USING MICROFLUIDIC IMPEDANCE CYTOMETRY TO IDENTIFY THE LIFE STAGES OF *C. ELEGANS* NEMATODES

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ABSTRACT

This paper presents a method to identify the life stages of *Caenorhabditis elegans* (*C. elegans*) by means of electrical impedance spectroscopy (EIS). A simple microfluidic chip, composed of a straight microchannel and a pair of coplanar electrodes was designed to form a microfluidic impedance cytometry. The electrodes were used to perform the impedance measurement of *C. elegans* nematodes when worms were flowing through the channel. Electrical signals and concurrent videos were recorded and analyzed to investigate the relationship between the impedance amplitude and the worm length. The result demonstrated that the length of nematodes is linearly correlated to the cubic root of EIS signal amplitude. Therefore, the life stages of *C. elegans* can be identified through this microfluidic impedance cytometry.

KEYWORDS

Caenorhabditis elegans, electrical impedance spectroscopy

INTRODUCTION

The nematode *Caenorhabditis elegans* (*C. elegans*), as a well-established model organism, has been widely used in the research of genetics, neurology, developmental biology and toxicology, for the sake of its fully mapped neural circuitry and well-sequenced genome. *C. elegans* can be easily cultured in many laboratories due to their unique features, such as short lifespan, transparent body and small size. To use *C. elegans* as a powerful tool in the relevant research, the nematodes are usually required to be synchronized. Traditionally, worms are isolated into populations at same life stages by manual selection, gravity stratification or chemical synchronization via bleaching [1]. In the case of chemical bleaching, the disadvantages cannot be ignored, such as long-time operation, variation of results and potential perturbations to worms [2]. The pursuit of higher efficiency to isolate the synchronized worms leads to the emergence of new methods in identifying the life stages of *C. elegans* nematodes.

To identify the life stage of *C. elegans*, commercially-available platform is a sorter named as COPAS (Complex Object Parametric Analyzer and Sorter). Worm samples are characterized by the optical density as the response signal of multiple laser excitations. Despite of high accuracy and efficiency, the unaffordable price blocks its use in many labs.

The advanced microfabrication technology enables the expansion of microfluidic systems in the field of biology and medicine. A microfluidic device, usually fabricated by micro-techniques and integrated various sensors and actuators can achieve multiple functions in the manipulation, cultivation and detection of biological organisms. In the past decade, several microfluidics-base methods have been proposed to identify the life stages of works and classify the worms into populations according to their life stages [3].

One microfluidic sorting method is based on the electrotaxis of *C. elegans*. By utilizing electrotaxis, Rezai *et al.* [4, 5], Maniere *et al.* [6], Han *et al.* [7] and Wang *et al.* [8] have developed various microfluidic systems to sort *C. elegans* nematodes. Under the effect of electrotaxis, the nematodes move towards to the cathode while being exposed to an electric field. The motility of nematodes at different life stages varies in angles which are proportional to the strength of electric field. According to this tendency, a mixed population of *C. elegans* nematodes can be classified into various groups to obtain synchronized stages of nematodes.

Another method is based on the filtration of nematodes. By using the flow filtration, Solvas *et al.* [9], Ai *et al.* [10] and Dong *et al.* [11] have developed microfluidic sorting systems to classify the worms into different groups with the same life stages. *C. elegans* nematodes have identical length and diameter at different life stages. Once the geometry of a microchannel is defined, only the nematodes with diameter smaller than the cross-section of the microchannel can pass. Such microchannels with defined sizes are filters to filtrate the nematodes at different life stages, thereby achieving the identification and classification of *C. elegans* nematodes. The throughput of such systems are high, but the portability is reduced due to the highly-customized parameters of these microfluidic devices.

Yan *et al.* [12] diverged from the above methods and used fluorescence to identify *C. elegans* nematodes. Two pairs of optic fibers were placed at both sides of a microfluidic sensing channel. The fluorescence of each passing worm can be detected. After obtaining the fluorescent signal of each worm, a Y-shaped channel in the downstream was used to sort the targeted worm out of the sensing channel. The microfluidic system can achieve high accuracy in sorting, but only at a low sorting rate of about 12 worms per minute.

Moreover, electrical impedance spectroscopy (EIS) can be integrated in the microfluidic system to sense the dielectric properties of biological samples. Once EIS is integrated in a flow-through microfluidic system, it can act as an impedance cytometry to detect each sample when passing the sensing zone. The impedance cytometry can provide a high-throughput and non-invasive method to identify the size, volume, morphology of cells, such as blood cells [13], cancer cells [14], stem cells [15] and sperms [16].

However, the microfluidic system combined the function of EIS to identify the life stage of *C. elegans* has

not yet appeared. Herein, we present a simple microfluidic chip, which consists a straight microchannel and a pair of coplanar electrodes. The electrodes are designed to perform the impedance measurement of *C. elegans* nematodes when worms are flowing through the channel. This microfluidic impedance cytometry can continuously transmit electrical stimulus to monitor the detection zone and obtain the variation in amplitude of response signal. Based on the change of amplitude, the life stages of worms can be identified.

MATERIALS AND METHODS

Design of the Microfluidic Impedance Cytometry

The microfluidic chip was designed to measure the change of EIS signals when worms flow through the micro-channel. Thus, a straight microfluidic channel with an inlet and an outlet and a pair of coplanar microelectrodes are integrated, as shown in Figure 1. When a worm flows through the sensing zone in the microfluidic channel, the measured impedance between the two electrodes increases since the impedance of worm is larger than the impedance of liquid buffer. Concurrently, real-time video is recorded for each passing worm so that the length of worms can be analyzed optically. According to data analysis of the recorded EIS signals and the images, the relationship between EIS amplitude and length of worms can be revealed.

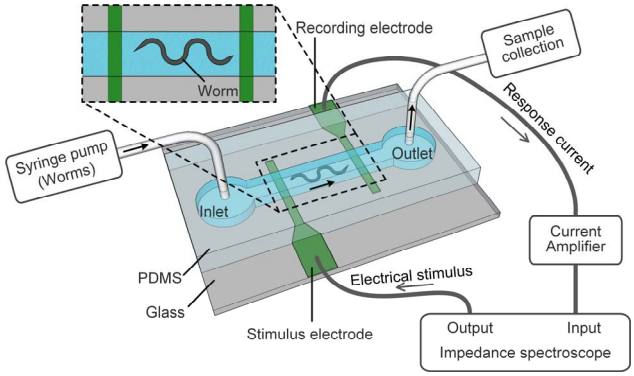

Figure 1: Schematic of the microfluidic impedance cytometry to measure and identify the life stages of C. elegans nematodes. Worms in buffer were first loaded in a syringe which was affixed on a syringe pump. By using the syringe pump, worms were infused into the microfluidic channel and were measured by electrical impedance spectroscopy when passing through the sensing zone. Stimulus signal was generated by an impedance spectroscope and was applied to the stimulus electrode. The resulting signal was received by the recording electrodes and was amplified via a current amplifier and finally was recorded by the impedance spectroscope.

To assemble the microfluidic impedance cytometry, the PDMS microchannel and the glass chip with electrodes were fabricated independently. For the glass substrate, 200-nm-thick Au electrodes were patterned on a glass wafer by a lift-off process, and then the wafer was diced into chips by using a dicing saw. The PDMS microfluidic channel was fabricated through softlithography as following steps. First, SU-8 3050 photoresist (MicroChem, CO., USA) were spin-coated twice on a silicon wafer to form a layer of SU-8 with the total thickness of 120 μm. Through the photolithography process, a SU-8 master was fabricated. The PDMS dies with microfluidic channels were replicated from the SU-8 master. Then, the inlet and outlet holes were punched on the PDMS dies, which were finally bonded to the glass chips by using oxygen plasma treatment of both sample surfaces. During the bonding, the microfluidic channel and electrodes were manually aligned under a stereomicroscope.

Experimental Setup

To assemble the experimental setup, the microfluidic chip was placed between a custom-designed aluminum holder and a PMMA cover by screws. A printed circuit board (PCB), containing golden spring-loaded pins to contact the electrode pads on the chip, was then placed above the PMMA cover and was fixed on the aluminum holder by screws as well. An impedance spectroscope (HF2IS, Zurich Instruments AG, Switzerland) and a current amplifier (HF2CA, Zurich Instruments AG, Switzerland) were connected to the electrodes via the PCB to apply the stimulus signals and record the response current signals. For the microfluidic access, PTFE tubing was inserted through holes in the PMMA cover into the inlet and outlet holes on the PDMS. Through tubing, worms in the buffer solution was infused into the microfluidic channel by using a syringe pump (LSP02-2A, Lange, China). The assembled setup was placed on an inverted microscope (IX53, Olympus, Japan) for observation and video recording. The whole experimental process was video-recorded by a camera equipped on the microscope.

C. elegans Culture and Preparation

The wild-type N2 *C. elegans* was used in this work. The nematodes were cultured on Nematode Growth Medium (NGM) plates and were fed with OP50 strain of *Escherichia coli* at 20 °C. To synchronize the life stages of worms, we mixed the pregnant adult worms and bleach solution containing 1% NaOCl, 0.1 M NaOH and 1x PBS at the ratio of 1:1:8 [17]. After centrifuging and cleaning the mixture, we collected embryos and hatched them in PBS. Then these embryos were cultured on NGM plates. Prior to the experiment, worms were washed and suspended in PBS buffer.

RESULT AND DISCUSSION

In the experiment, worms flowed through the sensing zone in the microfluidic channel, and were monitored optically by using the camera. At the same time, the impedance spectroscope transmitted alternative current (AC) signals (1.5 V_{pp} at 100 kHz) from the stimulus electrode to recording electrode, detecting the variation of impedance in the sensing zone. When a worm flowed in the sensing zone, the measured impedance rose, and the amplitude of the response signal dropped. When only PBS buffer was filled in the microchannel, the amplitude was stabilized at the baseline. While measuring a worm passing by, an EIS signal with a plateau was obtained (Figure 2).

For the recorded EIS signals, the original sampling rate was 1.8 kHz, with high-frequency noise and sharp peaks in rising and falling edges. To obtain the mean amplitudes of the baseline signals and the worm signals, a fixed length of the recorded EIS signal was analyzed by histogram. The probability density of amplitude was calculated and plotted, as shown in Figure 2. The two peaks of maximum probability were defined as Amp_{base} and Amp_{worm}. The difference between Amp_{base} and Amp_{worm} was defined as ΔAmp, which refers to impedance variation caused by the worm:

$$\Delta Amp = Amp_{base} - Amp_{worm}$$

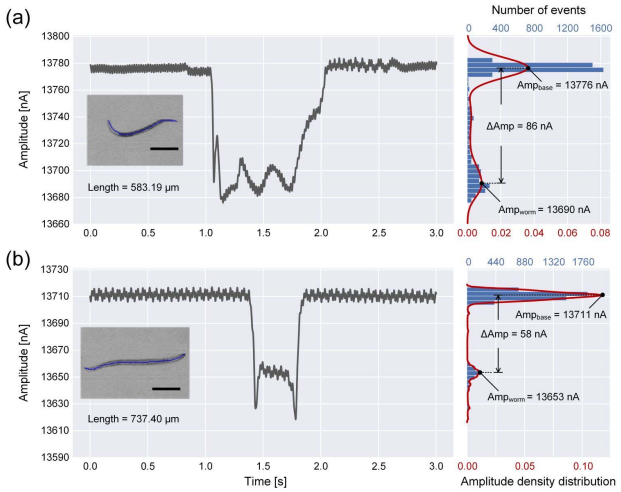

Figure 2: Real-time EIS signals and images of corresponding worms with different length. The plots of amplitude density distribution show two peaks, which are defined Amp_{base} and Amp_{worm} to calculate the amplitude variation ΔAmp. Scale bar is 200 μm.

When the worms were passing through the sensing zone, real-time video was recorded at a rate of 25 frames per second. The images were extracted from the video, and were adjusted in brightness and contrast to obtain the clear profile of worms. Thus, from the recorded video, the length of worms were measured (see Insets of Figure 2).

By using a linear regression calculation, we can clearly see a linear correlation between the cubit root of ΔAmp and the optically-measured worm length, as shown in Figure 3. The coefficient of determination R^2 is 0.93. According to the life stages of *C. elegans*, all worms were divided into four groups (L1, L2, L3, and L4&above) by using their optical length. In Figure 3, the data points in the color boxes represent the correctly-identified worms since their life stages identified by using EIS signals match well with those by using the optical length. The data points that are out of color boxes represent the worms that have mismatches in classification by using EIS signals and optical length. Then we evaluated the accuracy of impedance-based identification of life stages. The accuracy of each group was defined as the ratio of the number of worms identified correctly (data points in the color boxes) to the number of worms classified by using EIS signals (all data points in the same color). We can see that average accuracy of identifying the life stages of *C. elegans* nematodes is about 90% by using the microfluidic impedance cytometry, as shown in Table 1.

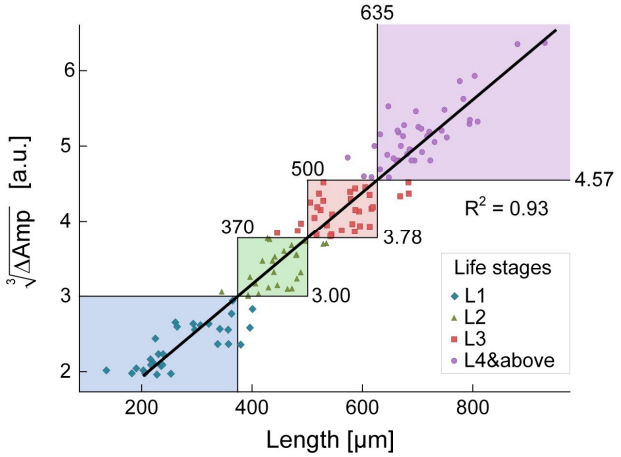

Figure 3: Linear regression between the cubic root of the EIS amplitude variation and the optical length of C. elegans nematodes. The coefficient of determination R^2 is 0.93. Worms are classified into four groups based on their life stages (L1, L2, L3, and L4&above). Each life stage of C. elegans is ended with a molt. The length range in classifying the life stages of worms is the average length at molting of one life stage to the next. The range of the cubic root of ΔAmp in classifying the life stages is calculated by using the classification range from optical length and the linear regression equation. Both ranges are shown in color boxes with corresponding values indicated. The data points in different colors represent the worms that are identified as different life stages by using the EIS signals. The data points located in each color box refer to the worms correctly identified by using the EIS signals and optically-measured length.

Table 1. Accuracy of identifying the life stages of C. elegans nematodes by using EIS signals.

	L1	**L2**	**L3**	**L4& above**
Length [μm]	<370	370~500	500~635	>635
ΔAmp [nA]	<27.0	27.0~53.6	53.6~94.8	>94.8
Accuracy	90.00%	88.00%	81.25%	90.24%

CONCLUSION

A microfluidic impedance cytometry that enables the identification of the life stage of *C. elegans* nematodes has been presented in this work. Worms were infused into the microchannel, and flowed through the EIS sensing zone one by one. The EIS sensing zone has a pair of coplanar electrodes to implement the impedance measurement of each passing worm. The cubic root of amplitude variation of recorded EIS signals has shown a linear correlation to the optical length of worms. Therefore, the life stages of *C. elegans* nematodes have been successfully identified by using the EIS signals. This microfluidic impedance cytometry has shown a high accuracy to classify the *C. elegans* nematodes into synchronized life stages.

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