Chapter 4

Advanced Microfluidic Assays for *Caenorhabditis elegans*

Natalia A. Bakhtina, Neil MacKinnon and Jan G. Korvink

Additional information is available at the end of the chapter

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Abstract

The *in vivo* analysis of a model organism, such as the nematode *Caenorhabditis elegans*, enables fundamental biomedical studies, including development, genetics, and neurobiology. In recent years, microfluidics technology has emerged as an attractive and enabling tool for the study of the multicellular organism. Advances in the application of microfluidics to *C. elegans* assays facilitate the manipulation of nematodes in high-throughput format and allow for the precise spatial and temporal control of their environment. In this chapter, we aim to illustrate the current microfluidic approaches for the investigation of behavior and neurobiology in *C. elegans* and discuss the trends of future development.

Keywords: *C. elegans*, chip-based, manipulation, microfluidics, model organism

1. Introduction

The invertebrate *Caenorhabditis elegans*, *Drosophila melanogaster*, and the vertebrate zebrafish (*Danio rerio*) are the most widely studied multicellular organisms. The *in vivo* analysis of these model organisms allows the understanding of many complex physiological processes, addressing many of the questions relevant to human biology. The choice of model organism depends on the biological question under investigation. For example, *C. elegans* is simple enough to be experimentally tractable. It has a short life cycle (3 days at 25 °C) and lifespan (15–17 days at 25 °C), passes through four larva (L1–L4) stages and an adult stage [1]. Its small size (1–1.2 mm long and 80 μm wide), transparent body at all life stages, and preferred food source (*Escherichia coli*) simplify its maintenance on agar plates or liquid cultures allowing visualization of individual cells and organs in intact animals. *C. elegans* possesses one of the simplest central nervous systems (the adult hermaphrodite has 302 neurons). Because it is so well studied, rapid identification of signaling pathways, for instance, in studies of aging, has

become possible. About 40 % of human disease genes have an orthologue in the genome of *C. elegans*, including those genes associated with Alzheimer disease, Parkinson's disease, Huntington's disease (HD), and many other neurodegenerative disorders [1–3]. This astonishing degree of correspondence permits the modeling of human ailments in a simple invertebrate without involving actual human subjects and provides a meaningful insight into the pathogenesis of a complex disease phenotype.

Traditionally, behavioral genetics is employed as a prime method for neurobiological studies in *C. elegans*. It is based on manual worm manipulation on a Petri dish or a multiwell plate, and monitoring the effects on various biological processes, such as growth and fertility, by visual inspection. Refreshment of old buffer solutions by a fresh solution is invasive and causes stress both to the larvae and adults. For drug screening, the concentration of the active compounds in the exposing solution might not be precisely controlled because of evaporation and non-selective adsorption effects on the wall of the wells. Permanent immobilization of the worm for further neuronal analysis is performed by means of glue or anesthetics. These methods are time consuming, expensive, tedious, prone to human flaws, and frequently result in failure. To address these problems, novel technologies for the manipulation of multicellular organisms are needed.

Microfluidics has recently been adopted as an instrument both to expand and accelerate progress related to the treatment of human diseases and injuries. Due to precise and automated manipulation of fluids and samples (e.g., single cell, multicellular organism, etc.) in a system of channels (10 –150 μm), a microfluidic-based approach is able to open up aspects that would remain hidden from traditional laboratory techniques. The technology provides a junction between engineering and pure sciences with an immense potential for offering simple and practical solutions. The unique properties of this technology are highlighted by several aspects. First, the dimensions of microfluidic channels perfectly match to the size of samples, allowing precise manipulation. With moving parts, flowing fluids, or other passive mechanisms, microsystems can be used to align samples with a particular orientation with ease as compared to hand-manipulations. Second, the ability to manipulate small amounts of liquid makes it suitable for the precise delivery of small amounts of reagent. Due to the laminar nature of the flow at the micro scale, efficient mass and energy transfer can be controlled in a completely predictable manner (e.g., diffusion of dissolved gases across tens of microns through fluids or polymer membrane materials). Third, based on relatively inexpensive polymer-based fabrication techniques, such as polydimethylsiloxane (PDMS) replica molding, it has become feasible to realize disposable, economic, and biocompatible systems [4]. Complex structures, adapted to different applications, can be easily fabricated in a short time. Finally, the capability to realize large-scale integration makes it possible to handle a large population of samples in parallel or in series for high -throughput assays. For example, COPAS BIOSORT high-throughput analysis system from Union Biometrica, Inc. (USA), enables the performing of high-speed imaging and offers the possibility of studying a large quantity of individual worms, thereby providing detailed statistical information on the biological variance within the same population.

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Substantial advances in microfluidic techniques and particular research interest in *C. elegans* have driven the development of numerous microchip-based systems. They have been reviewed a number of times focusing on various aspects of miniaturized systems, their advantages, application challenges, and scientific potential [5–19]. A summary of microfluidic-based systems with respect to the organism, organ, or tissue studies was presented by Sivagnanam et al. [5]. All available on-chip approaches for *C. elegans* investigations were systemized by the authors [6]. A classification diagram for structuring of approximately 100 references that simplifies their search according to five evaluated aspects (measured output data, and method for sorting, immobilization, stimulation, and detection of *C. elegans*) is included. In addition, we listed the relevant sorting, immobilization, and imaging methods that have been reported in recent literature, and indicated the main qualitative and quantitative characteristics for each.

This chapter provides a comprehensive overview of recent microfluidic-based approaches for investigations of worm behavior and neurobiology (Figure 1). This includes a discussion on tools and approaches needed to ensure high-throughput manipulation (culturing, sorting, and immobilization) and assaying for behavioral and neuronal studies. In addition, a perspective of novel methods for studies of metabolic activity facilitated by microfluidics is presented.

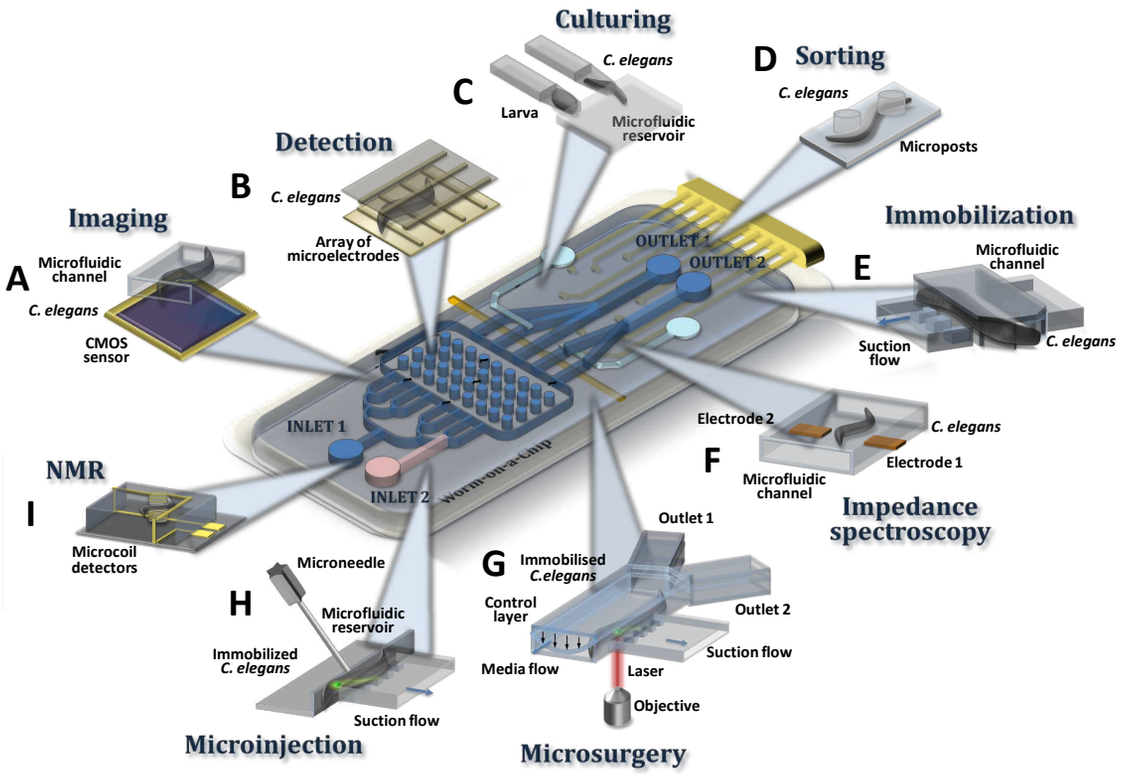

Figure 1. Schematic illustration of a microfluidic platform for *C. elegans* assaying. The structure of this chapter is as follows: in Section 2 we summarize the most frequently utilized imaging (A) and detection (B) methods; in Section 3 we concentrate our attention on different techniques for worm-on-a-chip manipulation, such as culturing, sorting, and immobilization (C, D, and E); Section 4 provides an overview of behavioral and neuronal phenotypes of *C. elegans* facilitated by different analysis techniques, including electrochemical impedance spectroscopy (F), microsurgery (G), and microinjection (H); additionally, Section 5 presents the methods utilized for metabolic activity studies (I).

2. Microfluidic approaches for *C. elegans* detection

Conventional optical imaging is an established detection technique for the observation of biological samples (e.g., cells, microorganisms, etc.). Microscopy-based (fluorescent, nonfluorescent, or their various combinations) systems can be used to extract valuable and unique data (e.g., image the activity of specific neurons) from biological samples. Combined with microfluidics, these systems offer several important advantages required for high-throughput screening [7–10]. Fully automated components, software control, and image processing tools make commercial confocal microscopes extremely versatile for real-time and high -resolution diagnosis. However, conventional optical imaging systems are quite expensive, bulky, and limit the miniaturization of chip-based systems. An overview of different optical imaging approaches in microfluidics (e.g., conventional optical imaging, lensless imaging, etc.) and their applications was recently presented by several research groups [20–22].

To overcome limitations mentioned above, researchers utilize on-chip or lensless imaging technologies. On-chip imaging systems for *C. elegans*, including contact optofluidic imaging [23, 24], direct shadow imaging [25], holographic imaging [26–28], in combination with automated data processing have enabled the observation and characterization of key behavioral parameters *in vivo* at micrometer and nanometer resolution (Figure 1A and B). Lensless imaging has the advantage of cointegration of microfluidics, microelectronics, and optical components into one platform. This has guaranteed an increase of the image quality, and has provided an ultimate spatial resolution of approximately 0.9 μm and a throughput approaching 40 worms per minute [23]. The combination with fluorescence imaging holds a great potential for screening of cellular processes [28].

Another promising approach is a lensless and sensor-less monitoring of the nematodes' movement in various microenvironments [29]. In a micro-electro-fluidic (MEF) grid, a moving nematode is detected by change in the electrical impedance at the intersection regions of the microelectrode grid, formed by two identical orthogonally arranged arrays of metal lines (Figure 1B). The approach ensured the real-time readout of the crawling nematode with a spatial resolution of 30 μm (the distance between grid lines) of the reconstructed images at the frequency of 174 Hz per readout.

Usually, the use of fluorescence-based techniques, such as calcium imaging or green fluorescent protein (GFP) expression, and microfluidics to image the activity of specific neurons requires chemically or genetically labeled animals to be immobilized for imaging at a cellular level [30–32]. An "immobilization-free" approach detection is achieved via two pairs of integrated optical fibers. Through the measurements of optical density and fluorescence, the fibers can detect and differentiate wild-type and green fluorescent protein (GFP)-type *C. elegans* even when they flow at high speeds (switching time of 1 s per worm) [33]. This has proven to be a well-controlled method for automated handling of worms in a high-throughput manner with a sorting accuracy of more than 96 %.

3. Microfluidic techniques for *C. elegans* manipulation

Environmental control and manipulation of whole animal poses significant challenges (e.g., animal's body orientation, precise delivery of chemicals, etc.). Transferring traditional neurobiology and behavioral investigation techniques to the microfluidic platform has the potential to overcome these challenges. This is driven by substantial progress in integration of functional components (e.g., valves, detectors, etc.) that allow the monitoring of various steps, such as administration, distribution, metabolism, and toxicity during drug screening. The advanced microfluidic approach offers both qualitative and quantitative data from a single organism by automatic high-throughput manipulation. For example, the worms can be oriented at regular positions on a substrate due to hydrodynamic forces in a microfluidic chip for the determination of gene function in a high-throughput manner [34]. In this section, we discuss general manipulation techniques, such as culturing, sorting, and immobilization.

Culturing. To interpret the underlying metabolic changes and specific developmental processes during nematode ageing, longitudinal experiments over the entire lifespan are necessary. Imaging and monitoring of the embryogenesis require specific techniques, which include single embryo isolation and mounting. Worm culturing can be dramatically improved using an automated microfluidic platform for culturing, phenotyping, and long-term live imaging of *C. elegans* embryo and larvae using microfluidic chambers (Figure 2A) and droplet encapsulation (Figure 2B) [35–47]. In combination with image recognition algorithms these "worm-chips" have successfully demonstrated their high potential at enhancing worm handling (e.g., automatic nutrient and waste exchange), accurate imaging, and automated analysis of embryonic morphogenesis during embryonic development [48]. Requiring the loading of only a few adult worms into the chip, the ensuing *C. elegans* embryo population could be processed at the same time.

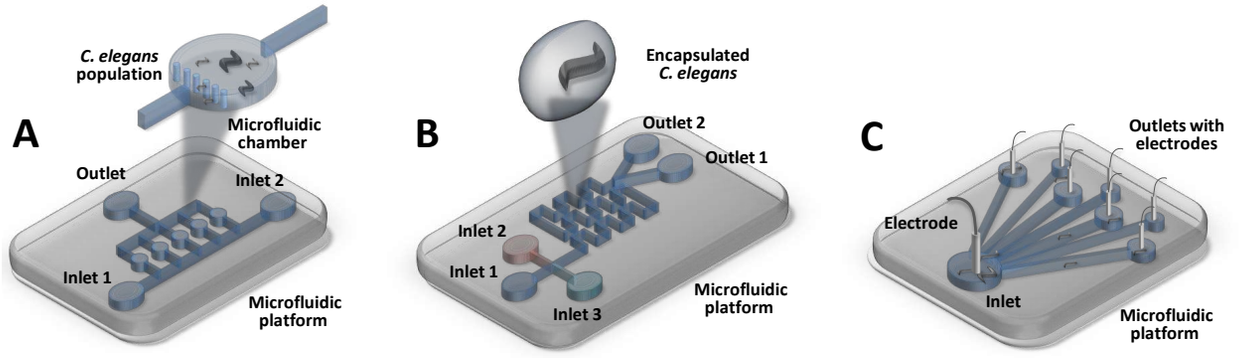

Figure 2. Schematic illustration of the microfluidic platforms for *C. elegans* culturing (A) [35] and sorting based on droplet encapsulation (B) [42–44] or electrotaxis (C) [49].

Sorting.*C. elegans* exhibits age-dependent specific neuron and behavioral responses. For instance, usage of both the early-stage and adult worms may increases the physiological relevance of drug candidates during the identification process and reveals potential toxic effects. Therefore, sorting (age or size synchronization) of worm populations or individuals is

often required for further diagnostics. For example, a passive sorting method is based on self-regulated worm distribution and loading into an array of narrowing channels [36, 50, 51] or microchambers [36, 37, 40, 52–55] with an average loading effectiveness rate of approximately 65 %. Once the worm enters the microchannel, the hydrodynamic resistance increases dramatically, thereby locking a single worm inside the chamber.

When considering high-throughput manipulation, automatic classification of worms (e.g., wild-type from mutants) becomes of high relevance. Typically, sorting involves individual *C. elegans* loading and separation, for instance, according to genetic phenotype for downstream analysis [31, 32, 34, 36, 56–60]. Together with real-time rapid image extraction and data processing, media flow in the microfluidic channel is driven by a syringe and is controlled by on-chip functional components, such as PDMS valves. Automatic sample positioning can guarantee rapid classification based on synaptic characteristics with sorting throughput at a rate of 900 worms per hour and an overall sorting accuracy of 96.5 % [32]. Depending on the extracted data, the worm could be flushed to either a waste or a sorting outlet by valve actuation.

Several other techniques have been successfully implemented in high-throughput studies [61–63]. In these systems, sorting is accomplished based on size difference in a passive, but extremely high throughput (up to 1200 worms per min) and selective manner (94 % of adults with 0.2 % larva contamination) [62].The device body contains an array of microstructured post (or filters) and a network of microfluidic channels allowing a large population of adult worms and larvae to be oriented in the desired direction.

Immobilization. Because of the high mobility, *C. elegans* immobilization is required for further developmental studies (e.g., neurosurgery). Manual handling and immobilization (e.g., by gluing or anesthesia) suffer from low throughput and is limited in long-term monitoring [1]. Immobilization in a microfluidic channel provides a simple well-controlled mechanism for automated handling of worms in a high-throughput manner. One of the widely used techniques is based on mechanical force. Several immobilization techniques are used to lock an individual worm against a microchannel wall in a robust and reversible manner. The first method involves microarrays of fixed-geometry clamps for *C. elegans* immobilization, which is a simple way to restrict the motion employing a single PDMS layer [36, 51, 64–67]. Using a constant pressure difference between the inlet and outlet of the device to drive fluid flow, an array of up to 128 wedge-shaped microchannels can be filled by nematodes with up to 90 % efficiency [68]. The second method includes a flexible PDMS membrane for squeezing the worm into the side of microfluidic channel under an external pressure through a control layer above the main chamber [51, 57–59, 67, 69, 70]. A third method is to trap of *C. elegans* by suction flow (Figure 1E), which is based on a vacuum-assisted restraint that aligns the worm along its axis [31, 71]. To highlight the utility of both immobilization techniques several research groups have combined suction posts with either flexible membranes [56, 65, 72, 73] or microchannel narrowing [30, 74–77] for extremely stable immobilization during microsurgery (Figure 1G).

Microfluidic devices offer advantages for both spatial and temporal control of the animal's position and microenvironment at the microscale. Based on acoustic wave in a single-layer microfluidic chip, on-chip manipulation technique permitted trapping and rotational manip-

ulation *C. elegans* regardless of shape and physical properties in the x- or y-directions for extended periods of time without inducing physiological damage [78, 79]. By implementation of a cooling liquid supply through a control layer to lower a worm's temperature down to 4 °C, *C. elegans* can be immobilized with a throughput up to 400 worms per hour for short-term cooling (~2 s) [32, 52]. Alternatively, light-induced sublethal heat can be used to increase the worm temperature (up to 31–37 °C) for its immobilization [80].

Gases, including carbon dioxide (CO_2) and nitrogen (N_2), are sensed by *C. elegans* and serve as a partial and complete method to eliminate worm mobility [67, 69, 76]. By passing pure gas from a control layer into a flow layer microenvironment, *C. elegans* can be immobilized in a channel with improved sensitivity and increased resolution.

4. Microfluidic approach for *C. elegans* sensing function and behavior

In this section, we review the use of microfluidic chips for *C. elegans* investigations under controlled physical and chemical conditions that have been advantageously used, for example, as integrated biosensors for toxicological experiments and drug screening. The two major methods of assaying are behavioral and molecular (or neuronal) studies. In behavioral studies, discussed in Section 4.1, the movement is generally analyzed by observation the animal's behavior in response to stimulation (e.g., touch, drugs, odorants, food, temperature, gases, osmolytes, or light). The key issues in neuronal studies, covered by Section 4.2, are the intracellular processes and neuronal signaling.

4.1. Behavioral studies of *C. elegans*'s responses to different stimuli

C. elegans explores its surrounding environment and moves according to environmental stimuli, including temperature, chemical, electric field, and light, which are detected by 24 sensilla organs and various isolated sensor neurons [1]. Obtaining meaningful data about the mechanism of environmental sensing requires strict control over the experimental conditions. Moreover, when a high number of identical biological samples are needed to be screened, a common need and challenge of the experimental procedure is the precise manipulation of worms with an emphasis on high throughput. Microfluidics offers a straightforward solution for automation and parallelization of screening in a rapid, sensitive, and accurate manner.

The environmental cues can be applied by devices, embedded in a chip-based microfluidic system, to analyze the behavioral response of the microorganism. For example, active and automated local manipulation and chemical stimulation of the individual worms can be achieved by implementation of multilayer PDMS layers. Because of *C. elegans'* small size and its ability to grow in liquid, on-chip imaging systems and automated data processing facilitate the observation and characterization of key behavioral parameters *in vivo* with micrometer and nanometer resolution.

Locomotion. Owing to the precision achievable by microlithographic techniques, researchers have been able to investigate in-depth different locomotion patterns (by varying the size and

spacing of posts), applied muscular forces (by measuring the deflection of posts) (Figure 1D) and motility quantification (time-averaged kinetic power over the swimming cycle) [81–90]. In contrast to traditional experimental techniques, these systems permitted straightforward dynamic force detection of moving nematodes. Whereas the motion of the animals in the artificial soil device exhibited the same principal characteristics of the motion of crawling on agar, the constraints on motion caused by the posts better mimicked the complexity of *C. elegans'* natural environment [91]. To further study crawling behavior, a number of PDMS microfluidic devices were presented that consisted of sinusoidal channels of varying wavelengths [84, 86, 87].

These devices allow researchers to manipulate the oscillating body motion of the crawling animals and investigate the biophysical and neuronal mechanisms of locomotion and proprioception. Microfluidics facilitates precise environmental control that was demonstrated by modifying the chemicals' concentration of the main chamber rapidly or and immediately observing the effect on locomotion [83]. Obviously, tracking animals through such a rapid media exchange would not be possible in a larger environment.

Electrotaxis.*C. elegans* exhibits responsive behavior to electric fields, mediated by certain amphid sensory neurons. These neurons are sensitive to both the direction and strength of the electrical signal, and forced the animal to move toward a negatively charged pole [1]. Analysis of the nematode's electrotaxis provides a detailed model of how neurons function together to generate a behavioral response to electric fields. When microfluidic chambers are combined with electrodes to deliver electrical stimuli, both behavioral and neuronal screening can be performed, providing the chance to elucidate potential treatment for human muscular disorders. Many microfluidic systems were proposed for fully automated control of electrotaxis, which overcame many of the inherent problems of manual operation [49, 92–98].

Normally, worms are exposed to a uniform electric field generated by two electrodes (e.g., platinum wires) embedded in inlet and outlet reservoirs and connected to external electrical drive circuitry (Figure 2C). Exposure to direct (DC), alternating (AC), and pulsed DC electric fields in a specified range of strengths has been employed as a means of guiding nematodes in a binary manner (e.g., start and stop), for sorting, and for immobilization, aiming to provide a close look at the mechanism of neuronal signaling transduced into behavioral responses [86, 92, 93, 98–100]. Such movement-based microfluidic devices permit the differentiation of worms according to locomotive abilities and similar physiological states, for instance, to distinguish adults from larva, or healthy worms from uncoordinated, and to locate individuals defective in electric field sensing. This guiding technique allows high throughput (up to 60 worms per min) and method selectivity of 70–90 %.

The progress achieved in microfabrication technologies has made monolithic integration of electrodes into microfluidic platform possible (Figure 1F). Micropatterned electrodes on the sidewalls of microfluidic channels (i.e., without blocking optical visibility) provides a simple means of creating electrofluidic glass chips to flexibly control the movement of *C. elegans* in a sensitive and reproducible manner [101]. Placing the microelectrodes inside the microfluidic environment as close to the animal as possible allows one to create transient pores in the cell membrane, which permits the diffusion of extracellular compounds that are present in the

vicinity of the pore into the interior of the cell [102]. All of these results demonstrate the potential of using active microfluidic devices as an alternative to Petri dishes for *C. elegans* assays.

Chemicals. Microfluidics is particularly attractive for many applications where *C. elegans* are used as integrated biosensors for toxicological experiments and drug screening. Behavioral investigations in response to chemical stimuli include real-time locomotion diagnostics of *C. elegans*. The effect on worm physiology to a variety of anesthetics, such as tricaine, muscimol, sodium azide, and levamisole [29, 55, 69, 93], odors, such as hermaphrodite-conditioned media and nicotine, and odors produced by pathogenic bacteria [55, 103–108], chemicals, such as zinc ion (Zn^{2+}) and glucose [109], different osmolarity levels [66, 71], was successfully examined by precise chemical control in a time- and dose-dependent manner. In most cases, pre- and postexposure locomotion phenotypes are compared by a variety of parameters (e.g., average velocity, individual head swing orientations, etc.).

Other stimuli. The ability of integration and individual worm manipulation makes microfluidic devices attractive platforms for understanding the correlation between *C. elegans*' neuronal and behavioral responses. Based on the properties of a microfluidic device, temperature stimuli could be delivered to individual worms accurately by flexible chip design and fluidic manipulation. Behavioral mechanisms in response to temperature change is quantified in terms of an average head angle of a semi-restrained animal [74] or swimming movements of the individuals heated in a microdroplet array [107].

Although the *C. elegans* has no light-sensing organs, it modulates a response to light known as phototaxis [50, 92, 109]. To analyze *C. elegans'* sensitivity to light, wild-type and mutant nematodes are illuminated with light and their behavioral response are examined. It was experimentally demonstrated that illumination to green light is preferable for animals, while blue light triggers muscle depolarization and further body contraction.

4.2. Neuronal studies

Behavioral studies, such as physiological responses, in a whole organism population include not only movement-based analyses but also monitoring of the *C. elegans'* neuronal activity in a confined space. Coupled with microfluidic-based systems, existing neuronal recording techniques (e.g., by calcium imaging or green fluorescent protein (GFP) expression) examine neuronal responses to sensory inputs of a single animal at a time under precise environmental control. For example, it was found that immobilizing a portion of the worm can directly override rhythmic activity and may cause changes in transport parameters of the touch neuron [69, 110]. In order to explore locomotive behavior and the underlying molecular mechanism, Wang et al. monitored a subcellular distribution of the DAF-16 gene that regulates different stress responses [91]. The experiments showed an increase of DAF-16 nuclear localization, attributed to crowding stress, in a microcolumn array with intervals from 40 to 200 μm between microposts (Figure 1D). As a result, a system-level understanding of the worm's motor circuit can be obtained.

One application where microfluidics and fluorescent-based imaging open up aspects that would remain hidden from traditional laboratory techniques is drug screening. *C. elegans* can be an effective test-bed for a wide range of water-soluble chemical compounds (e.g., glycerol [30, 66, 74, 75], anticancer drugs [48], heavy metals [54], sodium chloride NaCl [58, 65, 71, 83, 111, 112], copper(II) chloride $CuCl_2$ [66, 74], levamisole [70], manganese [102], antibiotics [104], isoamyl alcohol [113], cyanide [114], etc.). Microfluidic network manipulation allows the automation in a high-throughput manner and under reproducible experimental conditions while analysis of the nematode's chemosensitivity provides a detailed model of how neurons function together to generate behavioral response. For example, neurotransmitters and hormones, such as 1-methyl-4-phenylpyridinium (MPP+), 6-hydroxy dopamine (6-OHDA), and rotenone, have widespread effects as chemical regulators for coordinating physiological activity throughout the body of both nematodes and humans [1]. The microfluidic-based experiments proved that MPP +, 6-OHDA, and rotenone induce mobility defects in the animal (i.e., significant reduction in speed) after treatment and was potentially neurotoxic for dopaminergic neurons [43, 51, 112].

Due to PDMS microfluidic devices, much progress has been made to overcome the limitations of precise chemical control. The effect of ageing on physiological properties of the ASH chemosensory neuron can be characterized and quantified by the direct delivery of a chemical odor to the nose of *C. elegans* [30]. To emphasize the influence of different anesthetics on subcellular activity, a microfluidic platform was used for studying the contribution of vesicle transport to synaptic growth [70]. As a result, imaging of subcellular processes, such as pre-synaptic vesicle transport, intraflagellar transport (IFT), dendritic transport, and migration of neuroblasts during early developmental stages of the nematode, has become feasible. Monitoring of neuron activity (e.g., ASH neurons) with respect to osmotic gradient, can access the pattern-generating activity (e.g., individual head swing orientations) of the chemosensory circuit [66].

Another field where polymer-based fabrication techniques have already demonstrated themselves, is in investigations of gas sensing in nematodes [69, 76, 115–117]. In order to understand how oxygen level variation causes behavioral and physiological changes, freely moving adult animals were subjected to a gas-phase oxygen gradient. Experiments showed that specific soluble guanylate cyclase homologues (GCY-31, GCY-33, GCY-35, and GCY-36), located in URX, AQR, and PQR sensory neurons, activate hypoxia or hyperoxia avoidance [115, 116].

For many applications, such as characterizing stochastic neural responses, it should be beneficial to increase experimental throughput at the expense of image resolution. Microfluidics promotes simultaneous recording of calcium transients in individual neurons from multiple animals (up to 20), and increases experimental throughput [82, 118]. Thus, a systematic characterization of chemosensory neuron responses to multiple odors, odor concentrations, and temporal patterns, as well as responses to pharmacological manipulation can be performed.

The described experiments benefit enormously from the use of microfluidic technologies. The precise handling and chemical mixing of chemicals and neurotoxins in nanoliter volume droplets tremendously decreases reagent consumption and reaction time. The combination of brightfield imaging, fluorescent imaging, and microfluidics allows *in vivo* observation of biomolecules and automated analysis of protein aggregation phenomena in *C. elegans* for amyotrophic lateral sclerosis (ALS) at unprecedented resolution [119]. The level of precision that researchers have already achieved demonstrates the potential for the dissection of neuronal function and toxin-induced neurodegeneration *in vivo*.

4.2.1. Intracellular studying techniques

Several other techniques for studying and characterization of intracellular processes, including dielectrophoresis and electrochemical impedance spectroscopy, have been adopted by researchers for whole-animal drug screening (Figure 1F) [77, 92, 120, 121]. These methods use a noninvasive electrophysiological readout of neuromuscular function and can provide high-quality neurogenetic and neuropharmacological data on nematodes. Automatic real-time monitoring and parallelization (up to 8 worms simultaneously) with throughput of up to 12 worms per hour facilitate the rapid neuroactive drug screening, e.g., effects of drugs on neurons, as well as on muscles [77, 121].

4.2.2. Microsurgery and microinjection

In combination with microfluidics and optical image analysis systems, microsurgery and microinjection are employed for *in vivo* neuronal regeneration and cell-to-cell communication studies [52, 73, 80, 122–127]. Because the nervous system is described in great detail, the role of an individual neuron can be directly studied with laser ablation experiments (Figure 1G). Using a laser nanobeam in the UV wavelength region, fluorescent-labeled whole cell ablation is possible and a single synapse removal can be achieved [124–127]. Afterward, the resulting phenotypes (e.g., degeneration and regeneration) can be examined *in vivo*. Advances in optical imaging and microfluidic methods support this procedure. The capabilities of current chip-based systems are sufficient to perform precise animal manipulations, required for high immobilization stability of the worm, and complex image-based assaying with high throughput (up to 200 animals per hour with a success rate of 89 %) [52, 122]. This provides approximately one order of magnitude improvement over manually performed axotomies (when considering study of a single population) and gives an opportunity to perform genetic screening in a reasonable timeframe to identify the molecular mechanisms involved in nerve regeneration and degeneration.

The *in vivo* injection of chemical materials that have significant implications in genetics, drug discovery, and other biological applications is another way to study the mechanisms underlying intercellular communication in *C. elegans* (Figure 1H). Using a single needle tip of the micromanipulator, localized chemical stimulation can be delivered to a single intestinal cell of the immobilized worms [123, 128].

5. Conclusions and perspectives

The advances in microfabrication technologies have demonstrated the potential of using active lab-on-a-chip (LoC) devices as an alternative to microwell plates for worm-based assays. LoC technology offers a straightforward solution to all of the problems during manual manipulation. Complex three-dimensional (3D) microenvironments have been created, where a whole population of worms is cultured and analyzed in a reproducible way. Currently available microfluidic-based systems are capable of recording from sensory neurons in animals *in vivo*, whose neuronal responses could be correlated with behavior. Microsurgery and microinjection allow the investigation of many processes, including the role of individual neurons in neuronal networks and in cell-to-cell interaction. Obviously, this is pushing forward fundamental studies in biology and biochemistry.

The use of fluorescence-based techniques and microfluidics to image the activity of specific neurons requires that animals be labeled either chemically or genetically. However, for monitoring certain biological processes, fluorescent labeling might be inconvenient or may interfere with normal behavior. Moreover, many dynamic phenomena of motile samples might be missed during impedance spectroscopy, microsurgery, and microinjection because of the long-term immobilization required for subcellular-level stabilization of *C. elegans*. This makes monitoring of actual metabolic activity impossible.

Several other approaches can be used to study the neuronal and metabolic activity of a biological system. For example, nuclear magnetic resonance imaging (MRI) and nuclear magnetic resonance spectroscopy (NMR) are two of the most information-rich methods that provide a unique opportunity to link morphological, functional, and chemically specific spectroscopic information from small volume (e.g., μl) samples (Figure 1I). MRI and NMR uses strong time-varying radio frequency (RF) fields to generate a weak specific RF response from a certain tissue type [129]. Because the technology is noninvasive and only nonionizing radiation is absorbed and emitted, it might be especially suitable for the study of *C. elegans* in the identification and quantification of metabolites (intermediate products of metabolism) within the metabolic pathway *in vivo* [130–136].

In many of the reviewed research articles, the easy integration of microfluidic control and detection modules was a key factor in helping to link *in vitro* and *ex vivo* experimental investigations. The ability of *C. elegans* tracking in real time (i.e., with minimal latency) for further diagnostic applications could provide a close look at the cellular, molecular, and genetic levels. Consequently, an understanding of the underlying molecular mechanisms in multicellular model organisms would provide a unique opportunity to unthread analogous and complex biological processes in humans. This certainly will promote more automated and higher throughput applications in the future.

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Author details

Natalia A. Bakhtina[1,2*], Neil MacKinnon[2] and Jan G. Korvink[2]

*Address all correspondence to: natalia.bakhtina@kit.edu

1 Laboratory for Simulation, IMTEK – Department of Microsystems Engineering, University of Freiburg, Freiburg, Germany

2 Korvink's Group, Institute of Microstructure Technology (IMT), Karlsruhe Institute of Technology (KIT), Germany

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