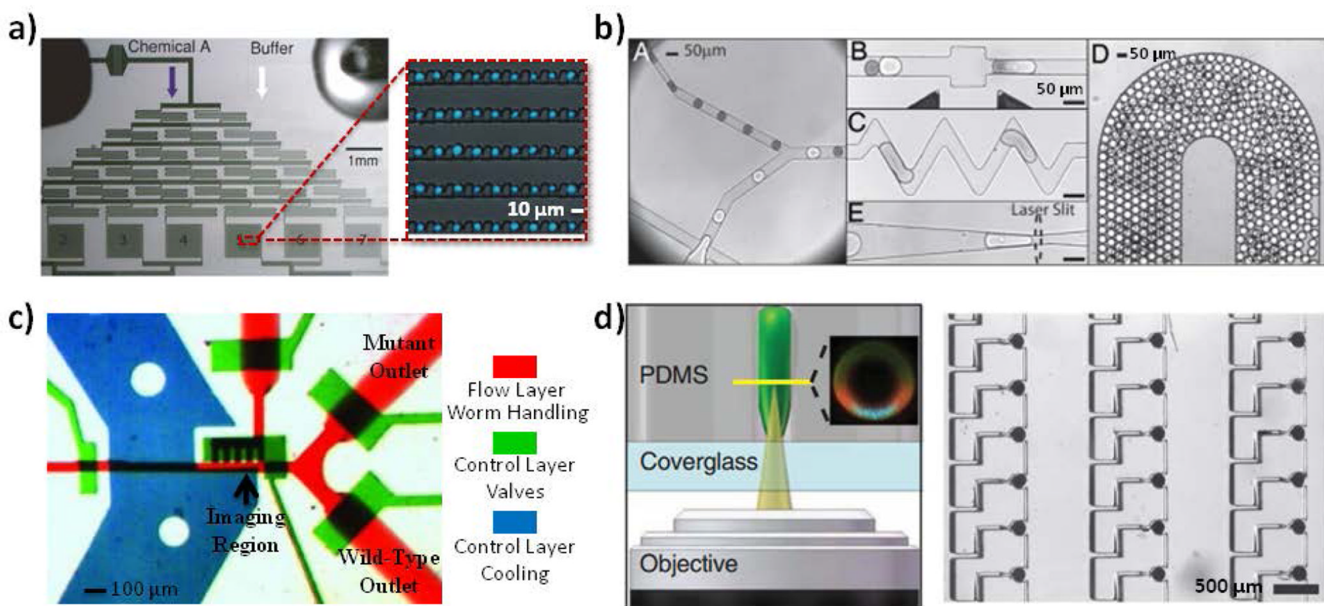

Figure 2.

Spatial positioning of biological specimens in microfluidic systems. a) A densely packed single-cell trapping array fabricated in PDMS. A microphotograph shows an overview of the device with multiple cell trap modules, which can be chemically stimulated at different concentrations. Inset shows individual fluorescently labeled cells trapped within a portion of one cell trap module[20]. Reprinted with permission. Copyright 2011 American Chemical Society. b) A droplet-based platform for single cell screening. The platform integrates five modules for performing common droplet functions including droplet generation (A), droplet merging (B), mixing (C), incubation and storage (D) and detection (E) [28]. Reprinted with permission from the National Academy of Sciences. (c) Microfluidic device for rapid immobilization, imaging and sorting of *C. elegans* [38]. Movement of worms in the flow layer of the PDMS device (red) are controlled by the state of the pressure activated valves in the control layer (green). Flow of chilled liquid through the cooling channel in the control layer (blue) facilitates rapid immobilization of the worm in the imaging region. Reprinted with permission from Nature Publishing Group. d) A PDMS-based device for the trapping and orientation of individual *Drosophila melanogaster* embryos by passive hydrodynamics. The positioning of the embryos in a typically physically unstable and difficult to achieve orientation (long anterior-posterior axis perpendicular to coverslip) single-plane imaging of patterning events along the dorsoventral axis[36]. Reprinted with permission from Nature Publishing Group.

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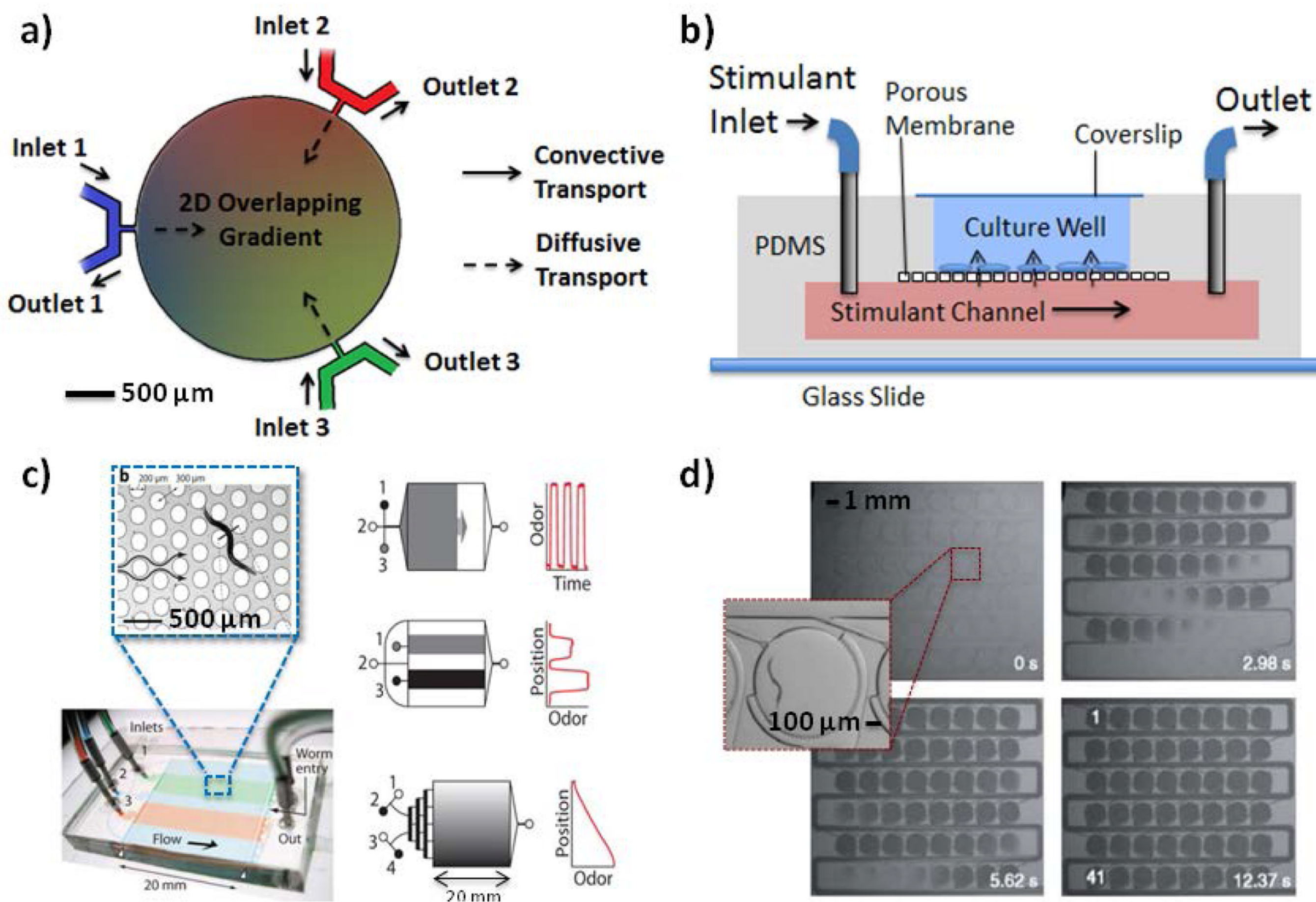

Figure 3.

Generating dynamic chemical stimuli for systems perturbation. a) A device for generating multiple overlapping gradients is used to probe the chemotactic response of the bacteria *P. aeriginosa* to glucose [57]. Three inputs diffuse radially into the culture chamber to create a two dimensional overlapping radial gradients. b) Device for delivering spatiotemporal signals to cells by diffusion from source bottom chamber through a porous polycarbonate membrane. Rapid convective transport in the stimulant channel permit high temporal resolution in stimulation while the porous membrane shields cells in the culture well from excessive shear [54]. c) A pillar array to accommodate the locomotive behaviors of *C. elegans* combined with spatiotemporal control of odorant stimulation to investigate behavioral response [62]. Reprinted with permission from Nature Publishing Group. d) Device for trapping individual *C. elegans* in discrete chambers and applying temporal chemical stimuli for longitudinal behavioral tracking [40]. Reprinted with permission from the Royal Society of Chemistry.

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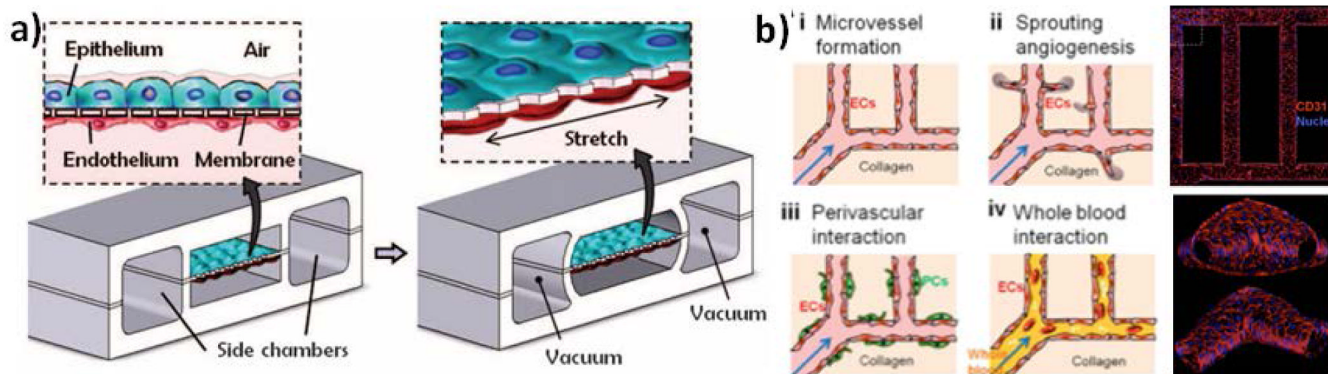

Figure 4.
Recapitulation of organs and tissue functions on microfluidic chips. a) Lung-on-a-chip. Endothelial cells and alveolar cells were seeded on either side of the thin perforated PDMS to mimic the endothelial-alveolar interface in the lungs. The device was made by sandwiching the perforated membrane between two PDMS chambers as shown in the figure, and flow could be introduced from the top or bottom of the chamber [94]. Reprinted with permission from The American Association for the Advancement of Science. b) Recapitulating microvasculature on a collagen matrix. Human umbilical vein endothelial cells were reconstituted onto a type I collagen matrix to demonstrate endothelial sprouting during angiogenesis in diseased and healthy microvessels, interaction of endothelial with perivascular cells and interaction of endothelial cells with whole blood. The microvessel network captures the branching nature of microvessels [88]. Reprinted with permission from the National Academy of Sciences.

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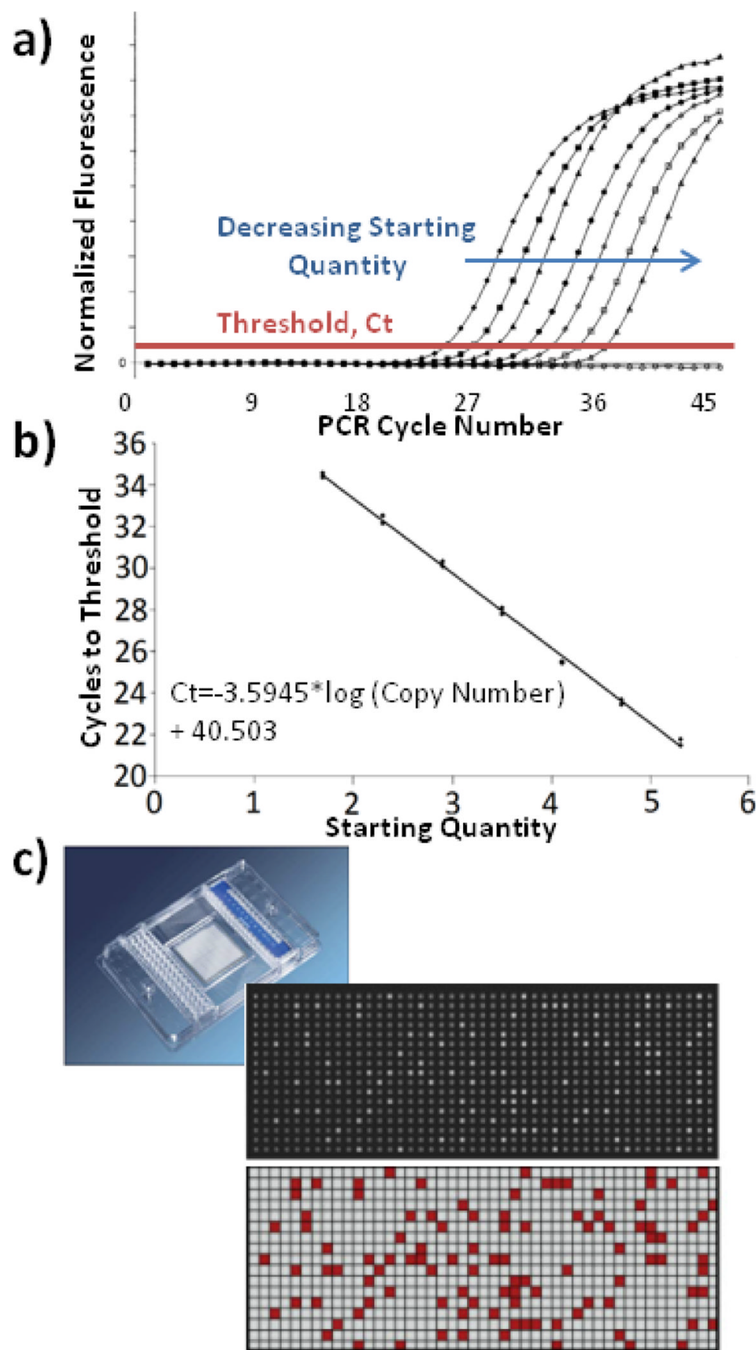

Figure 5.

Overcoming the exponential nature of PCR using digital PCR. a) Quantitative real time PCR relies on the ability to measure the progress of the PCR reaction via fluorescent detection of PCR products[96]. Since the PCR reaction is exponential, the fluorescence readout increases exponentially with cycle number. Reprinted with permission from Elsevier. b) The number of cycles to reach a fluorescence threshold can be used as an indicator of the starting quantity in the reaction. Due to the exponential nature of the PCR reaction (note logarithmic basis of the starting quantity axis), only two-fold differences in the starting quantity can only be resolved [96]. Reprinted with permission from Elsevier. c) A digital PCR microfluidic platform automates dilution and mixing of samples into 12 panels of 765 reaction chambers

using automated valve control. Due to the dilution of samples into many independent PCR reactions, quantities of the initial product can be counted with single molecule precision[97,98]. Reprinted with permission from John Wiley & Sons.

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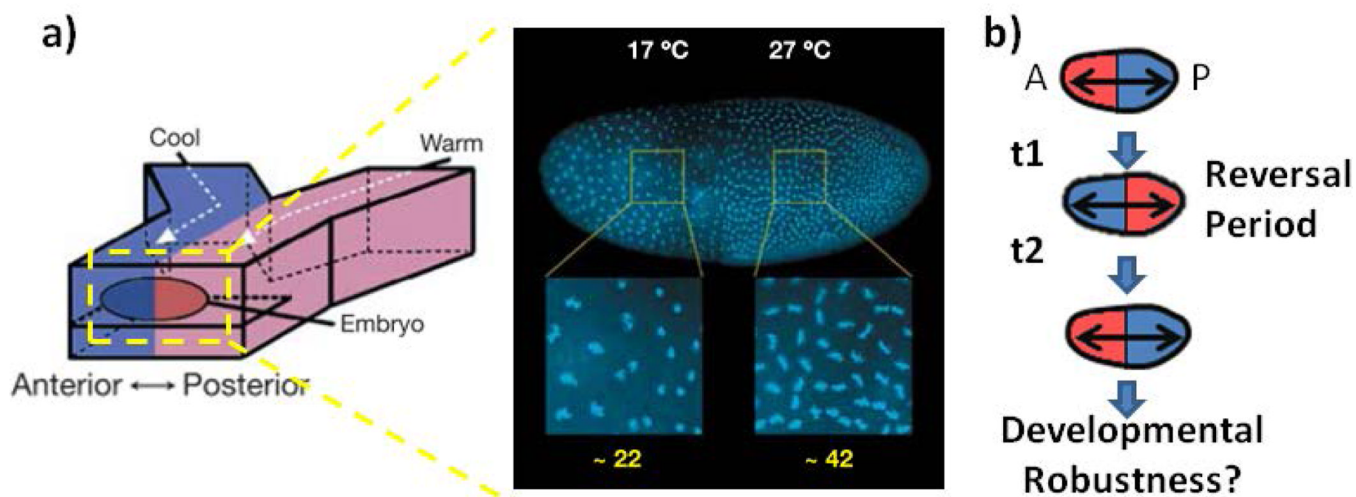

Figure 6.

Simple laminar flow for controlled disruption of developmental programs. a) A simple Y-channel microfluidic device is used to differentially control the temperature of two halves of a developing Drosophila embryo[64]. Reprinted with permission from Nature Publishing Group. b) This temperature difference allows for artificial disruption of developmental programs in a temporally and spatially controllable manner, testing the timing and nature of potential compensatory mechanisms for noise in development.