

# Microfluidics for mechanobiology of model organisms

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Anna A. Kim<sup>\*,†,‡,2</sup>, Adam L. Nekimken<sup>‡,2</sup>, Sylvia Fechner<sup>‡</sup>, Lucy E. O'Brien<sup>‡</sup>  
Beth L. Pruitt<sup>\*,†,1</sup>

<sup>\*</sup>University of California, Santa Barbara, CA, United States

<sup>†</sup>Uppsala University, Uppsala, Sweden

<sup>‡</sup>Stanford University, Stanford, CA, United States

<sup>1</sup>Corresponding author: e-mail address: blp@ucsb.edu

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## Abstract

Mechanical stimuli play a critical role in organ development, tissue homeostasis, and disease. Understanding how mechanical signals are processed in multicellular model systems is critical for connecting cellular processes to tissue- and organism-level responses. However, progress in the field that studies these phenomena, mechanobiology, has been limited by lack of appropriate experimental techniques for applying repeatable mechanical stimuli to intact organs and model organisms. Microfluidic platforms, a subgroup of microsystems that use liquid flow for manipulation of objects, are a promising tool for studying mechanobiology of small model organisms due to their size scale and ease of customization.

In this work, we describe design considerations involved in developing a microfluidic device for studying mechanobiology. Then, focusing on worms, fruit flies, and zebrafish, we review current microfluidic platforms for mechanobiology of multicellular model organisms and their tissues and highlight research opportunities in this developing field.

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## 1 INTRODUCTION

This chapter discusses the current state and future opportunities in the field of microfluidics for mechanobiological studies of multicellular model organisms. We define the concept of mechanobiology and describe several model organisms and their advantages for mechanobiological studies. We discuss how microfluidic platforms could be used to manipulate and apply repeatable stimuli, examine design considerations for devices, and review prior work on microfluidics for mechanobiology of model organisms and their tissues. We hope this chapter will be helpful for engineers interested in developing tools for model organisms, and for biologists interested in learning how microfluidic technologies can benefit their research goals.

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## 2 MECHANOBIOLOGY

Research in medicine and biology has largely focused on the biochemical nature of development, homeostasis, and disease, resulting in transformative insights (Broderick, Buchon, & Lemaitre, 2014; Ellis & Horvitz, 1986; Fire et al., 1998; Kok et al., 2015; Nüsslein-Volhard & Wieschaus, 1980; Walther et al., 2015). However, mechanical signaling also plays crucial roles in these processes (Janmey & Miller, 2011; Thompson, 1942; Vining & Mooney, 2017), including tissue patterning in development (Bardet et al., 2013; Hayashi & Carthew, 2004; Heisenberg & Bellaïche, 2013), balancing forces during homeostasis (Brown,

Prajapati, McGrouther, Yannas, & Eastwood, 1998; Cahalan et al., 2015; Gomez, McLachlan, & Yap, 2011), and cancer metastasis (Guck et al., 2005; Spill, Reynolds, Kamm, & Zaman, 2016; Wei et al., 2015; Wirtz, Konstantopoulos, & Searson, 2011). Mechanobiology is the study of how cells, tissues, and organisms convert mechanical stimuli into information, process that information, and respond accordingly. The sense of touch is a clear example of the conversion of mechanical stimuli into information. For the purpose of this chapter, we focus on mechanobiology in the context of multicellular model organisms.

One of the enablers of the proliferation of mechanobiology investigations is technology development. For example, the invention of atomic force microscopy (Binnig, Quate, & Gerber, 1986; Marti et al., 1988; Sonnenfeld et al., 1986) created the possibility of mapping the topography as well as mechanical properties of nanoscale biological samples (Bustamante, Erie, & Keller, 1994; Hansma, Elings, Marti, & Bracker, 1988). The capabilities of atomic force microscopy continue to grow, and now include high-speed nanoscale imaging (Ando, Uchihashi, & Kodera, 2013) and single molecule force spectroscopy (Neuman & Nagy, 2008). Advances in microfluidic technology have similarly enabled new experiments, including investigations of mechanobiology.

Importantly, the field of mechanobiology is not only focused on biomechanical properties of an organism but also involves understanding information processing and phenotypic change due to a mechanical stimulus. Our definition of mechanobiology thus sits at the intersection of phenotype, biomechanics, and information processing (Fig. 1). While biomechanical properties can be defined as phenotypes, we separate them out here because all mechanical stimuli are transmitted through physical materials. Thus, the biomechanical properties of an organism and its surroundings are inexorably linked to its response to mechanical stimuli. **Box 1** describes the basics of how mechanical stimuli are measured.

The field of mechanobiology has become more prominent in recent decades, but it is not entirely new (Wolff, 2011). The study of touch goes back at least to the time of Aristotle (1990) and continues today (Fehrenbacher, 2015; Singh, Kishore, & Kaur, 2014). In modern times, scientists began to explore the effects of mechanical stimuli in contexts other than touch sensation. Physicians in the 19th century observed that bones can remodel their structure to support mechanical loads after a fracture (Wolff, 2010a, 2010b, 2011). More recently, Carter and Hayes (1976) continued this work and renewed the focus on mechanobiology of bone (reviewed by Carter, Beaupré, Giori, and Helms (1998) and Wall et al. (2017)). The field has since expanded to study other tissues and cell types. Pelham and Wang (1997) demonstrated that altering the stiffness of polyacrylamide substrates changes the locomotion and gene expression of cells. Lo, Wang, Dembo, and Wang (2000) followed this result with a study showing that cell migration can be influenced by stiffness gradients (reviewed by Discher, Janmey, and Wang (2005)). Engler, Sen, Sweeney, and Discher (2006) discovered that the stem cell lineages can be altered by the stiffness of the substrate on which they are grown, leading to further investigation of stem cell mechanobiology (reviewed by Lee, Knight, Campbell, and Bader (2011) and Ireland

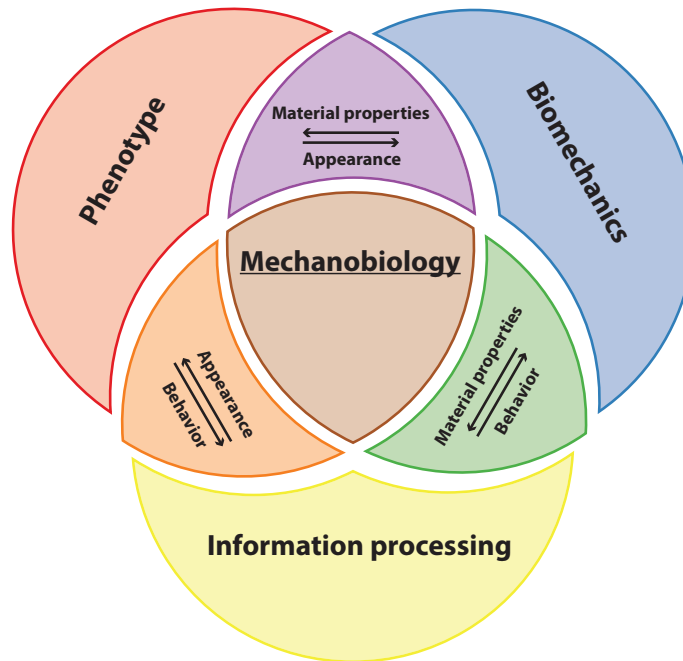
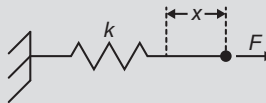


FIG. 1

Mechanobiology can be conceptually subdivided into biomechanics, phenotype, and information processing.

### BOX 1 A NOTE ON MEASURING FORCE AND DISPLACEMENT



In mechanobiology, mechanical stimuli are often measured as displacement,  $x$ , and reported as a measurement of force,  $F$ . In the simplest case, force and displacement are related by a spring constant according to Hooke's law:  $F = kx$ , where  $F$  is force in Newtons,  $x$  is the displacement of the spring in meters, and  $k$  is the spring constant in N/m (illustrated above). The spring constant is dependent on both geometry and material properties of the specimen, and mechanical models that take geometry into account can be used to derive the material properties (such as the elastic modulus,  $E$  with units of N/m<sup>2</sup>).

However, in many cases, displacement measurements between control and experimental conditions are sufficient for testing hypotheses and can be more accurate than force values. Each calibration measurement required to establish a mechanical model introduces uncertainty into the final force measurement. Moreover, [Eastwood et al. \(2015\)](#) found that the rate of displacement, rather than force, is more closely correlated to the response of touch receptor neurons in worms, and [Petzold, Park, Mazzochette, Goodman, and Pruitt \(2013\)](#) demonstrated that indentation of worms is a more proximal stimulus than force for touch receptor neurons. Together, these studies demonstrate cases where displacement is a more reliable indicator of stimulus strength than force. For more information, see reviews by [Schoen, Pruitt, and Vogel \(2013\)](#), [Moendarbary and Harris \(2014\)](#), and [Paluch et al. \(2015\)](#).

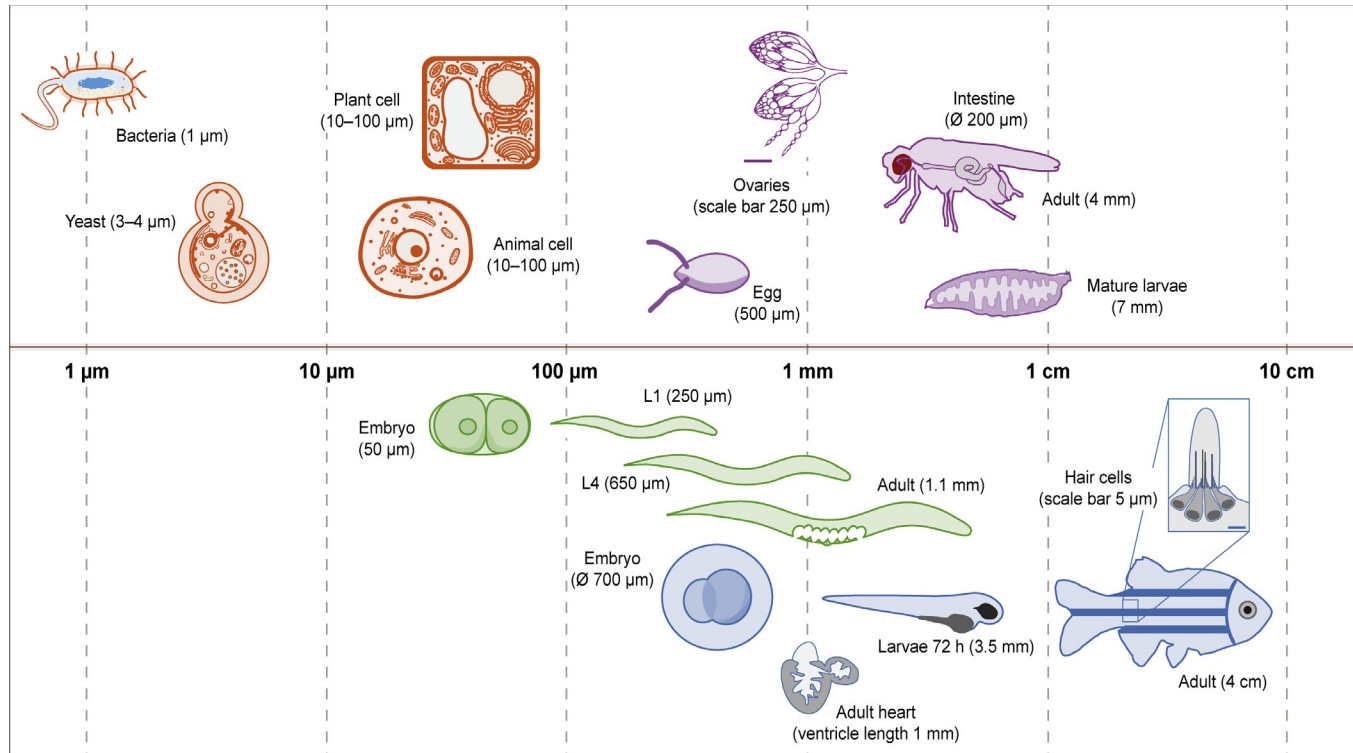
and Simmons (2015)). Paszek et al. (2005) demonstrated that the rigidity of tumors promotes the growth and malignancy of tissue. Thus, mechanobiology is relevant to cancer formation and propagation (reviewed by Huang and Ingber (2005), Jaalouk and Lammerding (2009), and Wirtz et al. (2011)). Aarabi et al. (2007) found that scar formation is initiated by mechanical forces applied to a wound, further underscoring the idea that mechanical signaling can affect clinical outcomes (reviewed by Gurtner, Werner, Barrandon, and Longaker (2008) and Ogawa and Huang (2016)). These milestones are not an exhaustive summary of mechanobiology, as the field encompasses a broad range molecular-, cellular-, tissue-, and organism-level investigation, which have been reviewed elsewhere (Ingber, 2003; Wang & Thampatty, 2006).

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### 3 MULTICELLULAR MODEL ORGANISMS

Model organisms are widely-studied nonhuman systems in biological research, ranging from simple single cells (e.g., *Escherichia coli*) to animals like mice. Selection of a model organism is dictated by the complexity required to address the research question and the convenience of the experiment. For investigations of responses to mechanical stimuli in the context of differentiated and distinct organs, higher-order multicellular organisms provide an attractive platform. *Caenorhabditis elegans* (worms) (Frézal & Félix, 2015; Hulme, Shevkoplyas, Apfeld, Fontana, & Whitesides, 2007; Kaletta & Hengartner, 2006), *Drosophila melanogaster* (fruit flies) (Jennings, 2011; Pandey & Nichols, 2011; Ugur, Chen, & Bellen, 2016), and *Danio rerio* (zebrafish) (Dahm, Geisler, & Nusslein-Volhard, 2005; Dooley & Zon, 2000; van der Sar, Appelmelk, Vandenbroucke-Grauls, & Bitter, 2004) offer a high level of biological complexity with comparatively low laboratory maintenance. These organisms are cost-efficient, experimentally tractable, and compatible with microscopy-based techniques. Moreover, these multicellular organisms come with well-established toolboxes of genetic techniques, sequenced genomes, and a varying number of genes homologous to humans. Communities of model organism researchers provide resources, accessible online (FlyAtlas, n.d.; FlyBase, n.d.; WormBase, n.d.; WormBook, n.d.; ZFIN, n.d.) that support information exchange and facilitate research.

In this chapter, we focus on worms, fruit flies, and zebrafish and describe their individual features in the following sections. These multicellular model organisms can benefit from developments in microfluidic technologies, because they are the appropriate size and compatible with immersion in liquids for part or all of their life stages. Our experience with the design process for a microfluidic device for studying worms can thus be extended to these other model organisms. Fig. 2 compares the size of model organisms and single cells during different stages of their respective life cycles. We exclude mammalian systems due to their comparatively large size.



**FIG. 2**

Approximate sizes of biological cells, tissues, and model organisms on a logarithmic scale. Biological cells (*orange*) are represented as a range because the size is dependent on cell type. Model organisms are organized by *color*, including nematodes (*green*), fruit flies (*purple*), and zebrafish (*blue*).

### 3.1 *Caenorhabditis elegans*

*C. elegans* is a species of small roundworms that develop from embryos to adults in less than 3 days when cultured at 20°C. It has four larval stages (L1–4) and lays eggs for 2–3 days after achieving adulthood (Corsi, Wightman, & Chalfie, 2015). Nearly all individuals in the species are hermaphrodites, making it easier to maintain homozygous genotypes, and worms can be recovered decades after freezing in liquid nitrogen, which can minimize the genetic variability of a population. Approximately 38% of worm genes are human orthologs, meaning they evolved from the same gene in a common ancestor (Hu et al., 2011).

Worms were the first animal to have a fully sequenced genome, and other genetic techniques, such as genetic mapping (Brenner, 1974), fluorescent protein expression (Chalfie, Tu, Euskirchen, Ward, & Prasher, 1994), and RNA interference (Fire et al., 1998) were pioneered in worms. One downside of using worms for mechanobiology research is their lack of a circulatory system (Corsi et al., 2015). Worms have a variety of mechanosensitive neurons and respond to mechanical stimuli by changing the direction of crawling (Goodman, 2006), making them useful for mechanobiology research.

### 3.2 *Drosophila melanogaster*

Similar to worms, fruit flies have a short generation time. The organism develops from embryo to adult in approximately 10 days when it is grown at 25°C. Fruit fly larvae go through three instar stages of increasing size before forming pupae, and eclose from pupae after metamorphosis is complete. Genetic crosses are facilitated because *Drosophila* are easily bred and a single female can lay 400 eggs (Greenspan, 2004). Approximately 40% of fruit fly genes are human orthologs (Hu et al., 2011).

Fruit flies are widely used for research, including studies in aging (Cannon et al., 2017; Fleming, Reveillaud, & Niedzwiecki, 1992; He & Jasper, 2014) and neurobiology (Feany & Bender, 2000; Nichols, 2006; Watanabe et al., 2017). In the context of mechanobiology, fruit flies are a promising model for investigations of organ architecture formation and function, as the animal is already a well-established model in developmental biology (Dai, Peterson, Kenney, Burrous, & Montell, 2017; Hiraoka et al., 1993; Pandey & Nichols, 2011). Research applications include morphogenesis and organogenesis using follicle cells (epithelium that encapsulates the developing egg), embryos, and imaginal discs (precursors to adult organs) (Dobens & Raftery, 2000; Haynie & Bryant, 1986; Lengyel & Iwaki, 2002). Other tissues of potential interest are the dorsal vessel and midgut. Recently, fruit flies became an invertebrate genetic model for cardiac disease (Cannon et al., 2017; Choma, Izatt, Wessells, Bodmer, & Izatt, 2006; Wolf et al., 2006) using the dorsal vessel that pumps hemolymph, although their open circulatory system does not entirely capture the complexity of vertebrate circulatory systems (Choma, Suter, Vakoc, Bouma, & Tearney, 2011). The adult midgut established the animal as a prime model for understanding principles of tissue-level processes that govern

homeostasis in adult organs, following the discovery of intestinal stem cells (Jiang & Edgar, 2011; Liang, Balachandra, Ngo, & O'Brien, 2017; Micchelli & Perrimon, 2006; Ohlstein & Spradling, 2006).

### 3.3 *Danio rerio*

Zebrafish have a similar cultivation cost in the laboratory as flies (Lieschke & Currie, 2007), but they require 2–3 months longer for development, a timeline more comparable to the generation cycle of mice (Dahm et al., 2005). The development of zebrafish, indicated by hours postfertilization, is divided into four stages: (1) embryo (ends at 72 h postfertilization at 28.5°C), (2) larva (an individual that is no longer an embryo but has yet to become a juvenile), (3) juvenile (most adult characteristics developed, but absence of sexual maturity), and (4) adult (defined by the production of viable gametes) (Kimmel et al., 1995; Parichy, Elizondo, Mills, Gordon, & Engeszer, 2009). Like humans, zebrafish are vertebrates, and approximately 55%–70% of zebrafish genes are human orthologs (Howe et al., 2013; Hu et al., 2011). Zebrafish are an emerging model to study human diseases because of this genetic similarity to humans, the relative ease of genetic manipulation, and the optical clarity of embryos and larvae.

Zebrafish have mechanosensitive hair cells in the inner ear and the lateral line organ. These hair cells can regenerate, an ability that humans lack. In humans, damage or destruction of sensory hair cells in the inner ear can lead to debilitating hearing or balance deficits, especially in older adults (Kniss, Jiang, & Piotrowski, 2016). Larvae, adult zebrafish, and isolated hair cells can be used to study hearing loss in vertebrates. Zebrafish have a closed circulatory system with many similarities to mammals, making studies of cardiac development and function possible (Chan & Mably, 2011). In addition to hair cells, the central nervous system and the heart can regenerate, which makes zebrafish an excellent model system for vertebrate tissue regeneration (Gemberling, Bailey, Hyde, & Poss, 2013).

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## 4 DESIGN CONSIDERATIONS FOR MICROFLUIDICS

The development of microfluidic devices began by taking advantage of fluid motion physics in the laminar flow regime (Whitesides, 2006). Devices capable of complex reactions that involve mixing and multiplexing were designed for chemical synthesis (Elvira, i Solvas, Wootton, & deMello, 2013; Hung et al., 2006; Lignos et al., 2016) and micro total analysis (Auroux, Koc, DeMello, Manz, & Day, 2004; Heiland et al., 2017; Patabadige et al., 2016).

Microfluidic devices for research involving biological cells soon followed (Chiu et al., 2017), because, in addition to fluid manipulation, microfluidics can manipulate objects at the scale of single cells (Fig. 2). Developments include microfluidic systems for cell culture (Halldorsson, Lucumi, Gómez-Sjöberg, & Fleming, 2015; Moreno et al., 2015; Rhee et al., 2005; Young & Beebe, 2010), sorting



(Henry et al., 2016; Krüger et al., 2002; Siedlik, Varner, & Nelson, 2016; Ung et al., 2017; Warkiani, Wu, Tay, & Han, 2015), manipulation (Applegate, Squier, Vestad, Oakey, & Marr, 2004; Chen, Chen, Chen, Jang, & Wang, 2014; Ding et al., 2012; Yi, Li, Ji, & Yang, 2006; Yun, Kim, & Lee, 2013; Zhao, Cheng, Miller, & Mao, 2016), and bioanalysis (Chen, Li, et al., 2014; Mirasoli, Guardigli, Michelini, & Roda, 2014; Wheeler et al., 2003). The research field of microfluidics continues to expand as novel strategies for stimulation and analysis continuously emerge. Microfluidics thus enables investigations on the effects of mechanical stimuli such as shear (Kim et al., 2014; Shao et al., 2009), compression (Guo, Park, & Ma, 2012; Kim, Kang, Park, Yoon, & Park, 2007), and tension (Douville et al., 2011; Gossett et al., 2012) for a range of biological applications (Kurth, Eyer, & Franco-Obregón, 2012; Polacheck, Li, Uzel, & Kamm, 2013; Zheng, Nguyen, Wei, & Sun, 2013).

Due to the size scale of many model organisms, microfluidics extended its applications further to include multicellular systems, among them worms, fruit flies, and zebrafish. The technology has the capability to address some of the limitations of classic techniques, with the main advantages being higher experimental throughput and integration of manipulation, stimulation, and readout. Devices can be designed to sort, trap, immobilize, and otherwise manipulate organisms within a single device. In addition, microfluidics offer more control over magnitude and reproducibility of mechanical stimuli in comparison to classic assays (Nekimken, Mazzochette, Goodman, & Pruitt, 2017). Other instruments with even more precise control of stimuli also exist (Mazzochette et al., 2018; Park, Goodman, & Pruitt, 2007; Park, Petzold, Goodman, & Pruitt, 2011), but they are more costly, difficult to use, and lower throughput compared to microfluidics.

We designed and published a microfluidic device for mechanical stimulation of worms during high-resolution imaging (Nekimken, Fehlauer, et al., 2017), as well as a detailed protocol for fabricating and operating our device (Fehlauer et al., 2018). The device integrated a trapping channel adapted from a microfluidic platform for studying chemosensation in worms (Chronis, Zimmer, & Bargmann, 2007) with six individually addressable mechanical actuators for stimulating touch receptor neurons. The actuators consisted of thin, deformable walls on either side of the trap channel. When the chambers on the other side of the walls were pressurized, the thin wall inflated, indenting the worm. Experimental results are described in [Section 5.1.1](#). The following sections focus on our design process for this microfluidic device, including actuator design, immobilization strategies, and fabrication constraints.

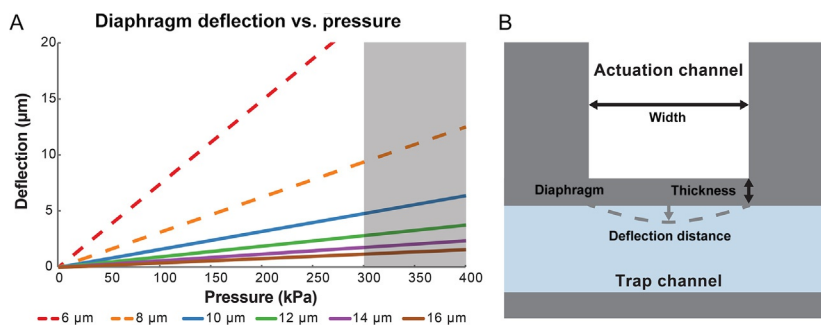
## 4.1 ACTUATORS

When designing the pneumatic actuators in our device, the key performance metric we chose was deflection distance of the actuation membrane, since this is often sufficient to quantify the magnitude of a mechanical stimulus ([Box 1](#)). Deflection distance is dependent on the pressure applied to channel, geometry of the actuators, and material properties of the device (Lee, Chan, Maung, Rezler, & Sundararajan,

2007; Nekimken, Fehlauer, et al., 2017). Material properties are discussed in Section 4.5. There are several practical challenges that limit these parameters.

The maximum deflection of an actuation membrane (diaphragm) varies with its thickness to the power of negative 3 ( $t^{-3}$ ), therefore minimizing the thickness of the membrane resulted in better performance (Fig. 3). However, successfully creating high-aspect-ratio membranes was the most challenging part of the fabrication process, and feature sizes depend on mask resolution and exposure system available. Transparency masks offered sufficient resolution for prototyping trap designs (Section 4.2), but not for reliable fabrication of actuation membranes. Using a chrome mask and a collimated UV light source, we were able to reliably fabricate 10- $\mu\text{m}$ -thick membranes with a 50  $\mu\text{m}$  by 50  $\mu\text{m}$  cross section. At times, we were successful in making 8- $\mu\text{m}$ -thick membranes, we decided not to use this design because some membranes would fail to create a seal between the trap and the actuation channel, rendering the entire device defective. A more advanced lithography system or other processes such as deep reactive ion etching might successfully produce thinner actuation membranes, but we elected to keep the fabrication process simple.

For our device, the pressure applied to the actuation channels was limited by the integrity of the tubing connecting the actuator and the pressure source. We punched holes in the PDMS device with a biopsy punch, then used polyethylene tubing with stainless steel pin connectors to interface with a pressure pump (Fehlauer et al., 2018). With this setup, the tubing reliably stayed in place when 300 kPa of pressure was applied, although we were able to increase this to 450 kPa in some cases (Nekimken, Fehlauer, et al., 2017). Actuation pressure could be increased further using a fixture and/or PDMS-compatible adhesive to hold the tubing in place.



**FIG. 3**

The design space for actuator geometry was limited by fabrication constraints and the maximum pressure we could apply without tubing failure. (A) Using our exposure system, we were unable to reliably fabricate membranes (diaphragms) with a thickness of 6  $\mu\text{m}$  or 8  $\mu\text{m}$  (red and orange dashed lines). The connections to the pressure pump sometimes failed above 300 kPa (gray zone). We thus chose 10  $\mu\text{m}$  as the thickness of the actuators because they met design constraints but still applied enough indentation to activate touch avoidance behavior. (B) Top-view schematic of one actuator with labeled geometric parameters, not to scale.

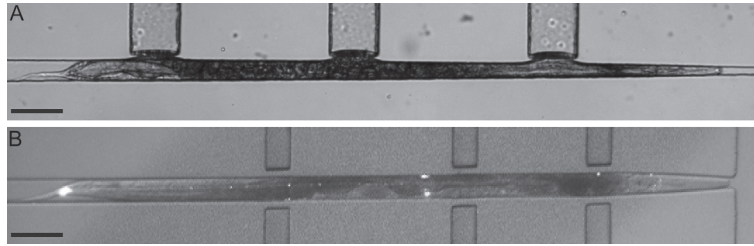
The width and height of the actuator also impact the maximum deflection distance. The height of the actuator was set by the height of the trap (Section 4.2), because we wanted to limit device fabrication to a single layer to avoid alignment challenges (Section 4.5). We decided to design actuators of equal width and height, since membrane deflection is primarily affected by the smallest dimension, resulting in 50 by 50  $\mu\text{m}$  actuators. For comparison, the size scale of this design fell between the diameter of a hair used in the classical hair touch assay (Nekimken, Mazzoquette, et al., 2017) and glass beads (10  $\mu\text{m}$ ) glued to actuators for applying touch stimuli to worms (Park et al., 2007; Petzold et al., 2013, 2011).

## 4.2 IMMOBILIZATION

Immobilization of an object of interest is critical because microscopy-based readouts remain an important approach for evaluation of biological responses. Immobilization of worms in microfluidic devices can be accomplished by directing a worm into a snug-fitting channel that hold the worm in place (Hulme et al., 2007), deflection of the channel's "ceiling" to compress a worm (Zeng, Rohde, & Yanik, 2008), or paralyzing a worm with carbon dioxide (Chokshi, Ben-Yakar, & Chronis, 2009), low temperature (Chung, Crane, & Lu, 2008), or anesthetic drugs (Morgan, Kayser, & Sedensky, 2007). Active immobilization strategies facilitate animal removal and accommodate size variations in a population, but require additional external components for implementation. We chose to use a passive approach that avoids the complications of additional moving parts as well as potential effects on the mechanics or information processing of the worm due to paralysis.

We tested two different strategies for passive immobilization of *C. elegans* during the development of our device (Fig. 4). Design A was a continuously tapering channel with evenly placed actuators along the full length of the channel. Design B was a form-fitting channel designed to accommodate the shape of a worm with a specific size. The height of the trap was optimized to a dimension approximately 5% less than the diameter of the worms (50  $\mu\text{m}$  for young adults), successfully minimizing their movement without getting them permanently stuck.

The main advantage of design A was its ability to accommodate size variations of the animal, thus enabling studies of *C. elegans* at different life stages using the same microfluidic device. The disadvantages were clogging and low experimental throughput as a consequence of the tapering feature. The narrow opening at the end of the trap was easily clogged with debris. To remove worms from this device, the flow of buffer in the device had to be reversed to push a worm back to the inlet of the device. With design B, animals could be preloaded into a larger entrance chamber, significantly reducing preparation time per worm (approximately sixfold improvement). The smallest constriction of the trap was approximately half of the worm width, facilitating removal of animals and minimizing the likelihood of clogging. Following on-chip experiments, it was possible to recover organisms, thus enabling long-term studies of individuals (Fehlauer et al., 2018; Hulme et al., 2007; Nekimken, Fehlauer, et al., 2017).

**FIG. 4**

Passive trap designs for worm immobilization with actuators positioned along the length of the worm. (A) Continuous taper channel and (B) form-fitting channel. In design A the worm pushes the diaphragms toward the actuation channels. Scale bars 100  $\mu\text{m}$ .

Design (A) adapted from Hulme, S. E., Shevkopyas, S. S., Apfeld, J., Fontana, W., & Whitesides, G. M. (2007). A microfabricated array of clamps for immobilizing and imaging *C. elegans*. *Lab on a Chip*, 7(11), 1515–1523.

<http://www.ncbi.nlm.nih.gov/pubmed/17960280>. <https://doi.org/10.1039/b707861g>; Design (B) adapted from Chronis, N., Zimmer, M., & Bargmann, C. I. (2007). Microfluidics for *in vivo* imaging of neuronal and behavioral activity in *Caenorhabditis elegans*. *Nature Methods*, 4(9), 727–731. <http://www.nature.com/doifinder/10.1038/nmeth1075>. <https://doi.org/10.1038/nmeth1075>.

### 4.3 FLOW RATE

The flow rate through the device can be important for (1) the sensitivity of the animal to shear stress and (2) the design of a fluidic network. Worms are not particularly sensitive to flow rates, so this was not a consideration for our device. However, other model organisms may be more responsive to shear flow. For example, as discussed in Section 5.3, mechanosensitivity of the lateral line of zebrafish larvae could be studied using this approach. In addition, the laminar flow profile and flow rate need to be considered in microfluidic platforms with an extended fluidic network, such as for drug or nutrient delivery.

### 4.4 MULTIPLEXING

Some devices for studying worms use multiplexing to increase experimental throughput by testing multiple worms in the same device (Hulme et al., 2007, 2010), but we found it difficult to remove worms from multiplexed devices. When one worm was successfully removed from such a device, the fluidic resistance of the vacant channel dropped. This redirected flow from channels that still had worms in them, so there was less pressure to push the remaining worms out.

### 4.5 FABRICATION

We fabricated microfluidic devices with the elastomer polydimethylsiloxane (PDMS) using well-established protocols (Jenkins, 2013; Xia & Whitesides, 1998). The process consists of two stages, (1) photolithography of the master mold

and (2) replica molding and bonding of PDMS devices. Details can be found in [Fehlauer et al. \(2018\)](#) and [Nekimken, Fehlauer, et al. \(2017\)](#). Ease of fabrication was an important consideration in the design of the trapping channel and actuators. We decided to restrict our microfluidic platform to a single layer to eliminate the need for alignment and maintain the simplicity of fabricating devices from a single master mold, instead of a mold for each layer. This fabrication process also made bonding devices to coverslips fast, simple, and reliable, because only one bonding step was needed and no alignment was required.

Mechanical properties of PDMS can be modified by changing the ratio between the base polymer and the cross-linking agent ([Wang, Volinsky, & Gallant, 2014](#)). The work by [Cho, Oakland, Lee, Schafer, and Lu \(2018\)](#) took advantage of this material property to influence membrane deflection of the actuators. We chose to use the standard 10:1 ratio of base:curing agent, but we used devices within 1 month of fabrication to limit stiffening from continued cross-linking over time ([Placet & Delobelle, 2015](#)).

#### 4.6 LAB AROUND THE CHIP

While microfluidic devices are sometimes touted as “lab on a chip” systems, this term neglects the external equipment that is required to operate the device, especially in a research environment. Operation of microfluidic devices with continuous flow requires external pressure pumps (e.g., syringe or solenoid) or integrated micro-pumps ([Laser & Santiago, 2004](#)). To apply mechanical stimuli in microfluidic devices to study mechanobiology in cells, tissues, and organisms, pneumatic or hydraulic equipment is required to generate the mechanical stimulus in addition to any pumps for flow control.

Our microfluidic device also required some external equipment for normal operation, and the detailed instructions can be found in [Fehlauer et al. \(2018\)](#). In this section, we describe why we chose to include the additional equipment in our experimental setup. We used a piezoelectric pressure control system (Elveflow OB-1 with 8 bar pressure unit) to control the pneumatic actuators because the system had a relatively fast response time and a simple user interface. As an alternative, we tested solenoid valves with a custom control system. However, they did not respond as fast as the piezoelectric technology, leading to attenuation of dynamic stimuli.

For fluid control, we used a gravity flow system supplemented with a peristaltic pump to provide a small amount of suction to the nose of the worm to hold it in place. The house vacuum with a simple regulator valve and liquid collector yielded similar results if a peristaltic pump was not available. The pump flowed buffer past the trap during the experiment, creating a small pressure drop in front of the worm from the continuously flowing fluid according to Bernoulli’s principle. The pressure drop had the added benefit of making it easy to dispose of worms, because the buffer carried the worm to the waste container when the worm was pushed through the exit of the trap. Alternatively, the worm could be collected for further experiments as it left the device.

Finally, a microscope was necessary for observing the results of an experiment. We measured the activation of mechanosensitive neurons using GCaMP (Chen et al., 2013), which changes fluorescence intensity when calcium concentration changes in the neuron. Mechanical stimulation of the neurons led to movement of the neuron away from the microscope's focal plane. To correct for defocusing, we used an additional fluorophore in our intensity measurements. This, in turn, required two excitation wavelengths and a beam splitter for imaging the intensities of both of the fluorophores simultaneously (Fehlauer et al., 2018; Nekimken, Fehlauer, et al., 2017). Different experiments require different optical setups, and the choices made when designing an imaging system can have a major impact on the results of an experiment.

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## 5 MICROFLUIDICS FOR MECHANOBIOLOGY OF MODEL ORGANISMS

Many of the design considerations discussed above apply to designing new microfluidic devices for mechanobiology as well as to adapting existing devices for studying other model organisms. Prior work can inform future developments in this field, so reviewing existing devices is an important part of the process for designing new ones. To this end, this section focuses on existing microfluidic platforms for mechanobiological studies, as defined above (Fig. 1). Many microfluidic devices have been used to study other aspects of model organisms, but the potential for using these devices to study mechanobiology is not yet fully realized. For reviews on microfluidic devices for sorting and imaging of multicellular organisms, readers are referred to works by Ben-Yakar, Chronis, and Lu (2009), Crane, Chung, Stirman, and Lu (2010), Hwang and Lu (2013), Sivagnanam and Gijs (2013), and Porto, Rouse, San-Miguel, and Lu (2016).

Each of the following sections features an overview of microfluidic devices used to study a model organism and then describes specific devices used to study mechanobiology in that organism. Sections are organized in the order of the animal's developmental stage: embryo, larva, adult, and explanted tissue. In contrast to worms, where the majority of research was performed on adults, microfluidic experiments on fruit flies and zebrafish focus on embryos and larvae. For fruit flies, this is a consequence of their physiological requirement for air, and, for zebrafish, it is due to their size. Each section concludes by highlighting some research opportunities tailored to each model organism.

### 5.1 *Caenorhabditis elegans*

#### 5.1.1 *Current state of the field*

Microfluidic devices for studying *C. elegans* have focused on (1) enabling imaging of worms and (2) creating specialized environments to study behavior of worms. These are reviewed as a whole elsewhere (Bakhtina & Korvink, 2014; San-Miguel & Lu, 2013). Both classes of devices have been used to study mechanobiology of worms. Devices designed for epifluorescence imaging of a worm require

some sort of immobilization, since the motile nature of worms makes high-resolution imaging of dim fluorophores difficult (Section 4).

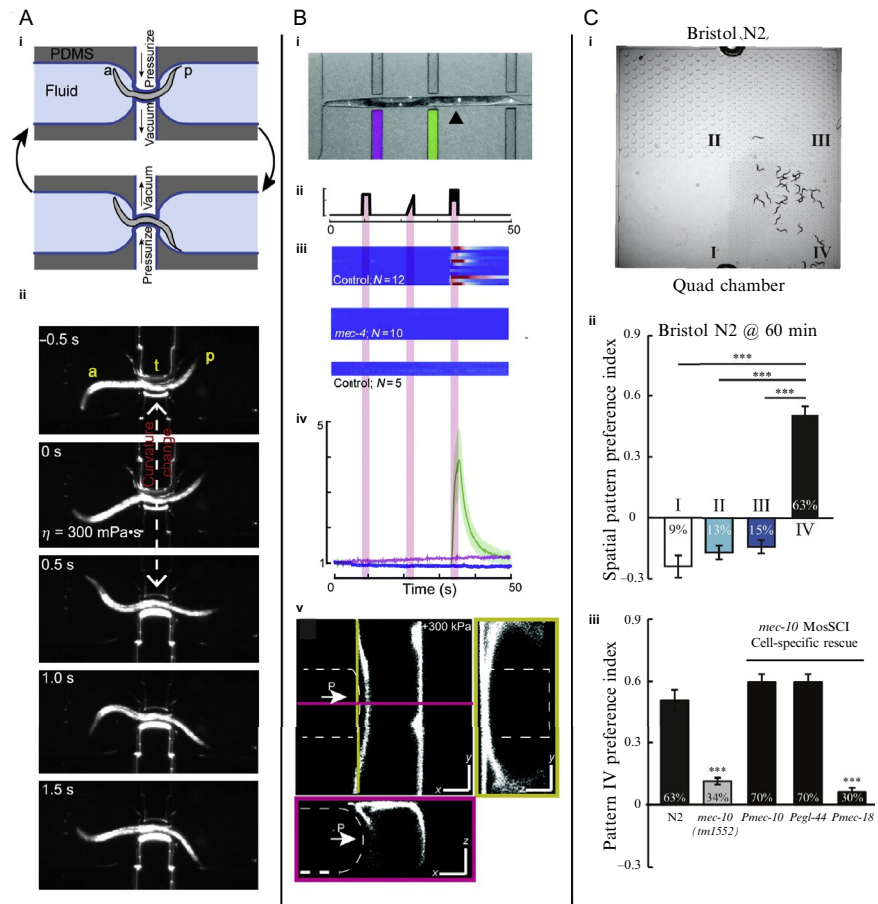
Devices for studying *C. elegans* behavior typically consist of a structured environment that presents mechanical, optical, or chemical stimuli to worms while providing a means (typically imaging whole worms) for researchers to observe the worms' behavior. Lockery et al. (2008) presented an early example of such a device where worms were placed in microfluidic chambers that contained micropillars of different size and density, then observed that the velocity of worms crawling through the device depends on pillar spacing. Subsequent researchers expanded on this device concept to measure the forces applied by worms during locomotion (Doll et al., 2009; Johari, Nock, Alkaisi, & Wang, 2013).

Initially, mechanobiological studies of *C. elegans* focused on the behavioral responses of worms to touch stimuli (Chalfie et al., 1985), but worms are now used to study mechanotransduction and mechanobiology of development on the cellular- and tissue-levels (Cram, 2014). The use of microfluidics for mechanobiology of worms is largely limited to studies of sensory neurons in adults rather than in developing larvae. To our knowledge, Cho et al. (2018) published the only device for testing touch sensitivity of larvae.

To study touch sensitivity of *C. elegans* larvae, Cho et al. (2018) presented a device adapted from previous devices for studying adult worms (Cho et al., 2017; Nekimken, Fehlauer, et al., 2017), with a fluorescent calcium sensor, GCaMP (Chen et al., 2013), as a readout for neuron activation. To mechanically stimulate a worm, the authors used actuators similar to those described in Section 3, but required a different polymer formulation, resulting in a lower modulus of elasticity to enable actuation suitable for small larvae. With this device, Cho et al. (2018) showed that the anterior ventral touch neuron can still be activated, despite the fact that the neuron is not incorporated into downstream neuronal circuits at the L2 stage. Similarly, a harsh touch neuron can be activated in L2 larvae even before its branched structure forms. Finally, the paper explores the mechanosensitivity of larvae during lethargus, a sleep-like state that occurs between larval stages in worms. In this case, anterior touch receptor neurons, as well as downstream interneuron, all failed to respond to a mechanical stimulus. Their findings indicate that lethargus involves inhibition of sensory neurons rather than attenuation of the signal by downstream neurons.

While no other microfluidic devices have been published for studying mechanobiology of worm embryos and larvae, other devices for sorting (Cornaglia et al., 2015; Sofela et al., 2018; Yang et al., 2017) and observing (Cornaglia et al., 2015; Hulme et al., 2010; Keil, Kutscher, Shaham, & Siggia, 2017) embryos and larvae have been presented. These devices have the potential to be used for studying mechanobiology if additional features are added to apply mechanical stimuli and observe the resulting information processing (Fig. 1). The devices described below accomplish this task for adult worms.

Wen et al. (2012) used microfluidics to study proprioception, a sensory system that uses mechanical cues to give organisms information about body position. In the study, the authors created microfluidic devices that control the curvature of a worm and then observed the worm's resulting movements (Fig. 5A).



**FIG. 5**

Microfluidic devices for *C. elegans* mechanobiology combine mechanical stimulation with behavioral and cellular readouts. (A) Device for controlling body curvature of worms (a: anterior, p: posterior). (i) Schematic of device and (ii) time series of a worm responding to change in body curvature. (B) Device for touch test of worms. (i) A worm immobilized in the device. (ii)–(iv) Stimulus profile, GCaMP fluorescence responses of individual worms, and average GCaMP response of touch receptor neurons. (v) Confocal images of a worm being indented in the device. (C) (i) Worms in quad-chamber for measuring texture preferences. (ii) Preference index for the spatial patterns. (iii) Disruption and rescue of preference for pillars in pattern IV.

Panel (A) reproduced and adapted from reference Wen, Q., Po, M. D., Hulme, E., Chen, S., Liu, X., Kwok, S. W., ... Samuel, A. D. (2012). Proprioceptive coupling within motor neurons drives *C. elegans* forward locomotion. *Neuron*, 76(4), 750–761. <http://linkinghub.elsevier.com/retrieve/pii/S0896627312008057>. <https://doi.org/10.1016/j.neuron.2012.08.039> with permission from Elsevier; Panel (B) reproduced and adapted from Nekimken, A. L., Fehlauer, H., Kim, A. A., Manosalvas-Kjono, S. N., Ladpli, P., Memon, F., ... Krieg, M. (2017). Pneumatic stimulation of *C. elegans* mechanoreceptor neurons in a microfluidic trap. *Lab on a Chip*, 17(6), 1116–1127. <http://xlink.rsc.org/?DOI=C6LC01165A>. <https://doi.org/10.1039/C6LC01165A> with permission from the Royal Society of Chemistry, authors' own work; Panel (C) reproduced and adapted from Han, B., Dong, Y., Zhang, L., Liu, Y., Rabinowitch, I., & Bai, J. (2017). Dopamine signaling tunes spatial pattern selectivity in *C. elegans*. *eLife*, 6(4), 431–436. <http://journals.sagepub.com/doi/10.1177/2211068216669688>; <https://elifesciences.org/articles/22896>. <https://doi.org/https://doi.org/10.7554/eLife.22896> with permission from eLife under the CC BY license.



In one of their devices, a worm traveled down a channel to a restriction flanked by two thin, deformable walls. These walls served as actuators when inflatable chambers on the opposite side of the wall from the worm were subjected to vacuum or positive pressure (Fig. 5Ai). By applying vacuum to one actuator and positive pressure to the other, the devices induced negative curvature on one side of the worm and positive curvature on the other. Using this device, Wen et al. (2012) observed that the induced curvature propagates toward the posterior of worms, independent of the curvature anterior to the actuators (Fig. 5Aii). They also used similar passive devices that constrain a worm to a straight posture or defined curvature, which prevented propagation of the worm's normal sinusoidal motion past the constraint. Additional experiments combining microfluidic control of body curvature with optogenetic manipulation or optical measurement of calcium concentration in neurons showed that B-type cholinergic neurons are responsible for this proprioceptive coupling (Wen et al., 2012).

Nekimken, Fehlaue, et al. (2017) and Cho et al. (2017) used similar lateral pneumatic actuators for stimulation and a fluorescent calcium sensor, GCaMP (Chen et al., 2013), for readout of neuronal activity. With our device, which is described in Section 4, we found that the touch receptor neurons were also activated with a blue light stimulus, such as the excitation light for green fluorescent proteins. Worms lacking the protein LITE-1 were not activated by blue light and were used for subsequent experiments. In this device, step function and ramp stimuli failed to elicit significant activation of touch neurons, but a buzz stimulus with a sinusoid superimposed on a step led to observable activation of touch receptor neurons (Nekimken, Fehlaue, et al., 2017).

The device presented by Cho et al. (2017) used an active trapping strategy consisting of two pairs of laterally deformable actuators that hold a worm in place while a third pair between the two sets of trapping actuators applies mechanical stimuli to the worm. Using step function stimuli, they demonstrated that increases in stimulus magnitude or duration can increase the activation of touch receptor neurons. Additionally, they observed activation of a nociceptor in the worm (Chatzigeorgiou et al., 2010) when stimuli of higher magnitude were applied. Finally, Cho et al. (2017) looked at how drugs modulate touch sensitivity by performing a pilot screen with small molecules from a library of ligand candidates. Most of the compounds screened had deleterious effects on touch sensation to some degree, but some increased the touch sensitivity of worms.

McClanahan, Xu, and Fang-Yen (2017) also recently described a device for testing the touch sensitivity of crawling worms with a behavioral readout. Unlike the devices designed by Nekimken, Fehlaue, et al. (2017) and Cho et al. (2017), the actuators in this device stimulated worms by deforming the top of the channel rather than the side. Another distinctive feature of this device is that it contained several parallel worm channels, each with several actuators, enabling higher-throughput experiments. Image processing then determined the velocity of each worm's centroid and an algorithm used a threshold to score changes in velocity as responses. Since worms were continuously crawling in the device, there was no

active control over stimulus location, yielding a variety of stimulus locations for each trial. This enabled [McClanahan et al. \(2017\)](#) to explore the effect of stimulus location by grouping the stimuli into five bins based on where an individual stimulus was applied. They showed that *egl-5* mutants, which lack functional posterior touch receptor neurons, tend to respond to posterior stimuli by moving toward the stimuli, rather than away from it like wild-type animals.

[Park et al. \(2008\)](#) used an agar device with micropillars to study how worms process information during locomotion. In contrast to other studies of worm locomotion in microfluidic chambers that focus on characterizing crawling and swimming in different environments ([Lockery et al., 2008](#)), this paper dissected mechanobiological pathways by showing the effect of knocking out genes that are essential for mechanosensation ([Park et al., 2008](#)). Worms crawling through the pillars lacking the ion channel subunits MEC-4 and MEC-10 moved less than half as fast as wild-type worms, on average. Their experiments thus quantified the dependence of worms' locomotion on touch sensation using a behavioral readout.

Similarly, [Han et al. \(2017\)](#) studied the preference of worms for different mechanical environments by presenting four regions with different pillar spacing to worms and observing where the worms accumulated ([Fig. 5C](#)). After an hour, 63% of worms in the device were found in the quadrant with the smallest pillars and tightest spacing (175  $\mu\text{m}$  pillar diameter, 116  $\mu\text{m}$  pillar spacing). Subsequent experiments showed that the preference is regulated by a dopamine pathway activated by a mechanosensitive ion channel, TRP-4. Some alleles of TRP-4 suppress the preference for the smaller, denser pillars. Thus, by comparing the behavior of worms with different genotypes in their device, [Han et al. \(2017\)](#) were able to elucidate a mechanosensory pathway in worms responsible for discrimination of texture.

### 5.1.2 Research opportunities

The current literature on microfluidics for *C. elegans* mechanobiology is largely limited to behavioral and calcium-dependent fluorescence readouts. Electrophysiology, biomechanical characterization, and advanced fluorescence measurements, such as fluorescence resonance energy transfer (FRET), have received less attention in combination with microfluidics. A microfluidic device for electrophysiology of worms was presented, but it did not incorporate mechanical stimulation ([Gonzales et al., 2017](#)). Using these alternative readouts in existing devices for studying mechanobiology could lead to novel scientific findings.

Another opportunity for expanding the experimental capabilities in this field is the combination of multiple sensing modalities on the same device. [Cho et al. \(2017\)](#) demonstrated this possibility by showing that exposing a worm to small molecules in their device can increase or decrease its touch sensitivity, depending on the particular chemical compound ([Cho et al., 2017](#)). The effects of drugs on touch sensation are particularly interesting given the deleterious effects of chemotherapy on touch sensation ([Fehrenbacher, 2015](#)). Including additional modalities such as temperature control might yield further insights into how cells integrate multiple stimuli.

Moreover, mechanobiology is concerned with not just sensory biology, but also the effect of mechanical stimuli on development. While many studies explore the effect of mechanical cues within an organism on development [Cram \(2014\)](#), little is known about how external mechanical stimulation might perturb development. For example, it is not known how a worm would develop if an embryo was placed under increased hydrostatic pressure or compression. Microfluidics are an ideal technology to aid in answering these open questions, combining application of mechanical stimuli with other important considerations like delivery of nutrients.

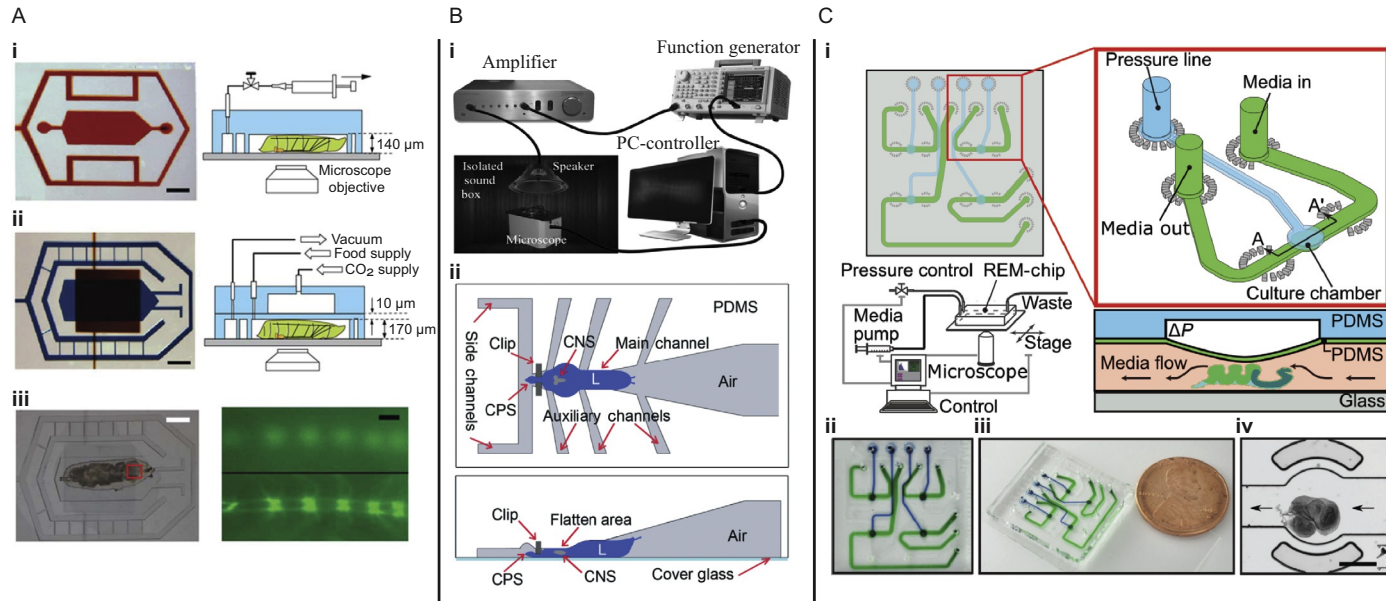
## 5.2 *Drosophila melanogaster*

### 5.2.1 Current state of the field

Microfluidic devices for studying fruit flies include platforms for automation of embryonic assays ([Chen et al., 2004](#); [Chung et al., 2011](#); [Levario, Zhan, Lim, Shvartsman, & Lu, 2013](#)), defining temperature environments for morphogenetic studies ([Dagani et al., 2007](#); [Lucchetta, Lee, Fu, Patel, & Ismagilov, 2005](#); [Lucchetta, Munson, & Ismagilov, 2006](#)), and automating injections ([Delubac et al., 2012](#)). Recently, to study the mechanical properties of fruit fly follicle cells during oogenesis, [Crest, Diz-Muñoz, Chen, Fletcher, and Bilder \(2017\)](#) designed microscale flexible “egg cartons” to immobilize the cells while measuring the stiffness of basement membrane in live follicles using atomic force microscopy.

Drawing inspiration from devices originally designed to study *C. elegans*, such as [Chokshi et al. \(2009\)](#) and [Wen et al. \(2012\)](#), microfluidic platforms to study mechanobiology in *Drosophila* larvae are starting to emerge. Some of these devices immobilize fruit fly larvae for imaging purposes using mechanical constraints ([Ghaemi, Rezai, Iyengar, & Selvaganapathy, 2015](#); [Ghaemi, Rezai, Nejad, & Selvaganapathy, 2017](#)), temperature ([Chaudhury et al., 2017](#)), or carbon dioxide ([Ghannad-Rezaie, Wang, Mishra, Collins, & Chronis, 2012](#)). Differences between worm and fruit fly larvae need to be considered for facile adaption of devices from one model organism to another. Fruit fly larvae are larger with a stiffer cuticle, and, moreover, the larvae are not as readily maintained in a fluid suspension and require some access to air ([Chaudhury et al., 2017](#)).

[Ghannad-Rezaie et al. \(2012\)](#) demonstrated two different designs of microfluidic devices for short- and long-term studies of cellular responses to neural injuries in larvae. The authors developed a novel approach for immobilization of larvae that reduces stress in the larval body by employing mechanical forces and/or carbon dioxide gas. To deliver carbon dioxide, they implemented a second layer on top of the microfluidic chamber ([Fig. 6A](#)). Both devices for short- and long-term investigations sealed reversibly to a coverslip. For long-term studies, the device enabled time lapse imaging of axonal sprouting following injury to dendrites with laser axotomy. Recently, the two-layer architecture was adapted to develop a device that combines liquid coolant with mechanical compression for immobilization of larvae for high-resolution imaging in vivo ([Chaudhury et al., 2017](#)).



**FIG. 6**

Microfluidic devices for mechanobiology of *Drosophila melanogaster* combine mechanical stimulation with imaging. (A) Photographs and schematics of microfluidic devices for

(Continued)

To study the auditory response of larvae, [Ghaemi et al. \(2015\)](#) demonstrated two designs, one for automated loading and one with a manual loading option. The manual FlexiChip is shown in [Fig. 6B](#), along with their experimental setup for auditory stimulation. Their devices reduced movement of the central nervous system to permit monitoring of the response to acoustic signals using fluorescence imaging. The authors proposed further study of neuronal responses to other external cues with this platform.

[Zhang, Dong, and Liu \(2017\)](#) reported a system setup for robotic micromanipulation of fruit fly larvae, consisting primarily of a fluorescence microscope, an immobilization device, micromanipulators, and a glass pipette mounted on a microscale force sensor. The manipulation procedure was automated to recognize the pipette tip and the centroid of the first larva's head in the immobilization device as the target location for applying mechanical stimulation. The platform was developed to facilitate identification of new mechanotransduction mechanisms in larvae.

[Narciso, Contento, Storey, Hoelzle, and Zartman \(2017\)](#) demonstrated one of the first microfluidic devices for probing mechanosensitive responses in fruit fly organs. The microfluidic device was used to create a "Regulated Environment for Microorgans," termed REM-chip, and it was designed to support studies on explanted organs by mimicking the *in vivo* environment. The detailed schematic of the REM-chip is depicted in [Fig. 6C](#). Briefly, the organ tissue was gently loaded into a fluidic channel

#### FIG. 6—Cont'd

(i) short- and (ii) long-term immobilization of larvae for studying cellular responses to neural injuries (scale bars 1 mm). (iii) Bright-field (left, scale bar 1 mm) and fluorescent images (right, scale bar 20  $\mu\text{m}$ ) of the larva body with a fluorescently (GFP) labeled ventral cord. (B) (i) Experimental setup for examining the auditory response of fruit fly larvae. (ii) Schematic top and side views of the FlexiChip used to immobilize larvae for quick manual animal loading. (C) Regulated Environment for Microorgans (REM) chip, (i) schematic design of the chip with the *inset* showing detail of a single chamber unit, and the operation diagram for wing disc compression, (ii) and (iii) photographs of the device with individual fluidic (*green*) and pressure (*blue*) channels, and (iv) wing disc loaded into a culture chamber of the device (scale bar 400  $\mu\text{m}$ ).

*Panel (A) reproduced and adapted from reference Ghannad-Rezaie, M., Wang, X., Mishra, B., Collins, C., & Chronis, N. (2012). Microfluidic chips for in vivo imaging of cellular responses to neural injury in Drosophila larvae. PLoS ONE, 7(1), e29869. <https://doi.org/10.1371/journal.pone.0029869> with permission from PLoS ONE under the CC BY license; Panel (B) reproduced and adapted from reference Ghaemi, R., Rezaei, P., Iyengar, B. G., & Selvaganapathy, P. R. (2015). Microfluidic devices for imaging neurological response of Drosophila melanogaster larva to auditory stimulus. Lab on a Chip, 15(4), 1116–1122. <http://xlink.rsc.org/?DOI=C4LC01245C>. <https://doi.org/10.1039/C4LC01245C> with permission from the Royal Society of Chemistry under the CC BY license; Panel (C) reproduced and adapted from reference Narciso, C. E., Contento, N. M., Storey, T. J., Hoelzle, D. J., & Zartman, J. J. (2017). Release of applied mechanical loading stimulates intercellular calcium waves in Drosophila wing discs. Biophysical Journal, 113(2), 491–501. <http://www.sciencedirect.com/science/article/pii/S0006349517306264>. <https://doi.org/10.1016/j.bpj.2017.05.051> with permission from Elsevier.*

where growth media could be delivered. The second layer of the chip applies compressive stress at intersections across deformable diaphragms. Interestingly, the authors found that mechanical unloading can initiate intercellular calcium waves in fruit fly wing discs, similar to those observed *in vivo* during organ development.

He, Si, Huang, Samuel, and Perrimon (2018) investigated mechanoregulation of stem cell differentiation in the adult midgut. To examine cellular responses, the authors presented an *ex vivo* approach. By compressing explanted midguts in a microfluidic device, He et al. (2018) studied calcium signaling in cells expressing an ion channel sensitive to mechanical stimuli, PIEZO. The design of the chip was previously applied to study *C. elegans* by Wen et al. (2012), described in Section 5.1.1 and shown in Fig. 5A.

### 5.2.2 Research opportunities

Microfluidic devices to study mechanobiology in fruit flies have followed in the footsteps of those for *C. elegans*. However, because adult fruit flies breathe air and fly, devices for whole-organism studies are restricted to embryos and larvae. An interesting opportunity is the development of devices that not only combine microfluidics with mechanobiology but also adapt to the model organism as it grows in the different stages of its life cycle, facilitating development studies of multicellular organisms.

Microfluidic technologies for *ex vivo* mechanobiological studies are an important future direction, since explanted tissues and organs can provide valuable knowledge on biological processes, as already illustrated in works by Narciso et al. (2017) and He et al. (2018). This particular area would benefit from standardized protocols for maintaining healthy explants.

Furthermore, as technological developments in the field of microfluidics continue to advance, they enable integration of new readouts and stimuli, such as exposure to chemical gradients or electrical fields. Since, in principle, it is possible to recover larvae for subsequent analyses following stimulation, this opens up new doors for studies on aging, genomics, and proteomics.

## 5.3 *Danio rerio*

### 5.3.1 Current state of the field

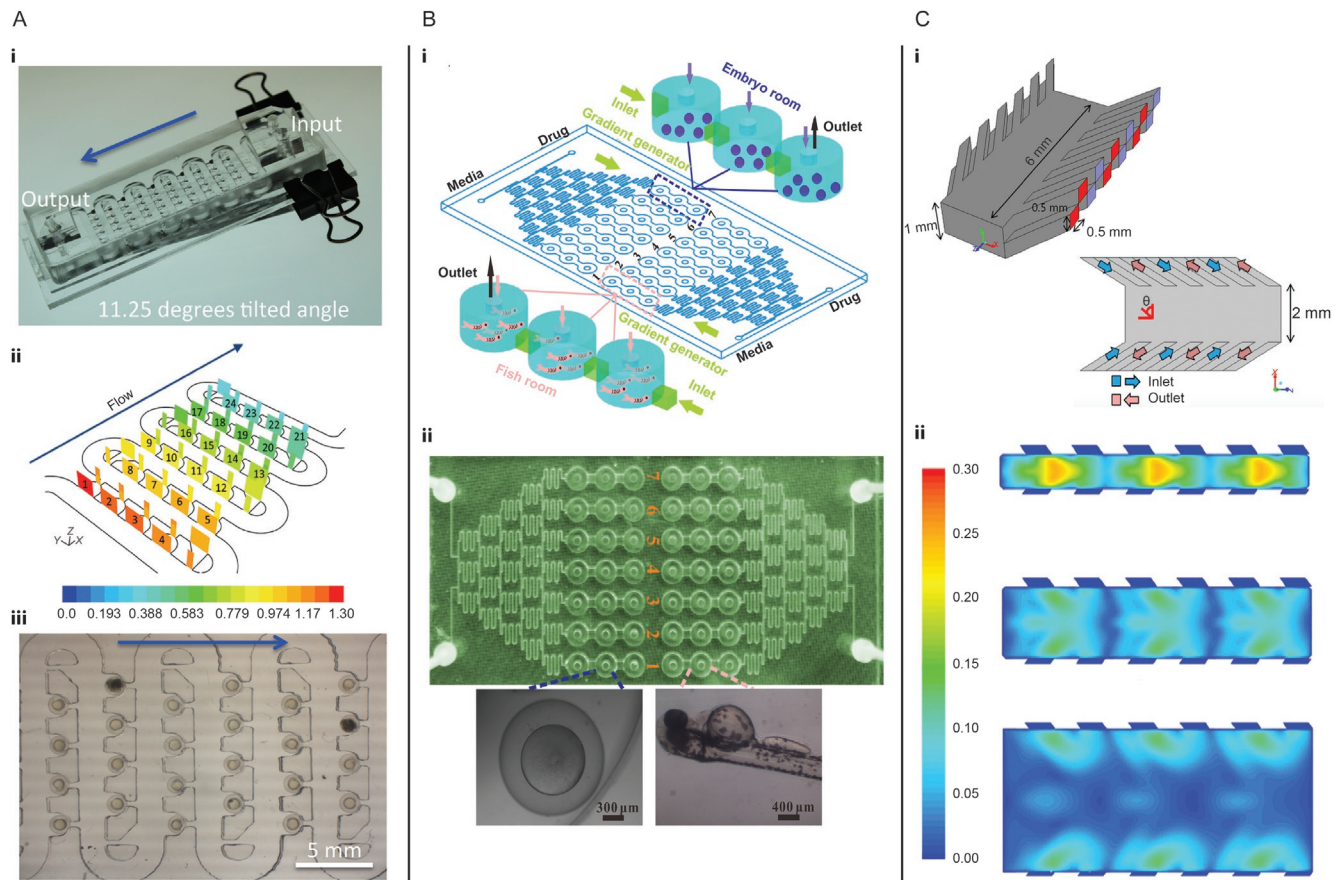
The small freshwater vertebrate *Danio rerio* (zebrafish) is an emerging model to study mechanobiology in microfluidic devices. So far, microfluidics for studying zebrafish focus on methods for high-throughput drug screening. One pair of adult fish can produce many eggs, allowing a high number of replicates for drug screening with automated microfluidics (Gibert, Trengove, & Ward, 2013; Parnig, Seng, Semino, & McGrath, 2002). The fish embryo toxicity assay is a promising alternative approach to classical ecotoxicity testing with adult fish (Lammer, Carr, et al., 2009; Lammer, Kamp, et al., 2009; Strähle et al., 2012). The transparency of embryos and larvae makes the effects of drug application on the developing animal easy

to monitor. Heart rate can be tracked in larvae 22–24 h postfertilization and the cardiovascular system is fully developed 36 h postfertilization (Kimmel et al., 1995; Yanik, Rohde, & Pardo-Martin, 2011). Mechanosensitive hair cells in the lateral line are fully developed and visible 48 h postfertilization (Kimmel et al., 1995), making zebrafish an ideal model to study mechanobiology.

Most microfluidic devices for zebrafish have focused on trapping embryos or larvae for long-term imaging (reviewed by Yang et al. (2016)). The biggest challenges in the design of a zebrafish microfluidic-based chip compared to other model organisms are (1) the zebrafish is a freshwater organism and needs sufficient perfusion for fresh water exchange to deliver oxygen and maintain consistent pH without damaging the animal, and (2) embryos develop at an optimal temperature of 28–29°C (Westerfield, 2000), so a heating system may be necessary for the health of the fish.

Akagi et al. (2012) developed a microfluidic device to study a large number of embryos simultaneously (Fig. 7A). The device consisted of several connected rows with trapping channels containing hydrodynamic deflectors and a small suction channel. Embryos were loaded one-by-one via hydrodynamic forces. The first embryo was trapped in the first empty trap channel, blocking that channel so subsequent embryos rolled freely toward the next available trap channel. Mounting the device on an elevated stage improved the trapping success from 80% when the hydrodynamic forces alone trapped the embryo, to nearly 100% when a combination of both hydrodynamic and gravitational forces held the embryos in place. In this device, the embryos can be kept for long-term imaging until they hatch and leave the trap. The device was positioned on a stage heater to maintain optimal culturing temperature. With the same device, Akagi et al. (2012) performed an angiogenesis assay to measure the development of new blood vessels and tested the effect of a selective blocker for angiogenesis, the inhibitor AV951. The transparency of the larvae allowed for a clear visualization of the presence or absence of newly forming blood vessels.

To study different drug concentrations in both embryo and larvae, Li et al. (2014) developed a device consisting of two identical units each divided into two parts: (1) three replicates of seven open culture rooms for embryos or larvae and (2) a concentration gradient generator for dose-dependent drug application (Fig. 7B). Yang et al. (2011) and Choudhury et al. (2012) previously described similar designs with concentration gradient generators. They all had a perfusion system to replace manual exchange of media and prevent pH changes. Li et al. (2014) increased the number of replicates per well. Each well was connected to a plug, allowing easy removal of dead embryos or larvae. Indicators of health were recorded by imaging the device, including the lack of a heartbeat, blood circulation, and motility, which together characterize a lethal or teratogenic effect. The goal of Li et al. (2014) was to investigate the toxicity on embryos and larvae of an antiasthmatic drug which increases zebrafish heart rate with increased drug concentration. In parallel, they also investigated the effect of perfusion velocity on hatching success of embryos, estimating a flow rate of 5  $\mu\text{L}/\text{min}$  for optimal hatching success. Although their main goal



**FIG. 7**

See legend on opposite page.



focused on the effects of drugs on healthy embryos, this device is also suitable to study the effect of shear stress on hatching and organ development.

Kwon et al. (2014) presented a microfluidic device to apply shear stress to the lateral line of larvae (Fig. 7C), which detects mechanical stimuli induced by movements of the surrounding fluid. The hair cells in the lateral line system are functionally analogous to hair cells in the inner ear of mammals, except they have the capacity to regenerate. Better understanding of the molecular and cellular basis of hearing loss and regeneration of hair cells in zebrafish may aid in designing therapies to induce regeneration in mammals (Kniss et al., 2016). The design of Kwon et al. (2014) focused on manipulating boundary layers using sidewall inlets and outlets. They used computational fluid dynamics simulations with and without a fish-shaped obstruction to optimize the flow profile in their device. Their simulations showed that a 2.0 mm wide channel with the inlet/outlet pairs set to 45 degrees was optimal without a fish, and a 1.0 mm wide channel with a 60 degrees inlet/outlet angle created the lowest pressure (0.3 Pa) on the fish head while maintaining a reasonably strong shear stress (1.9 Pa) on the lateral line hair cells. To study hair cell damage, shear stress at the boundary must be sufficient to induce damage with a reduced flow rate in the center of the device to prevent damage to the rest of the fish.

#### FIG. 7—Cont'd

Microfluidic devices for studying zebrafish have the potential to be used for mechanobiology, but have mostly been used for other applications. (A) Tilted device for combined gravitational and hydrodynamic trapping. (i) Photograph and (ii) simulation of hydrodynamic forces for trapping embryos. (iii) Embryos trapped in the device for long-term imaging. (B) Microfluidic device for drug-delivery to embryos and larvae. (i) Schematic of the device with media inlet, drug inlet, gradient generator and seven series of fish tanks. (ii) Photograph of the device. (C) Microfluidic device to apply shear stress to zebrafish larvae. (i) Isometric and top-view schematics of optimized device design ( $\nu = 60$  degrees). (ii) Simulation of fluid velocity magnitude in m/s at channel widths of 0.5, 1.0, and 2.0 mm with an inlet and outlet angles  $\nu$  set to 45 degrees.

Panel (A) reproduced and adapted from reference Akagi, J., Khoshmanesh, K., Evans, B., Hall, C. J., Crosier, K. E., Cooper, J. M., ... Wlodkowic, D. (2012). Miniaturized embryo array for automated trapping, immobilization and microperfusion of zebrafish embryos. *PLoS ONE*, 7(5), 12–15. <https://doi.org/10.1371/journal.pone.0036630> with permission from *PLoS ONE* under the CC BY license; Panel (B) reproduced and adapted from Li, Y., Yang, F., Chen, Z., Shi, L., Zhang, B., Pan, J., ... Yang, H. (2014). Zebrafish on a chip: A novel platform for real-time monitoring of drug-induced developmental toxicity. *PLoS ONE*, 9(4), e94792. <https://doi.org/10.1371/journal.pone.0094792> with permission from *PLoS ONE* under the CC BY license; Panel (C) reproduced and adapted from reference Kwon, H.-J., Xu, Y., Solovitz, S. A., Xue, W., Dimitrov, A. G., Coffin, A. B., & Xu, J. (2014). Design of a microfluidic device with a non-traditional flow profile for on-chip damage to zebrafish sensory cells. *Journal of Micromechanics and Microengineering*, 24(1), 017001. <http://stacks.iop.org/0960-1317/24/i=1/a=017001?key=crossref.898bd18771a566d89de4176cbca5c> <https://doi.org/10.1088/0960-1317/24/1/017001> with permission from IOP publishing.

### 5.3.2 Research opportunities

Microfluidic devices have potential for studying mechanobiology of zebrafish, especially larvae. So far, the effects of shear stress on zebrafish have only been studied to identify optimal flow rates to avoid damaging larvae (Li et al., 2014). Thus, future studies could address the effect of shear stress on cell damage, regeneration, and organ development more thoroughly, as suggested by Kwon et al. (2014). Organ development has also been studied under static mechanical input using microfabricated platforms (Chen & Chen, 2013). Chen and Chen (2013) studied the effects of silicon nanowires on heart rate and organ development. Using silicon substrates with and without hydrophobic or hydrophilic nanowires, they identified an increase in heart rate on substrates with rougher textures. These textures and other mechanical stimuli could be integrated into a microfluidic device to study their impact on embryo development. Adapting devices originally designed for *C. elegans* and *Drosophila* will also advance experimental capabilities for zebrafish. Although progress in studying zebrafish mechanobiology with microfluidics is so far limited, microfluidic platforms have the potential to advance the study of zebrafish mechanobiology.

## 5.4 OTHER MODELS

While the three model organisms discussed above are some of the most commonly used, there are other model organisms that are suited for addressing other mechanobiological questions. Below, we highlight two microfluidic devices, one designed to study root growth in the plant *Camellia japonica* and another for monitoring muscular activity in *Hydra vulgaris*, and comment on how these devices could be used for further mechanobiological studies.

Agudelo et al. (2013) designed a microfluidic device to study the pollen tube growth of the plant *C. japonica*. Their device distributes pollen grains to microchannels of varying geometries. The pollen grains then extended growth tubes into the channels with different growth characteristics due to the geometry of the channel, flow rate through the channel, and chemical composition of fluid in the channel. The authors found that high flow rates can damage the pollen grain, and that kinks in the growth channels can help the pollen grain anchor itself in place. Either of these observations could be the basis of a mechanobiology study on root development or mechanosensing at the tip of pollen tubes, but the authors focused on the effect of air-water interfaces and chemical cues in this study.

*H. vulgaris* is a freshwater polyp and model organism for tissue regeneration. Hydra are small (0.5–15 mm) and transparent. Their neurons continually replenished and are more dynamic than in other model organisms (Badhiwala, Gonzales, Vercosa, Avants, & Robinson, 2018). Badhiwala et al. (2018) presented three polymer-based microfluidic devices to study the fragile *Hydra*: (1) an hour-glass chamber for electrophysiology that constrained the body column from large movements, (2) a wheel-and-spoke perfusion chamber that constrained locomotion, and (3) a behavioral micro-arena with micropillars that limited movements and locomotion in the vertical plane but allowed all movements in the horizontal plane. They observed that the *Hydra* reacted to shear stress induced by a high flow rate with

body contractions or tentacle swaying. Here, they tried to avoid mechanical stimulation by using a reduced flow rate and instead studied the response to different chemical stimuli. However, this design seems suitable to investigate shear stress responses and future designs could integrate microactuators for applying mechanical compression in addition to shear stimuli.

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## 6 CONCLUSION

Microfluidic technologies are opening doors to mechanobiological studies of multicellular model organisms and their tissues. Worms, fruit flies, and zebrafish are excellent candidates for mechanobiological study in microfluidics because they share fundamental characteristics with more complex organisms, are amenable to genetic techniques, can be cultivated in the lab with relative simplicity, and have the proper size to fit in microfluidics. Further integration of mechanical actuation into existing devices will be key to supporting many more applications. Devices and features developed for one model organism can be easily adapted to another, expanding experimental capabilities without starting from scratch. Thus, microfluidic technologies will continue to contribute to advancing our understanding of mechanobiology in development, homeostasis, and disease.

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