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Perspective

Microfluidics as an Emerging Precision Tool in Developmental Biology

Katharina F. Sonnen[1] and Christoph A. Merten[2,*]
[1]Hubrecht Institute, Utrecht, the Netherlands
[2]Genome Biology Unit, European Molecular Biology Laboratory (EMBL), Heidelberg, Germany
*Correspondence: merten@embl.de
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Microfluidics has become a precision tool in modern biology. It enables omics data to be obtained from individual cells, as compared to averaged signals from cell populations, and it allows manipulation of biological specimens in entirely new ways. Cells and organisms can be perturbed at extraordinary spatiotemporal resolution, revealing mechanistic insights that would otherwise remain hidden. In this perspective article, we discuss the current and future impact of microfluidic technology in the field of developmental biology. In addition, we provide detailed information on how to start using this technology even without prior experience.

Development requires tight regulation of thousands of cell fate decisions with high spatial as well as temporal control. This is guided by intrinsic factors, such as signaling pathways or mechanical cues, but also influenced by extrinsic factors from the environment. How these affect development and what the molecular mechanisms are remain key topics in the field of developmental biology. Conventional tools and methods often allow only averaged measurements, are challenging to use for analysis of dynamic systems at the multicellular level (e.g., imaging of certain motile specimen, modulation of oscillating pathways), and lack throughput. In brief, they frequently do not offer the precision required to gain mechanistic insights. This is exactly where microfluidics can make a significant difference by exploiting miniaturized dimensions and unique features of microscopic flow (such as laminar streams, stable gradients, monodisperse emulsions) for perturbing systems with extraordinary control. The aim of this Perspective article is to give an overview of the many exciting possibilities offered by microfluidics (Figure 1) and to encourage the readers in taking first steps toward the implementation of microfluidic technology in their own research. Based on length limitations, we cannot comprehensively discuss all the interesting applications of microfluidics and/or compare its benefits with that of other highly promising technologies such as optogenetics, advanced imaging, and imaging mass spectrometry (which all can potentially even be combined with microfluidic approaches) in full. Readers interested in these topics are pointed to more specific reviews (Buchberger et al., 2018; Canaria and Lansford, 2010; Kowalik and Chen, 2017; Passarelli and Ewing, 2013; Rost et al., 2017).

Defining Microfluidic Devices

Microfluidic devices typically consist of channels with diameters of tens to hundreds of micrometers (smaller dimensions can be realized in the z-dimension or when using high-end equipment). Most academic labs produce them by soft-lithography, a method pioneered in George Whitesides' lab (Qin et al., 2010). This procedure allows the preparation of customized chips made of transparent, flexible polydimethylsiloxane (PDMS; often bonded to a microscopic glass slide) within approximately 2 days and can be easily learned by novice users (see section below on how to access to the technology).

Different categories of microfluidic chips exist (Figure 2; Box 1), each with its own benefits and limitations, briefly introduced in the following paragraphs. In the simplest experimental setup (termed "continuous flow"), channels are filled or perfused with a single liquid, which is typically an aqueous solution such as culture medium for cells. These continuous flow devices already offer some remarkable advantages over conventional systems. For example, the channels can be designed so that individual specimens (e.g., single cells, embryos, or multicellular organisms) are trapped in a particular orientation for imaging. This process can also be parallelized (i.e., imaging multiple specimen within an array in parallel) to increase the throughput of image acquisition. Another unique feature of continuous flow at the microscale is the absence of turbulences or vortices. This means that different reagents can be infused in the form of laminar streams that do not mix (except based on diffusion, which as a relatively slow process can be ignored in continuously perfused systems). Such setups can be used to expose specific subregions of a single cell, a cell population, or a multicellular organism to particular reagents (e.g., inhibitors or stimuli). Continuous flow systems also allow pulse infusion of reagents in a precisely timed fashion or the generation of gradients that are stable over time.

Complementary to such applications, many biological studies require the compartmentalization of different samples, similar to parallelized assays carried out in microtiter plates. Using a microfluidic format has several advantages: Due to the small volumes (typically in the pico- to nanoliter range), high concentrations of analytes such as DNA, RNA, proteins, or metabolites can be obtained from single cells. Furthermore, miniaturization enables a much higher sample density, which in turn results in strongly increased throughput. The easiest way of generating microfluidic compartments is to simply shrink microtiter plates. Up to several ten thousand wells hosting individual cells or organisms can be fitted onto a single chip, herein referred to as "nanowells." These are typically loaded by pipetting a cell suspension under limiting dilution conditions (less than one cell or organism per well volume) onto the chip surface.

A more sophisticated way of generating compartments is the use of multilayer chips and valves. In this setup, perpendicular "control channels" are implemented on top of the fluidic

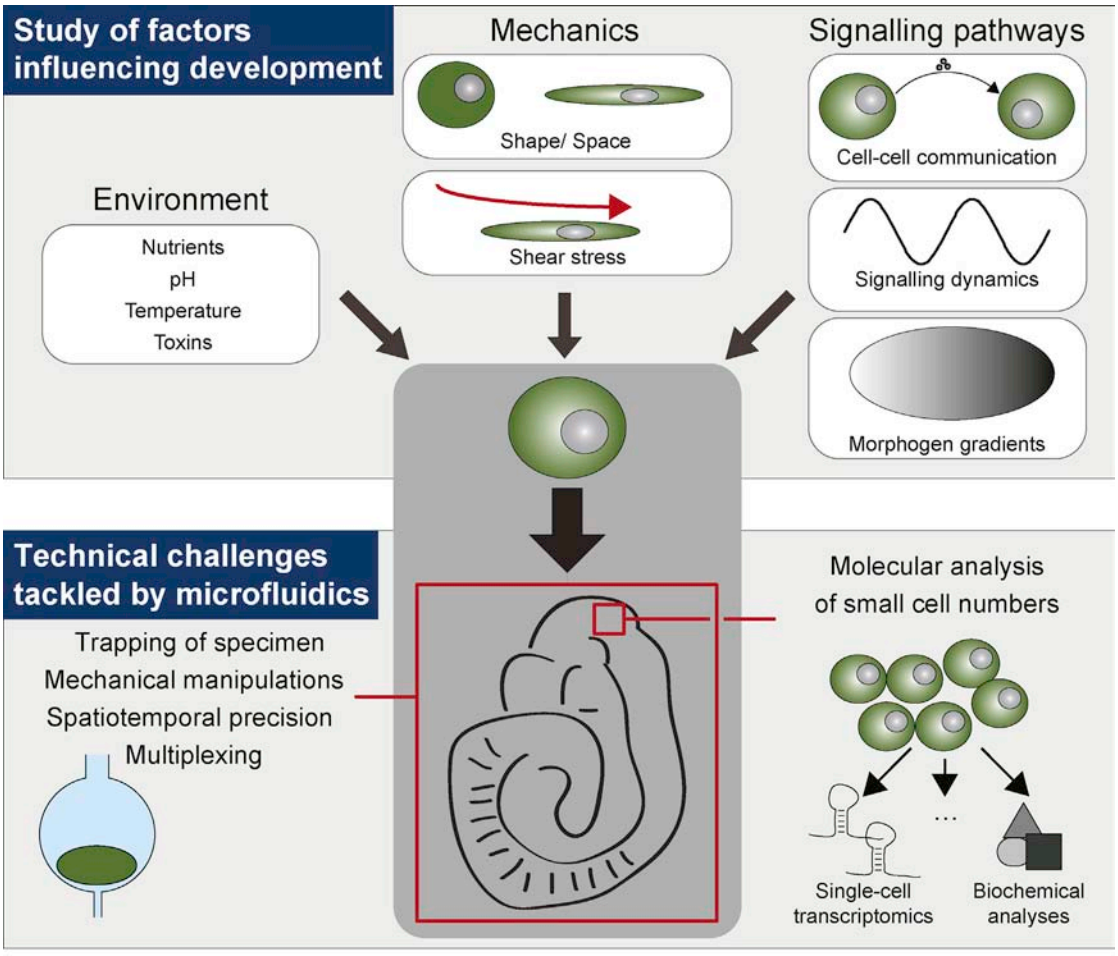


Figure 1. Microfluidic Applications in Developmental Biology
The upper panel summarizes factors influencing development that can be specifically analyzed by microfluidics, such as environment, mechanics, and signaling pathways. The lower panel highlights benefits of microfluidic technology for studying developmental processes.

channels (Unger et al., 2000). Given that the PDMS composing the chips is highly flexible, filling the control channels with pressurized air results in the pinching off of a section of the fluidic channels below, which is then no longer in fluidic connection with the remaining channel network. Moreover, pressurizing three serial control channels in a peristaltic fashion (sequentially one after another) can be exploited to pump reagents through the fluidic channels below. Further reagents can be added in a programmable way and compartments can be generated, fused, and resolved on demand. This way entire experimental workflows can be automated to allow the processing of several hundred to a few thousand samples in parallel (Thorsen et al., 2002). The Fluidigm single-cell transcriptomics and qPCR platforms are based on this type of microfluidic chip.

Yet another way of generating compartments for biological assays is the use of droplet microfluidics (Umbanhowar et al., 2000). In this method, aqueous droplets surrounded by oil are generated by co-injecting the sample phase and an immiscible oil phase into the device. When using special geometries such as a T-junction or flow-focusing channel networks (Shembekar et al., 2016), this results in the generation of hundreds to thousands of droplets per second, typically with picoliter volumes. Cells can be encapsulated together with different reagents, and the droplets can even be incubated off-chip (due to the use of surfactants, no or only very few droplets fuse) (Clausell-Tormos et al., 2008). The most prominent application for this type of technology is single-cell transcriptomics and genomics (e.g., the commercial 10x Genomics platform, Table 2), but phenotypic assays can also be carried out (Shembekar et al., 2016). This is facilitated by the fact that droplets can be fused to add further reagents and sorted based on fluorescence. In contrast to fluorescence-activated cell sorting (FACS), not only cells but rather entire assay compartments containing additional soluble factors (assay reagents, metabolites), secreted proteins (such as antibodies), or different cell types (e.g., for interaction studies) can be sorted.

In summary, many different microfluidic formats exist, each with specific advantages and different requirements in terms of equipment. For example, experiments exploiting continuous flow devices, nanowells, or simple droplet makers can be carried out in non-specialist labs and do not require particularly expensive instrumentation. In contrast to this, automated valve- or droplet-based microfluidic platforms necessitate

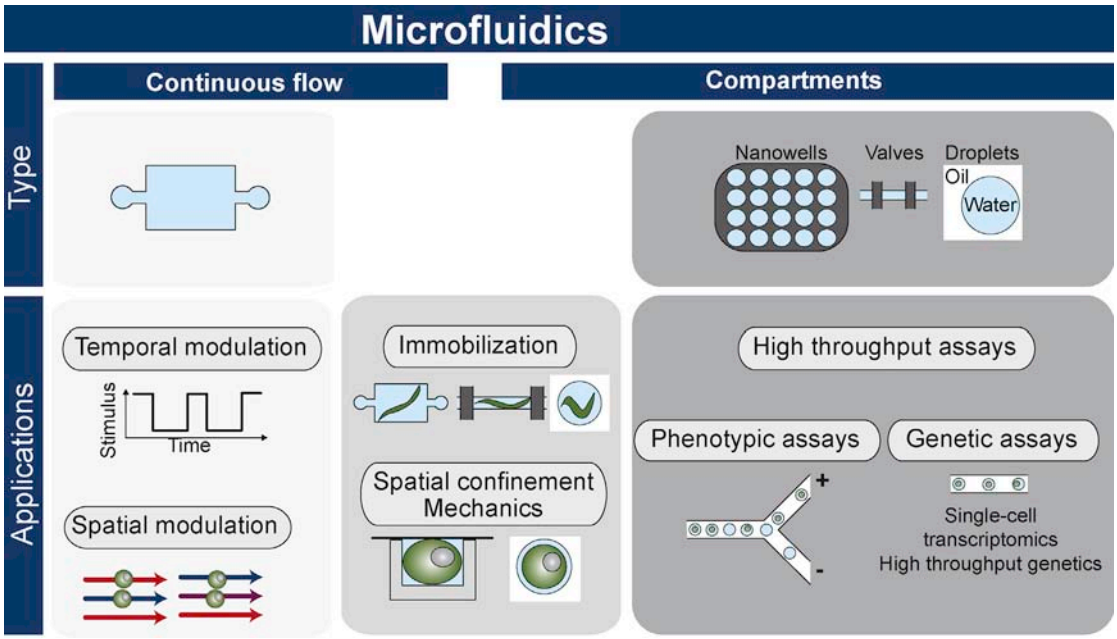


Figure 2. Different Microfluidic Formats and Application Fields

Microfluidic systems can be subdivided into continuous-flow and compartmentalized formats. Continuous-flow microfluidics indicates constant flow of a liquid, generally an aqueous solution. These are, for instance, used for temporal and spatial modulation of cellular parameters. In contrast, compartments imply the generation of small reaction chambers either by miniaturizing microtiter plates down to the nanowell format, by closing a channel with valves, or by using water-in-oil droplets. Note that the simplest way of generating valves is to use a multi-layered elastic-chip design with orthogonal control channels (orange) above the fluidic channel (light blue); the main channel is made of flexible polymer and closed by applying pressure to the above-lying control channels, which in turn expand and squeeze off the fluidic channel below. Compartmentalized microfluidic formats have specific advantages for high-throughput assays. Both "continuous-flow" microfluidics and "compartments" are used for spatial fixation and confinement.

more sophisticated and costly devices. This should be taken into account when implementing the technology in the lab and addressing questions in developmental biology as detailed below. Microfluidic applications can be broadly subdivided into two categories:

(1) Enabling technology: precise modulation of biological parameters; microfluidics allows the testing of hypotheses on the function of these parameters, such as signaling dynamics (see below) or the influence of cell size on the size of the mitotic spindle (Box 2).
(2) Multiplexing: microfluidics allows parallelization and automation of procedures and experiments for high-throughput approaches, such as trapping and orientation of embryos (Box 3) or single-cell sequencing (see below).

In Table 1 we have highlighted advantages, disadvantages, and challenges of microfluidic applications.

On-Chip Culture of Embryos and Tissues

Before discussing specific applications of microfluidics in developmental biology, we want to emphasize that the possibility of culturing whole organisms or selected tissues of various model organisms on-chip has been demonstrated many times. For instance, *Drosophila melanogaster* embryos have been cultured in a continuous flow device for several hours (Lucchetta et al., 2005). Similarly, *Caenorhabditis elegans* can survive on a continuous-flow microfluidic chip for several days and develop from larval stages, starting from the L1 stage, toward adulthood (Chokshi et al., 2009; Gilleland et al., 2010; Keil et al., 2017). Complementary to this, we have demonstrated the possibility to grow live *C. elegans* in water-in-oil droplets (Clausell-Tormos et al., 2008). Starting from eggs, these organisms undergo a complete life cycle, generate offspring, and survive within droplets for 6–9 days.

Dissected tissues and *in vitro* tissue models can also be grown on-chip. We have cultured primary mouse embryo tissue (from E10.5 embryos) on continuous-flow chips to study signaling dynamics governing segmentation during embryogenesis (Sonnen et al., 2018). Similarly, *in vitro* model systems or organoids of several organ types have been grown on chip (reviewed in Bhatia and Ingber, 2014; van Duinen et al., 2015).

Thus, developing tissues and organisms can survive and physiologically develop in microfluidic systems. This emphasizes the feasibility to combine the technical advantage of microfluidic precision with the investigation of multicellular phenomena. In the following sections, we will discuss examples of how microfluidics has advanced the investigation of development in recent years, and we will also highlight its potential for future studies.

Microfluidic Device Applications

In-Toto *Imaging of Developing Embryos*

Technological advances in the field of microscopy allow imaging at high spatiotemporal resolution. These methods have been used to follow development of selected organisms, such as flies (Huisken et al., 2004; Keller and Stelzer, 2008), fish (Keller et al.,

Box 1. Microfluidics Glossary

Barcode. Unique DNA sequence which is used to label individual cells in droplet microfluidics-based screens.
Droplet microfluidics. Microfluidic approach in which water-in-oil droplets are used as small experimental units.
Flow-based microfluidics. Microfluidic approach where aqueous solutions flow through microfluidic channels, in which experiments, e.g., cell culture, are performed.
Flow-focusing design. A channel geometry in which the central stream is focused by the infusion of two perpendicular streams. When using two immiscible phases, this can be used to produce droplets.
Laminar flow. Non-turbulent flow, which is characteristic for microfluidic systems. Parallel liquid streams do not mix, except based on the diffusion of molecules.
Lithography. A process in which high-resolution photomasks are used to pattern photoresists.
Monodisperse. Equal or similar in size and volume.
Optical tweezer. A highly focused laser beam allowing to drag dielectric objects with a refractive index higher than that of the surrounding media into the focal point.
PDMS. Polydimethylsiloxane is a transparent and flexible polymer widely used for the production of microfluidic chips.
Photoresist. A compound that polymerizes either upon exposure to (negative photoresist) or shielding from (positive photoresist) a light source. Using a high-resolution photomask with channel patterns allows the manufacture of molds for microfluidic chips.
Reynolds number (*Re*). Describes the ratio of inertial forces to viscous forces (the latter dominating in microfluidic systems).

$$Re = \frac{pvl}{\mu},$$

with p being the density of the liquid, v = speed of the fluid, l = travelled length of the fluid and μ = dynamic viscosity of the fluid. At low *Re*, flow is laminar. In microfluidic systems, *Re* is typically <<2,000, and hence all flow is laminar.
T-junction. A channel geometry that allows the generation of droplets when co-infusing two immiscible phases.

2008), and mice (Strnad et al., 2016; Udan et al., 2014). However, in practice, real-time imaging of living multicellular organisms and developing embryos remains challenging. One key issue is that often a live sample will be constantly in motion, making long-term imaging at high spatial resolution, let alone single-cell tracking, challenging. For zebrafish or medaka, for example, this problem was tackled by chemical inhibition of movement (Readman et al., 2013, 2017). This has proven to be useful to address many scientific questions in the field of developmental biology. However, permanent chemical inhibition of movement might have adverse or detrimental effects on development (see study of mechanics using microfluidics below), and for several organisms, useful chemical compounds for immobilization have not yet been identified. For these reasons, microfluidic devices have been utilized extensively for imaging of both *C. elegans* and *Drosophila*. This has been done for two aims: to enable high-resolution imaging at single-cell resolution and to multiplex such long-term imaging.

Toward the first aim, development of single *C. elegans* has been followed by high-resolution imaging for up to 72 h (Chokshi et al., 2009; Gilleland et al., 2010; Keil et al., 2017). *C. elegans* can be fixed in a microfluidic chip by physical pressure, which pushes the organism against the wall of the microfluidic device. However, permanent pressure is deleterious. To circumvent this, in a recent study, Keil and colleagues cultured *C. elegans* on chip and gently immobilized the worm for 10 s every 8 min. During each immobilization step, real-time imaging was performed. In combination with automatic image registration, this even allowed single-cell tracking to follow, for instance, neuronal differentiation and neurite outgrowth in the developing animal (Keil et al., 2017). Microfluidic devices have also been used to immobilize zebrafish for manipulation and long-term recording of neuronal activity (Candelier et al., 2015).

For the second aim, a challenge for real-time imaging in developmental biology is limited throughput. In this regard, researchers have used microfluidic devices to parallelize trapping and real-time imaging of organisms, such as *C. elegans* (Cornaglia et al., 2015; Mondal et al., 2016) or *D. melanogaster* (Chung et al., 2011; Levario et al., 2013). This is achieved by immobilizing multiple animals in neighboring channels or fluidic traps (Box 3). For instance, Cornaglia et al. have generated a single microfluidic device with the possibility to culture both adult worms and larvae within different compartments of the chip (Cornaglia et al., 2015). A suspension of differently sized worms was loaded onto the chip and sorted by age, making use of hydrodynamics and on-chip filters. While adult worms were trapped in the first chamber, smaller animals could pass through the narrow channels (which functions as filter) to reach the array of embryo traps. By fluid flow, L4 larvae were pushed into the traps with a diameter of 35 μm. Such parallelization of on-chip immobilization and imaging can also be combined with high-throughput screens, which will be discussed below.

A commercial system for parallelized embryo trapping and imaging is available from Dolomite (Table 2). This chip harbors 252 well chambers with a diameter of 120 μm, within which, for instance, early mouse embryos can be cultured. In the future, similar microfluidic devices will certainly make long-term imaging of other organisms possible, such as *Nematostella* or *Hydra*, for which immobilization remains a challenge to date. This way, microfluidics will enable the investigation of the dynamics of development of further complex organisms.

Studying Mechanical Forces and Spatial Constraints

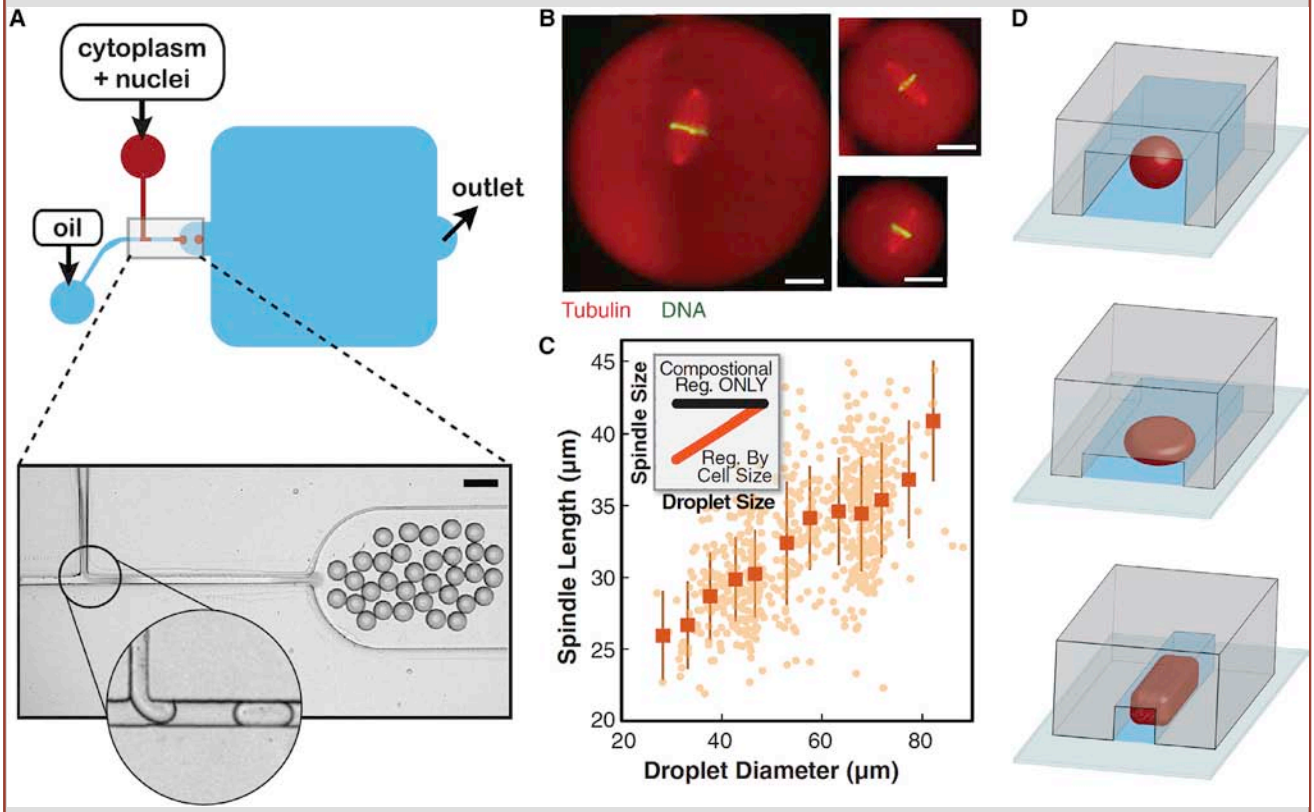
Mechanical cues play an important role in embryonic development (Heisenberg and Bellaïche, 2013). For example, lateral cell deformation by only about 10% is sufficient to induce the

Box 2. Controlling Cell Size and Cell Shape with Microfluidics

How the size of macromolecular structures like the mitotic spindle is controlled within animal cells has been a long-standing question in the field. Especially during early embryonic development, cell size decreases quickly and is correlated with a reduction in spindle size (Wühr et al., 2008). A correlation between spindle size and cell size or shape has been observed in various species (e.g., Courtois et al., 2012; Hara and Kimura, 2009). Yet, whether cell morphology has a functional impact on spindle size can only be tested if cell size or cell shape are specifically altered without affecting the chemical composition of the cell. Thus, genetic or chemical modulations of intracellular structures do not unequivocally establish a functional link between cell morphology and spindle size.

Good et al. (2013) and Hazel et al. (2013) have used droplet-microfluidics to spatially confine *Xenopus* egg extracts including nuclei within droplets of defined size. Within these ''artificial cells,'' spindle size depended on the volume of confined cytoplasm. Moreover, they used microfluidic channels of different dimensions to additionally control the shape of their defined ''cells.'' This indicated that spindle size depended on cell volume rather than cell shape (in the absence of cytoskeletal attachments to the cell cortex).

Thus, the high precision with which cytoplasmic volume and shape can specifically be changed by microfluidics, allowed to provide direct evidence for mitotic spindles scaling to cell volume. To reveal the molecular mechanism of spindle size control, such microfluidic experiments have to be complemented with perturbations of the intracellular machinery using chemical or genetic means.



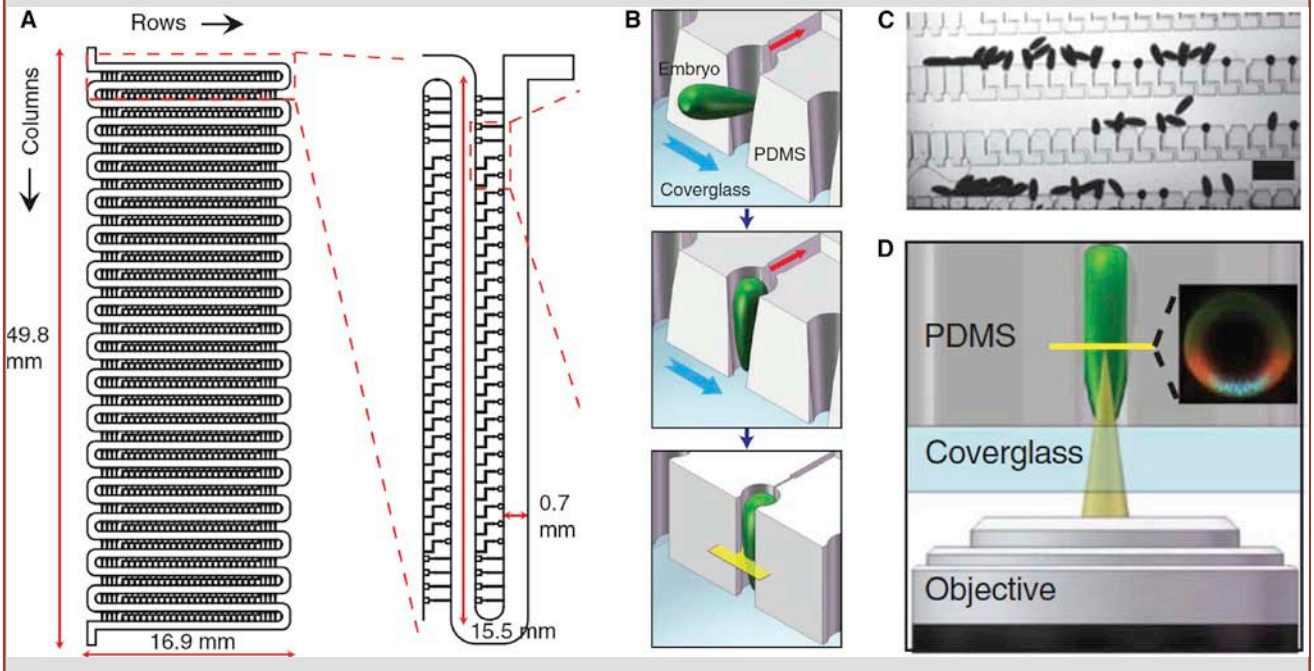
(A) Droplet microfluidics to encapsulate *Xenopus laevis* cytoplasm and nuclei into droplets of defined size. Upper panel: schematic representation of experimental setup with a droplet generator, in which cytoplasm-in-oil droplets are generated, and collection chamber. Lower panel: picture of microfluidic chip. (B) Scheme of experimental design to change shape of generated droplets within microfluidic channels. (C) Images of encapsulated spindles within droplets of different diameter. (D) Quantification of spindle length relative to droplet size revealed scaling of spindle-to-cell volume. Scale bar represents 20 μm. Adapted from Hazel et al. (2013) (A) and Good et al. (2013) (B–D), reprinted and modified with permission from AAAS.

expression of specific genes in *D. melanogaster* (e.g., Twist), ultimately mediating the ventralization of the embryo (Farge, 2003). Similarly, it is well known that shear forces have direct impact on the differentiation of stem cells into endothelial cells (Datta et al., 2006; Wang et al., 2005; Yamamoto et al., 2005). These insights are a result of pioneering mechanobiology studies in the

Box 3. Multiplexed Phenotypic Analyses of Developing Embryos

Morphogen gradients guide spatial patterning in developing embryos. A model system for the study of morphogen gradients is *Drosophila melanogaster* in which multiple gradients determine, e.g., the dorsoventral and posterior-anterior axes (Gilbert, 2010). For visualization of these gradients and the resulting patterns by microscopy, embryos have to be specifically oriented. Manual orientation is a tedious process, low in throughput, and prone to variability. This is especially true for end-on orientation of egg-shaped fly embryos, which is required to analyze dorsoventral patterning. To tackle these problems, Chung et al. have established a microfluidic system that allows the automatic end-on orientation and trapping of more than 700 embryos, which allows quantification of dorsoventral processes at high throughput (Chung et al., 2011). Traps along a serpentine channel have the dimensions to exactly accommodate a single embryo with the posterior-anterior axis in vertical position (see A). They are simply loaded by flushing an embryo suspension through the chip using a syringe pump. Interestingly, due to the flexibility of the PDMS chip, the hydrodynamics during the loading process induce a transient opening of the traps and allow the embryos to enter. After loading, the openings reverse in size and trap the embryos even without continuous flow (see B and C). This is very useful for handling of the chip and imaging since embryos are stably localized.

The setup allows precise quantification of molecular processes in hundreds of developing embryos in parallel using fluorescence microscopy (see D). For instance, the dynamics of the ERK (extracellular signal-regulated kinase) signaling pathway have been analyzed in the developing embryo (Goyal et al., 2017; Lim et al., 2015). One can also envision a chip design with multiple separate channels. This enables the comparison and quantification of cellular parameters of, for example, genetically or chemically perturbed embryos. In principle, this approach can be used for various non-motile or immobilized embryo types, but it has to be tested for individual species whether the hydrodynamic pressure required for trapping can be tolerated by the embryo. *Drosophila* has been the standard model system for identifying the genes required for development using genetic screens (e.g., Nüsslein-Volhard and Wieschaus, 1980). To also implement high-throughput phenotypic analysis, one has to be able to automatically orient and image the embryos. However, the current chip design does not yet allow recovering individual embryos for characterization to correlate phenotype and genotype. Nonetheless, such a system would be highly useful for many researchers. Loading of the chip seems to be straightforward and only requires a simple syringe pump (Levario et al., 2013). However, the authors also note that designing such chips is difficult for novices, since hydrodynamic flow conditions essential for trapping depend greatly on the exact chip dimensions (Levario et al., 2013). Therefore, commercialization of the molds and/or chips or deposition of the designs at dolomite or others could bring this system to the standard developmental biology lab.



(A) Top-view of the chip design including a serpentine channel with traps for individual embryos along the channel. (B) Scheme illustrating the embryo trapping principle: flow directs the embryo into the trap in a vertical orientation (upper and middle panel). When hydrodynamic pressure is reduced, the "trap contracts," which maintains the embryo stably in this position. (C) Loading of the chip is achieved by flushing embryos through the serpentine channel. Scale bar represents 800 μm. (D) Embryos can be imaged from below the chip by, for example, fluorescence microscopy to visualize dorsoventral gradients. (A), (B), and (D) adapted from Chung et al. (2011), and (C) adapted from Levario et al. (2013), reprinted and modified with permission from Springer Nature.

Table 1. Challenges and Possibilities of Different Microfluidic Production Methods and Applications

	Advantages	Disadvantages
Manufacturing molds by soft lithography	resolution to μm scale possible. allows generation of multi-layered designs or curved channels. fast prototyping and optimization of chip designs. channel height limited to hundreds of μm.	requires specific equipment and educated personnel. expensive and tedious.
Manufacturing molds by 3D printing	resolution to tens of μm possible. cheap and easy. easy exchange of designs within the community.	channel height no less than ~50 μm.
3D-laser lithography (e.g., Nanoscribe)	unprecedented resolution (sub μm in all dimensions), generation of true 3D-features including gradually changing channel dimensions.	expensive (~$220,000). slow. poorly suited for deep channels (>100 μm).
Continuous-flow microfluidics	allows high temporal and spatial precision of flow conditions. can easily be adapted for manipulation of cellular events with different molecules. commercial solutions for simple applications available.	low throughput: multiplexing and parallelization difficult. temporal resolution is limited by removal of substances from cells or tissues. spatial precision typically not at single-cell resolution. laminar flow requires high flow rates, which can be detrimental for cells. Optogenetics could be an alternative.
Droplet microfluidics	enables high-throughput screens. commercial systems for some applications available (see below).	establishment of new approaches requires expensive laboratory equipment and technically skilled personnel.

pre-microfluidics era, using experimental setups that generate directional forces with piezoelectric actuators (e.g., pushing cover slides onto cells) or shear forces within parallel plate systems and perfusion reactors. This nicely illustrates the need for customized tools to obtain details on mechanotransduction pathways.

Microfluidic technology can simplify mechanobiology experiments and significantly increase flexibility. For example, PDMS chips with custom dimensions can be used to expose entire tissue sections or even subcellular organelles to mechanical forces: Ingber and co-workers used a relatively large, macroscopic PDMS-stamp to apply forces of about 1 kPa to mesenchymal cells, thus mimicking mesenchymal condensation (Mammoto et al., 2011). They found that a mechanical stimulus is sufficient to suppress the signaling molecule RhoA in mice, which in turn induces tooth-specific cell fate switching. Similarly, elastic PDMS chips can also be used to measure mechanical forces (e.g., based on the deformation of PDMS posts) or to stretch entire cell populations (e.g., by applying pressure to channels below a PDMS surface on which cells are grown). Jianping Fu and coworkers have used such approaches to show that mechanical forces are sufficient to induce differentiation of human pluripotent stem cells into neuroectoderm, based on BMP-SMAD signaling (Xue et al., 2018). Using much smaller PDMS chips, the Whitesides group could quantitatively determine the minimal surface area required by individual adherent cells to survive. They could even reveal preferred geometrical shapes of these minimal footprints (Chen et al., 1997; Singhvi et al., 1994), something that is entirely impossible using conventional technologies.

While those studies were based on microscopic patterns generated by lithographic methods (on the surface of a chip) and hence on mechanical forces generated by the cells themselves (upon attachment), mechanical forces in the range of picoNewtons can also be applied directly to cells and parts thereof using optical tweezers (Kuo, 2001; Zhang and Liu, 2008). Such devices allow the researcher to grab individual cells (also in a tissue context) or beads bound to particular membrane regions and pull them into any desired direction. The technology is based on highly focused laser beams and an optical effect dragging dielectric objects with a refractive index higher than that of the surrounding media into the center of the laser beam (in a 3D space). While this allows maximal flexibility, the technical requirements are significant. Commercial optical tweezers cost about 50,000 US dollars (e.g., the Thorlabs system), and quantitative force measurements require further upgrades. As an alternative to using microfluidic technology, the role of mechanical forces in developmental biology can also be studied using optogenetic approaches. These allow perturbation of cell contractility or mechanotransduction pathways in individual cells of a developing embryo at very high spatiotemporal resolution, thus revealing mechanistic insights into morphogenetic processes (Guglielmi et al., 2015; Guglielmi and De Renzis, 2017; Izquierdo et al., 2018). Optogenetic approaches seem to be particularly beneficial for whole-organism studies, while microfluidic technology is potentially advantageous in applying precise quantitative forces to individual cells.

Many developmental biology studies do not require this level of control and only require imposing spatial constraints or providing 3D scaffolds, which can easily be implemented using microfluidic technology. For example, we have previously used microfluidic approaches to reveal how scaling of the cell nucleus with the cytoplasmic volume is achieved in *Xenopus laevis* (Hara and Merten, 2015). It has long been known that during early embryonic development, rapid reductive cell divisions cause the generation of very small cells, whose nuclei are scaled down accordingly so that the ratio of the cytoplasmic to nuclear volume remains unaffected (Box 2). However, the exact

Table 2. Commercial Solutions for Microfluidic Applications

Biological Question	Microfluidic Format	Company
Chemotaxis, perfusion	continuous-flow, spatial gradients	Gradientech, Ibidi
Mechanical force measurement, manipulations with optical tweezers	continuous-flow	Elliot Scientific, Lumicks, Thorlabs
Cell/embryo immobilization, compartmentalization, mechanical constraints	continuous-flow	Dolomite, Fluigent, Ibidi
Single-cell transcriptomics	droplet, nanowells or valves	10x Genomics, BD, Fluidigm
High-throughput phenotypic droplet assays	droplet	Sphere Fluidics
Single-cell western blotting	nanowells	Zephyrus Biosciences

mechanisms of this remained largely elusive. Microfluidics allowed the trapping of *in vitro* reconstituted nuclei in channels of different diameters for assessment of how they grow under different conditions. It also allowed excess material for nuclear growth to be provided independent of the diffusion limit of biomolecules (by constantly perfusing trapped nuclei with egg extract). In consequence, any remaining biological factors could be analyzed in detail, revealing microtubular transport as the limiting process: whenever large cytoplasmic volumes are available, the microtubular network expands and recruits more material from even more distant spaces. In this way, the size of the cell can be sensed and translated into suitable nuclear dimensions.

In the future, it will be very interesting to determine if the expression of particular genes is also coupled to cellular or nuclear size, which might reveal additional, active regulatory mechanisms. Such studies could greatly benefit from droplet-based microfluidic systems for transcriptomic analyses, as discussed further below.

Microfluidics has also helped reveal the effect of spatial constraints and cell deformation on cell migration. For example, it has been shown that confining immune cells in narrow channels increases their migratory phenotype in an adhesion-independent manner (Irimia et al., 2007; Lämmermann et al., 2008). Similarly, the ability of cancer cells to enter narrow microfluidic channels has been correlated with their ability to form metastases (Lautenschläger et al., 2009; Rolli et al., 2010).

Having these microfluidic tools on hand makes it now seem straightforward to also use them for analyzing cell migration during embryonal development and organ formation. This is not only possible for assays at the single-cell level but also for studies looking at cellular behavior within small-cell populations. For example, the Viasnoff lab established a microfluidic assay to study lumen elongation in a minimal organ approach (Li et al., 2016). In particular, they established nanowells in which liver cells could attach at the bottom and/or the side of the wells. By optionally also overlaying the cell cultures with extracellular matrix (ECM), the authors generated a variety of different geometrical setups and quantitatively measured parameters such as lumen size and shape and the position of the lumen edges. The results clearly showed that the ECM guides lumen elongation in a directional way by inducing anisotropic intercellular mechanical tension.

Investigating Signaling Pathways in Developing Embryos

Signaling pathways are central in guiding development and coordinating cell fate decisions within the developing organism. However, to study signaling during development, two major challenges have to be tackled. First, for functional investigation of signaling, it is critical to recapitulate or only subtly modulate the spatial as well as temporal organization of signaling pathways. Second, to dissect the function at the cellular or even molecular level, one has to be able to perform biochemical or genomic assays with very low input levels. Both challenges can be addressed by microfluidics.

Spatially Modulated Perturbations

The role of spatially organized signaling pathways, e.g., local availability of ligands or gradients, has been studied in the context of developmental biology for decades. In 1952, Alan Turing coined the term ‘‘morphogens,’’ which provides spatial information to the embryo (Turing, 1952). However, their precise mode of action remains difficult to decipher to this day. To investigate the effect of spatially confined signaling proteins, researchers have, for example, placed agonist-soaked beads into developing tissues (Niswander et al., 1993). However, exact concentration and spread of the molecule are difficult to control. In contrast, microfluidics allows manipulation with high spatial precision: Due to the small dimensions of microfluidic devices, liquid flow is usually laminar rather than turbulent. In fluid dynamics, laminar flow describes liquid flow without lateral mixing of neighboring layers (Figure 2). This is dependent on flow rate, viscosity of the liquid, and length of the channel, which is summarized by the Reynolds number (see Box 1). Making use of laminar flow within the microfluidic chamber is a key advantage for developmental biologists, since asymmetries are prerequisites for spatial organization of development. With microfluidics, signaling modulators can even be applied with subcellular precision, depending on chip dimensions and flow conditions (Takayama et al., 2001). For instance, in a proof-of-principle experiment Takayama et al. have applied MitoTracker Green to one side of the cell and MitoTracker Red to the other side, which resulted in the staining of the two sides of a single cell in different colors (Takayama et al., 2001, 2003) (Figure 3A). However, modulation of signaling pathways with cellular or even sub-cellular precision within a 3D tissue or even a whole organism remains very challenging using microfluidics, since molecules in the liquid flow do not penetrate deep into the tissue. Furthermore, large objects might require channel dimensions and flow rates for which laminar flow is no longer obtained. For such applications, optogenetics in combination with multi-photon microscopy is the better choice (de la Cova et al., 2017; Guglielmi et al., 2015; Imayoshi et al.,

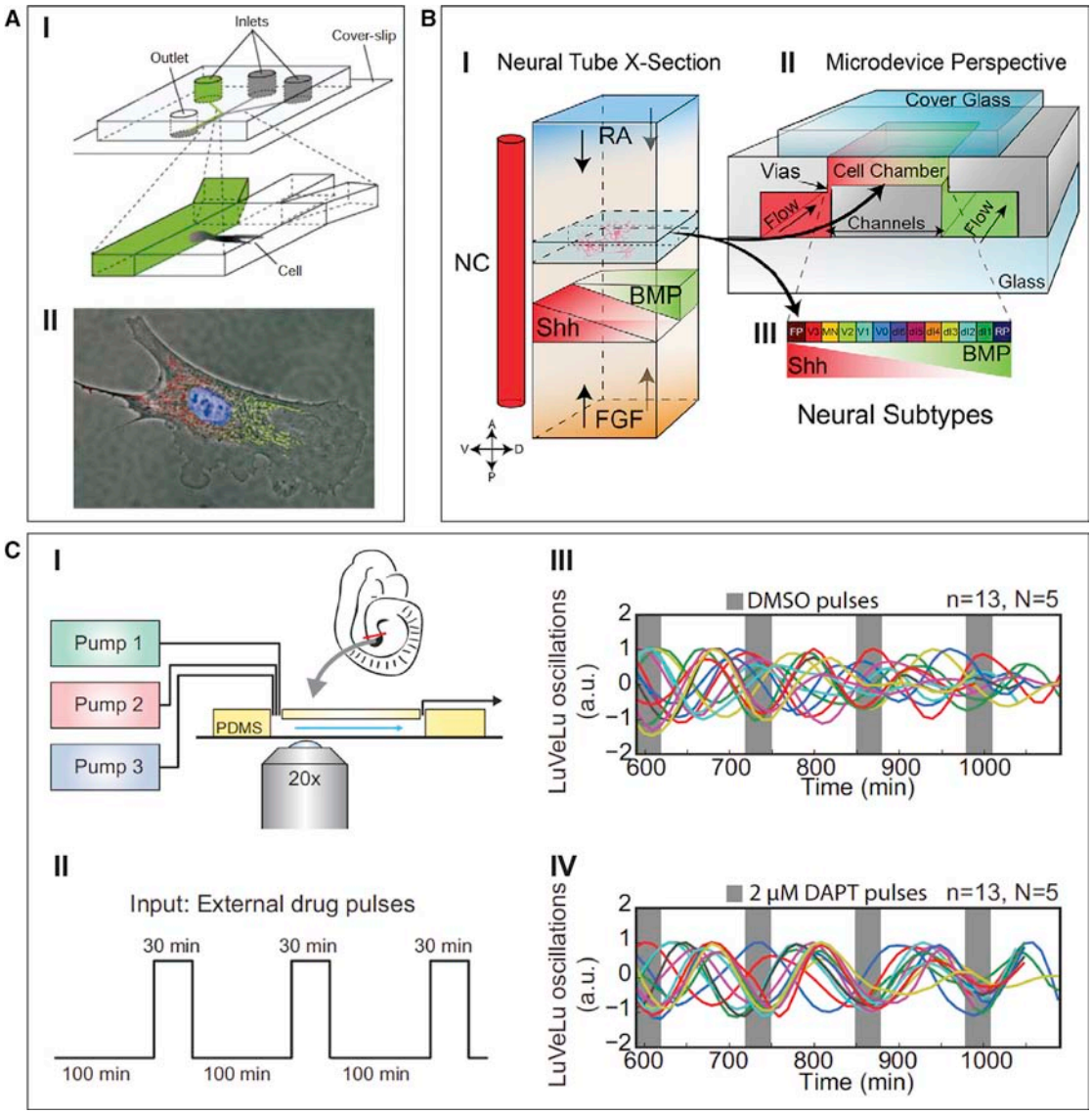


Figure 3. Spatial and Temporal Modulation of Signaling Pathways

(A) Subcellular targeting of drugs by laminar flow. (I) Schematic representation of chip design. The lower panel is a magnification of the microfluidic channel. Laminar flow within the main channel leads to neighboring streams that do not mix. (II) This setup allows staining of a single cell with MitoTracker red or green at opposite sides, respectively (adapted from Takayama et al., 2001). Reprinted and modified by permission from Springer Nature.

(B) Microfluidics has been used to recapitulate opposing and orthogonal signaling gradients in the developing neural tube (I.). (II) The microfluidic chip has a multi-layered design with flow channels on either side and a cell incubation chamber in the middle. Within the main chamber, gradients of signaling modulators are generated by diffusion. (III) The chip was used to study the effect of signaling gradients on differentiation into different cell types (adapted from Demers et al., 2016). Reprinted and modified from *Development* according to Creative Commons 4.0.

(C) Study of signaling dynamics by microfluidics-based entrainment. (I) Mouse embryonic tissue is grown on a microfluidic chip, which allows real-time imaging and simultaneous manipulation of signaling pathways using programmable pumps. (II) To control signaling oscillations, periodic pulses of pathway modulators are applied. (III. and IV) Detrended Notch signaling oscillations in control (III) and treated (IV) samples are shown. Note that in treated samples oscillations become synchronized, while oscillations are not synchronized in control samples (adapted from Sonnen et al., 2018). Reprinted and modified from *Cell* according to Creative Commons Attribution CC BY-NC-ND 4.0.

2013). For a review on optogenetic systems, see Toettcher et al. (2011) or Tischer and Weiner (2014).

Cells can also be cultured in spatial gradients of signaling molecules. Generation of graded molecule concentrations with defined upper and lower limits can be achieved using two approaches: flow based or diffusion based. In flow-based devices, liquid streams from separate inlets are mixed partially before entering the main incubation chamber: This is done by either using a simple T-junction or a network of intersecting delay lines (Dertinger et al., 2001; Irimia et al., 2006; Jeon et al., 2000). The resulting gradient is then maintained within the main experimental chamber by laminar flow (see above). In contrast, in diffusion-based devices, there is no active flow in the main chamber, where cells are cultured. On either side of this chamber, there are flow channels with constant liquid flow. From there, molecules can diffuse into the main chamber.

Thus, a gradient is generated by diffusion of molecules into the central culture chamber (Frank and Tay, 2013) (see Figure 3B). Today, commercial solutions (e.g., from ibidi or Gradientech, Table 2) are available, which allow gradient generation and chemotaxis to be studied without the need for microfluidic pumps. In addition, newer developments combine on-chip gradient generation with surface immobilization of signaling molecules, which allow study of the influence of both bound and soluble components at the same time (Schwarz et al., 2016), or with ECMs to study cellular processes in 3D structures (Frank and Tay, 2013; Frick et al., 2018).

One application of microfluidic gradient generation is the investigation of chemotaxis and cell migration (reviewed in Wu et al., 2013). Li Jeon et al. (Nature Biotechnology 2002) have made use of flow-based gradient generation to investigate chemotaxis of neutrophils within an interleukin-8 gradient (Li Jeon et al., 2002). Interestingly, the microfluidic device allows for the dynamic modulation of a molecular gradient to test how cells react to sudden changes in the gradient. Such setups are also highly valuable for unraveling the impact of chemotaxis on development. For instance, a microfluidic approach was applied to study growth and axon guidance of hippocampal neurons (Bhattacharjee and Folch, 2017). To this end, the authors cultured dissociated E18.5 mouse embryonic tissue on a laminin-coated substrate in an open cell chamber on chip. From opposite sides, gradients were applied by a flow-based approach. Each chip consisted of an array of 1,024 gradient generators for high-throughput analysis of axon guidance. To validate functionality of the setup, the influence of Netrin-1 on axon guidance and its concentration dependence were investigated in detail.

Microfluidic devices for gradient generation have also been used to study the function of morphogen gradients during development. Already in 2009, Park et al. had applied alternating gradients of Shh and Bmp4 or FGF8 using a flow-based microfluidic system to differentiate human neural progenitor cells into neurons (Park et al., 2009). However, within the developing embryo combinations of multiple signaling gradients control establishment of the different body axes. To unravel the function and interaction between these signaling pathways, one would have to be able to externally apply or modulate all pathways simultaneously. Demers et al. have used a diffusion-based approach to generate a microfluidic device, which allows the external application of opposing and even orthogonal gradients of signaling agonists (Demers et al., 2016). The multilayered design includes a layer with flow channels, from where nutrients and agonists are provided to the incubation chamber in the upper layer. As proof-of-principle, a combination of retinoid acid and opposing gradients of Bmp and Shh signaling modulators were used to recapitulate neural tube patterning on chip. This way, embryonic stem cells were differentiated into roof and floor plate cells as well as somitic cells, dependent on spatial gradient application (Demers et al., 2016) (Figure 3B).

Thus, microfluidics allows for spatial control over fluid streams, which has been used to investigate asymmetric distribution of signaling molecules on cellular behavior. In the future, the combination of gradient generation and cultivation of embryos or tissues will further advance our understanding of how signaling gradients interact within 3D multicellular systems.

Temporally Modulated Perturbations

How biological information is accurately encoded and transmitted between cells is a long-standing question in biology. The importance of temporally modulated signaling in this regard has been highlighted before (reviewed in Sonnen and Aulehla, 2014). For instance, biological information can be encoded in the duration or the fold-change of a signal or the frequency of an oscillatory signal. However, to understand the function of such signaling dynamics and to reveal what information is encoded by them, it is essential to be able to subtly modulate the dynamics rather than constitutively perturbing them. New developments in optogenetics (de la Cova et al., 2017; Guglielmi et al., 2015; Imayoshi et al., 2013) and microfluidics now allow us to precisely unravel how signaling dynamics control multicellular biology.

The laboratory of Stephen Quake has generated a microfluidic system with which dynamics of multiple single cells could be detected and modulated simultaneously (Gómez-Sjöberg et al., 2007). The microfluidic chip consists of 96 incubation chambers, within each of which small cell populations can be cultured for weeks. Due to a combination of 16 inputs and a multiplexer, chambers can be infused with specific reagents individually. Simultaneous imaging allows quantification of the signaling activity. This automated setup allows the perturbation and analysis of signaling in high throughput. However, it is questionable whether such a sophisticated system is indeed required to answer the biological questions that were addressed after initial publication of the setup. A flow chamber within which cells are trapped and syringe pumps for flow control would in principle be sufficient to apply periodic pulses of pathway modulators, a setup more accessible to a standard developmental biology lab. The automated system was applied to investigate oscillatory nucleocytoplasmic shuttling of nuclear factor κB (NF-κB) (Kellogg et al., 2014; Kellogg and Tay, 2015; Tay et al., 2010). Sorre et al. (2014) have used an adapted system to investigate how a steadily increasing morphogen gradient is decoded. By applying the agonist transforming growth factor β1 (TGF-β1) in specific concentrations using temporal treatment regimes and analyzing intracellular signaling activity, they found that cellular response depends on the fold-change of the signal (Sorre et al., 2014). This could only be revealed by combining precise modulation of dynamics with real-time imaging.

We have recently established a microfluidic system that allows for the external control of signaling oscillations in primary tissue cultures (Sonnen et al., 2018). Mouse somitogenesis was used as a model system for the study of signaling dynamics during embryonic development. Somitogenesis describes the periodic segmentation of the presomitic mesoderm (PSM) into somites (precursors of vertebrae) during embryogenesis. It is controlled by signaling gradients and a network of Wnt, Notch, and FGF signaling oscillations (Hubaud and Pourquié, 2014). To analyze the function of these signaling oscillations, we cultured primary embryo tissue (Lauschke et al., 2013) on a microfluidic chip and entrained signaling oscillations to periodic pulses of pathway modulators (Figure 3C). This entrainment approach revealed a functional link between Wnt- and Notch-signaling oscillations and even allowed for changing of the phase-relationship between the two oscillatory signaling pathways. This demonstrated the importance of phase-shifts for embryonic development (Sonnen et al., 2018).

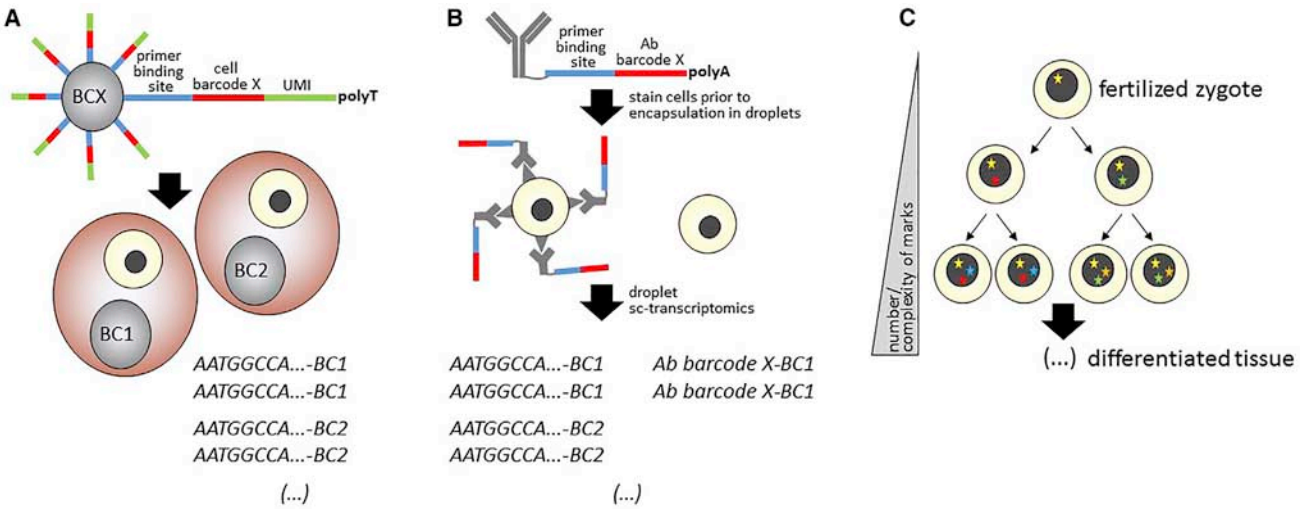


Figure 4. Microfluidic Single-Cell (SC) Analyses

(A) sc-Transcriptomics. Beads displaying barcoded (BC) polyT-primers are encapsulated into droplets, together with individual cells. Upon lysis, cellular mRNA hybridizes with the barcoded primers and the barcodes (a different one for each bead and hence for each cell) get incorporated into all newly synthesized cDNA. Data obtained from NGS can therefore be clustered according to the barcodes, revealing which sequences originate from the same cell.

(B) Combined transcriptome and protein expression analysis. Oligonucleotide-labeled antibodies are used to stain cells prior to encapsulation into droplets. The oligonucleotides comprise an antibody-specific barcode and a polyA tail enabling their amplification by barcoded polyT-primers as used in (A). In consequence, protein expression data (optionally based on multiple differently labeled antibodies against different proteins) and transcriptomes can be obtained for individual cells.

(C) Cell lineage tracking. Enzymes introducing mutations or barcodes (e.g., using CRISPR/Cas or transposases) are injected into the fertilized zygote. The labeling reaction continues during cell proliferation, resulting in increased numbers and complexities of marks within the genome (shown as colored stars). This way lineage trees can be reconstructed after sequencing.

While optogenetics allows very fast modulation of signaling dynamics (de la Cova et al., 2017; Guglielmi et al., 2015; Imayoshi et al., 2013), generation and optimization of particular optogenetic tools for usage in multicellular systems remains challenging. Moreover, for targeting specific signaling pathways, optogenetic systems have to be established for each pathway individually and can often not be combined. Finally, the dynamic range of optogenetic tools is often limited and does not allow a fine manipulation of the pathways. In contrast, microfluidic systems are highly versatile; perturbations can be adapted to target various different signaling pathways, either individually or in combination; and a fine titration of pathway modulators is possible. Therefore, if the dynamics to be perturbed are in the range of multiple minutes or even hours, we recommend the use of microfluidics because this leaves enough time for substances to be washed out of the tissue culture. In contrast, for very fast processes, manipulation of very specific cellular reactions or for spatially very confined manipulations, optogenetics is more suitable.

Biochemical Investigation

The investigation of developmental mechanisms at the molecular detail remains a challenge in developmental biology. Since developing organisms consist of small numbers of heterogeneous cells, proteomic and transcriptomic analyses should ideally be performed at single-cell resolution. However, quantification of these, especially the proteome, remains difficult for very small sample amounts (for transcriptome analysis of single cells, see below). Immunohistochemistry is commonly performed to visualize protein expression, but analysis is limited to a few proteins and throughput is low. Flow cytometry in contrast has a higher throughput, but again the number of analyzable proteins is limited. An approach to address this problem is mass cytometry, in which—similar to flow cytometry—proteins are labeled with antibodies, enabling their quantitative detection at the single-cell level. However, in contrast to flow cytometry, antibodies are not tagged with fluorophores but with "multiatom" elements containing different isotopes, which are detected by mass spectrometry (Bandura et al., 2009; Spitzer and Nolan, 2016). Rather than using a small number of different fluorophores, this method allows detection of more than 40 different cellular properties. However, even though new tags are continuously being developed, the number of possible tags is still limited.

Very recently, this was tackled by the development of new approaches. Methods such as AbSeq (Shahi et al., 2017), RNA expression and protein sequencing assay (REAP-seq) (Peterson et al., 2017), or cellular indexing of transcriptome and epitopes by sequencing (CITE-seq) (Stoeckius et al., 2017) use antibodies that are neither labeled with fluorophores nor isotopes, but rather with unique DNA sequences (Peterson et al., 2017; Shahi et al., 2017; Stoeckius et al., 2017). In case of REAP-seq and CITE-seq, the DNA sequences consist of a unique antibody-specific DNA barcode, a primer or "PCR handle" for amplification and sequencing, and a poly(dA) sequence (Figure 4). This poly(dA) sequence allows detection of antibody and mRNA levels in a single experiment, making use of the tools already available for single-cell sequencing. In a proof-of-principle experiment, Peterson et al. determined the levels of 82 proteins and more than 20,000 genes simultaneously (Peterson et al., 2017). Similar

to flow cytometry, these methods are limited by the availability of specific antibodies that additionally have to be barcoded. So far, the technique is restricted to cell surface proteins and requires further optimization to allow detection of intracellular proteins. It remains to be seen whether approaches such as CITE-seq, REAP-seq, and AbSeq will become standard methods for high-throughput protein detection.

Cytometry methods for protein detection always include some cell loss during experimental setup. This is critical if only a limited number of cells are available, for instance in the case of circulating tumor cells (Sinkala et al., 2017) or developing embryonic tissues. Therefore, another approach to quantify protein expression in single cells, which does not lead to high cell loss, is single-cell western blotting (Hughes et al., 2014) (scWestern blotting). In this approach, polyacrylamide gel microwells are generated by soft lithography. By micromanipulation, single cells are loaded into individual wells and then lysed within the wells. Proteins are then separated by gel electrophoresis within the surrounding gel and afterward immobilized by UV crosslinking. Primary and secondary antibody incubation is performed within the gel and fluorescently labeled antibodies are then detected. By combining multiple fluorophores, 5 different proteins can be detected at a time. In addition, just like standard western blotting, the number of detected proteins can be further increased by stripping and re-probing. This technique is ideally suited for investigating protein expression in a small population of specifically selected cells, but throughput is very limited.

In summary, microfluidic approaches can advance the proteome analysis of a small number of cells or even single cells, which is key for molecular investigation of developmental processes. Importantly, the scWestern blotting system has already been commercialized by Zephyrus Biosciences and is available for standard developmental biology labs (Table 2).

Genetics

In parallel to proteomic analyses, genetic tools have been a major driver for revealing key molecular players in development. Genetic knockouts have been used for a long time to elucidate the role of particular proteins in developmental processes (Kutscher and Shaham, 2014; Lin et al., 2014; Shalem et al., 2015). While individual knockouts can be tested easily using standard lab equipment, global knockout screens require high-throughput screening facilities. These are usually based on microtiter plates (e.g., for *C. elegans* RNAi screens) but could strongly profit from microfluidic technology in the future. As mentioned above, *C. elegans* can undergo a complete life cycle in droplets, fed exclusively by co-encapsulated bacteria (Clausell-Tormos et al., 2008). Furthermore, the encapsulation of individual bacteria into droplets and their subsequent clonal amplification is easily possible. Taken together, these techniques can be exploited to compartmentalize individual clones of a bacterial RNAi library, amplify them in droplets, and assess their effect on offspring of co-encapsulated worms. While such approaches would demand very large droplets whose number is typically restricted to several thousand per experiment (Clausell-Tormos et al., 2010; Eduati et al., 2018), the required equipment and handling steps are rather simple. One could start off with a diverse bacterial RNAi library in a single tube and use a microfluidic setup consisting of no more than a droplet maker, a standard microscope for sample imaging, and a cheap droplet sorter (>4,000 US dollars) (Utharala et al., 2018), enabling the isolation of particular phenotypes for downstream sequencing of the corresponding RNAi constructs. Similar approaches could potentially also be implemented for species other than *C. elegans*, replacing co-encapsulated bacteria by alternative gene vehicles (e.g., a lentiviral producer cell library generating particles transferring RNAi or CRISPR/Cas constructs).

Apart from just qualitatively assessing the effect of particular genes, quantitative transcriptomics has become the method of choice for understanding mechanisms of development. Transcriptomic patterns represent a blueprint for all cellular processes, and single-cell technologies allow to reveal the different cell types and functions within a tissue or a developing embryo. While such assays have been carried out using conventional microtiter plate formats, e.g., to obtain transcriptomic patterns of cells exhibiting pluripotency and committing to particular lineages (Semrau et al., 2017), microfluidic technology offers higher throughput and increased levels of automation (Figure 4). Furthermore, the number of genes that can be detected per cell typically improves using microscopic volumes. The first microfluidic systems for single-cell transcriptomics experiments were based on chambers generated by valves (Toriello et al., 2008), a technology that has also been commercialized in form of the Fluidigm C1 platform (Table 2). However, an inherent limitation of this format is the maximal number of cells that can be processed per experiment, which typically does not exceed a few hundred. Furthermore, valve-based platforms are very sensitive to differences in cell sizes and require many more (>100-fold) cells as starting material than what can be processed. Systems based on nanowells and droplets can overcome many of these limitations. In 2015, Fan et al. published a setup ("CytoSeq") in which barcoded beads displaying uniquely barcoded polyT primers are seeded into microwells together with the cells of interest (Fan et al., 2015). Subsequent to cell lysis in the wells, all cellular mRNAs hybridize with the polyT primers and, upon reverse transcription, are barcoded. Based on the fact that the barcode is different for different beads (while it is the same for all polyT primers on the same bead), the sequencing reaction itself can be carried out after pooling all samples without losing single-cell information: by clustering all sequences showing the same barcode, the transcriptome of individual cells can be reconstructed. This way the transcriptome of tens of thousands of individual cells can be obtained in a single experiment, with only minimal equipment requirements. Refined academic systems (Gierahn et al., 2017) as well as commercial platforms (BD Rhapsody™) based on this setup have been introduced over the years, now making the technology broadly available. Instead of using only nanowells, one can also co-encapsulate barcoded polyT-beads and single cells in droplets, as implemented in the "DropSeq" and "InDrop" approaches (Klein et al., 2015; Macosko et al., 2015). This strategy is also exploited in the commercial 10X Genomics platform, which performed particularly well in terms of read and gene numbers per cell in the first comparative studies carried out (Lake et al., 2015, 2018; Zhang et al., 2019). However, it should be noted that "home-made" platforms such as DropSeq and InDrop offer a higher level of flexibility for experienced users, e.g., for implementing targeted sequencing of particular genes (Saikia et al.,

2019; Zilionis et al., 2017). Taken together, a variety of microfluidic, high-throughput single-cell genomics platforms are readily available and could boost new discoveries in developmental biology.

However, to really get insights into development, one has to merge single-cell-transcriptomic data with spatiotemporal information of the cells within the embryo. This is not trivial, but several alternative methods have been established. For example, transcriptomic analyses can be performed at different developmental stages with relatively high temporal resolution, by preparing sequencing libraries from replicate samples that have been cultivated for different time periods after fertilization. Using complex data analysis workflows for pseudotemporal arrangement (Trapnell et al., 2014), such data have been used successfully to derive transcriptional trajectories of zebrafish embryos (Farrell et al., 2018; Wagner et al., 2018) and to follow differentiation of neuromesodermal progenitors (Gouti et al., 2017).

Alternatively, one can use relatively sparse spatial gene expression data from fluorescence *in situ* hybridization experiments (typically less than 100 genes) to map scRNA-seq data to particular positions (Achim et al., 2015; Karaiskos et al., 2017). While this does not provide true single-cell resolution, it is at least sufficient to identify tissue types and the neighborhood of the sequenced cells. Spatial information on individual cells can also be obtained by clonal lineage tracking. To do so, the Klein lab combined single-cell transcriptomics with TracerSeq (Wagner et al., 2018). The latter method uses a transposase for randomly introducing 20 bp barcodes into the cellular genomes of the developing embryo over time. This way, individual cells can be distinguished, and their offspring can be mapped back to the parental clone. Combining this approach with scRNAseq analysis reveals a matched dataset of clonal lineage and transcriptome for each individual cell, ultimately allowing to reconstruct the entire embryonic development starting from the fertilized egg. Klein and coworkers used this approach to study the first 24 h of zebrafish development in great detail. Analyzing almost 100,000 cells, the authors observed not only simple tree-like hierarchies but also clonally related cells diverging into distant states and distant clones converging into similar states. These observations most likely represent asymmetric cell divisions and similar differentiation events in distant domains of the embryo. Clonal lineage tracing has also been implemented using CRISPR/Cas9 technology to generate double-strand breaks (“scars”) in the genome (Alemany et al., 2018; McKenna et al., 2016; Spanjaard et al., 2018). These scars are randomly introduced in the zygote and further accumulate over time (due to continuing activity of Cas9) so that subsequent multicellular stages exhibit cells with different scar patterns. While this approach has not yet been combined with microfluidic droplets, it demonstrates that a variety of single-cell technologies for developmental biology studies are readily available.

We believe that these technologies will, in the near future, generate datasets that could be used as a kind of “kinetic cell atlas,” similar to the human cell atlas initiative (www.humancellatlas.org), but with developmental time as an additional dimension. Furthermore, we envisage the inclusion of further single-cell omics datasets. This could be facilitated by the use of oligonucleotide-labeled antibodies, whose binding to the surfaceome can be detected in parallel to the amplification of cellular mRNAs (Stoeckius et al., 2017) (Figure 4B; as described above in detail).

Environmental Influence on Development

While development follows genetically determined programs intrinsic to the organism, proper progression is affected by the environmental conditions, namely temperature, pH, gas levels, or nutrient concentrations, but also by the presence of chemical compounds or toxins. Study of the influence of such external factors is difficult, as they are usually not uniform in time and/or space. Microfluidics provides the means to modulate environmental conditions in a highly defined manner.

In a pioneering study, Lucchetta et al. used a microfluidic system to investigate temperature compensation during embryonic patterning (Lucchetta et al., 2005). They cultured single *D. melanogaster* embryos at two different temperatures simultaneously, with half of the embryo at a different temperature than the other half. This was achieved by using two different culture medium portions at different temperatures as input into the microfluidic chip. The temperature difference was maintained by laminar flow (see above) with each stream of culture medium having and maintaining a different temperature. They found that the organism can even compensate for a temperature step from 20°C to 27°C between posterior and anterior embryo. Using the same experimental approach, cell cycle progression has been artificially slowed down in half of a fly embryo compared to the other half to study the impact of cell-cycle rate on developmental progression (Esposito et al., 2016).

Instead of applying inputs with different temperatures, researchers have also used microfluidics to test the effect of different chemical compounds in the culture medium. For instance, Albrecht and Bargmann have investigated the influence of odors in the culture medium on the behavior of *C. elegans* worms (Albrecht and Bargmann, 2011). They used a 2- × 2-cm chamber to culture and observe the worms and applied odors either as separate streams by laminar flow, as a gradient or as temporally modulated pulses. Similarly, we made use of a microfluidic setup to study the role of pH and salt concentration on marine zooplankton by applying different sea water conditions in a spatially controlled manner (Ramanathan et al., 2015).

PDMS, which is mainly used for microfluidic chip generation, is gas permeable. However, permeation through PDMS and exact gas concentration within the chip are difficult to predict. As flow-based microfluidics allows a constant exchange of culture medium, gas concentration can also be modulated easily (Halldorsson et al., 2015). Thomas et al. guided culture medium through a gas exchange chamber, in which a specific gas mixture is provided, before entering the microfluidic chip (Thomas et al., 2011). This way a more exact gas concentration is maintained within the incubation chamber by perfusion.

The advantage of the microfluidic setup is that environmental conditions can be varied exclusively in the fluid phase without temporal or spatial boundaries. Such an approach now allows investigation of the impact of external factors on development—not only temperature or pH but also the influence of drugs, toxins, or nutrients. In recent years, the impact of metabolism on development has been highlighted (reviewed in Miyazawa and Aulehla, 2018). In the future, it will be exciting to study

the effect of transient or periodic changes in glucose concentration or oxygen levels on embryonic development. This will give insight into the effect of, for instance, diabetes or transient hypoxia on embryonic development during pregnancy.

How to Access Microfluidics Technology?

As discussed above, the new possibilities brought up by microfluidics are numerous and likely mostly limited by a perceived lack of access to the technology. However, preparing a microfluidic chip is probably no more difficult than doing a western blot. It all starts off with the design of a 2D-channel network on a lithography mask (as illustrated in Figure 5). This step is usually done using computer-aided design (CAD) software such as AutoCAD (free trial versions are available at www.autodesk.com/products/autocad-lt/free-trial), which is somewhat similar to basic drawing programs, just with more precisely defined dimensions and helpful additional options (e.g., scaling, arraying, extending lines to the next feature). The final design can then be sent by email to specialized companies that provide high-resolution printing services (>25,000 dpi; e.g., Outputcity, Selba, or Micro Lithography Services). In return, the user obtains a high-resolution photomask in which the channel features are usually transparent on black background (or vice versa when using positive photoresist for manufacturing valves). Using a UV light source, this mask is then used to project the channel patterns on a wafer coated with photoresist of specified thickness. Only below the transparent parts of the photomask will the photoresist polymerize, resulting in a negative mold of the channel structures upon treatment with chemical developer. Subsequently, the mold can be filled with transparent PDMS, which is cross-linked by baking at 65°C for a few h. The PDMS is then cut out (now having an imprint of the channel structures) and bonded in a plasma oven to a microscope slide or a PDMS membrane. Subsequently, tubing is connected to pre-defined inlets and outlets (cut using biopsy punches and self-sealed based on the flexibility of PDMS), through which reagents can be injected. Comprehensive protocols for all these steps exist (Lake et al., 2015; Qin et al., 2010), and many universities even offer 2 to 3-day hands-on training courses (such as the Brandeis Microfluidics Course, the Georgia Tech IEN Soft Lithography for Microfluidics Short Course, or the University of Toronto Microfluidics Professional Course).

The only requirement for transferring the method into the home lab is access to a clean room or the will to set up a more improvised do-it-yourself environment including at least a laminar flow hood, a spin coater, several hot plates, a UV light source (e.g., a handheld UV lamp or even a gel nail curing device), and a plasma oven. Given that laminar flow hoods can be found in almost any biology laboratory, such do-it-yourself solutions can be realized at costs of less than 20,000 US dollars for the additional equipment. Once established, they allow the production of customized microfluidic devices for all kinds of applications at consumables costs of less than 40 US dollars per chip. All that is further needed for conducting experiments are a couple of syringe- or pressure-driven pumps (e.g., New Era or Fluigent pumps, starting at a cost of several hundred US dollars per pump), disposable materials such as syringes and tubing, and a microscope. Compared with any commercial solution, the establishment of microfluidic chip production in one's own lab offers the highest flexibility in design, fastest turnaround time from a biological question to a tailored microfluidic solution, and significant cost savings over time.

Users who want to bypass the manufacturing process completely can also obtain customized devices from commercial providers such as Dolomite, Flowjem, Microfluidic Chip Shop, Microliquid, or Simtech. Depending on the complexity of the design and the number of chips ordered one has to calculate between ~50 and several thousand US dollars per chip.

In parallel to ordering customized chips from external companies, many commercial platforms for specific applications exist, as summarized in Table 2. Taken together, it is easy to get started with microfluidics, and we envisage rapid spreading of the technology in the developmental biology community.

Concluding Remarks

Microfluidics is revolutionizing developmental biology studies. While classical developmental biology approaches were limited by low throughput and/or low precision analysis of developmental processes, microfluidics allows high-throughput analysis and perturbations with high spatiotemporal resolution. This makes the precise manipulation of a variety of environmental factors, e.g., chemical and mechanical cues, possible. Additionally, quantitative multidimensional omics data can be obtained with high throughput, if required, at the single-cell level. This strongly facilitates highly precise functional investigations and mechanistic insights into the otherwise inaccessible aspects of developmental regulation. We believe that further spreading of the technology and further innovations will enable and accelerate discovery. Today, microfluidics is already being used to study the complex interactions within tissues or organs on-chip to recapitulate the long-range coordination that occurs within an organism (Bhatia and Ingber, 2014; Huh et al., 2010).

A very interesting trend on the technology side is the use of plastic printers for the production of microfluidic chips. While current systems do not offer the resolution that can be achieved by lithographic methods, there is continuous improvement on this front. Channel diameters of ~50 × 50 μm can already be achieved. Although the channel walls and edges are less smooth compared to PDMS chips, this is often negligible, as flow-based microfluidics for cultivation of tissues or whole organisms often do not require high-resolution chips with μm details. Furthermore, even complex systems including valves have been achieved using this method of production (Beauchamp et al., 2017; Bhattacharjee et al., 2016; Gong et al., 2016), and it is foreseeable that such approaches will ultimately be able to compete with soft lithography. A clear advantage is the minimal equipment required (plastic printers are available starting from a few hundred US dollars) and the high level of automation (designs from other groups can simply be imported and manufactured without any additional manual work).

Complementary to this, very expensive, high-end 3D lithography stations such as the Nanoscribe Photonic Professional are also gaining momentum. Similar to plastic printers, they can generate complex structures in a fully automated way (by polymerizing a photoresist with a highly focused laser beam). However, their resolution is even better than that of conventional lithography methods and enables submicrometer features. The only significant drawback is the instrument cost

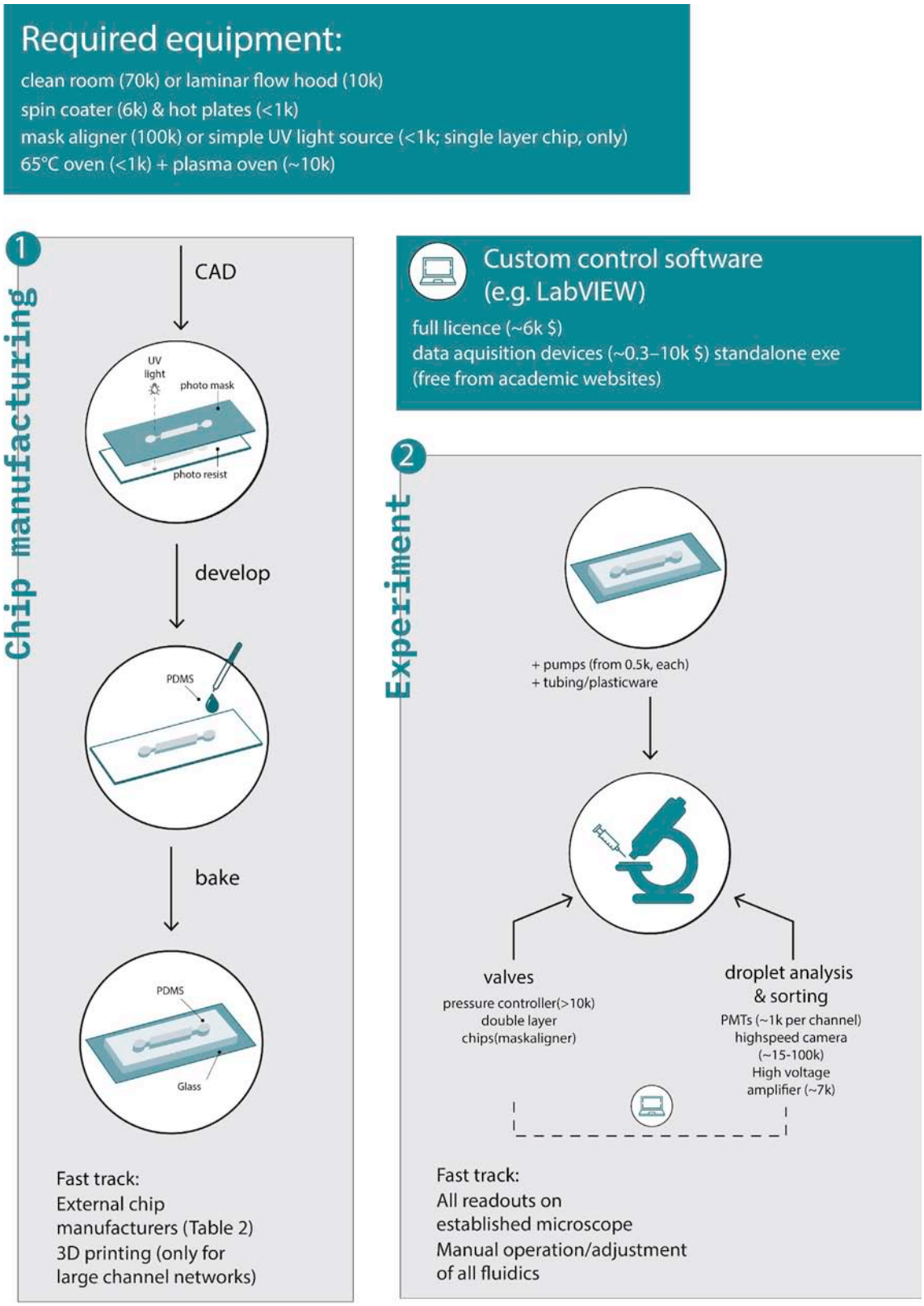


Figure 5. Performing Microfluidic Experiments
Overview of the process of chip manufacturing, including the required equipment (left panel), and of microfluidic experiments, including use of custom control software (right panel).

(>200,000 US dollars), which restricts the user community mostly to specialized facilities. Nonetheless, technical innovations such as these will push the limits further and enable experiments that have been unthinkable using conventional equipment.

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