Microfluidic Platform for Analyzing the Thermotaxis of *C. elegans* in a Linear Temperature Gradient

Sunhee YOON,*** Hailing PIAO,*** Tae-Joon JEON,*** and Sun Min KIM***,****†

* Department of Biological Engineering, Inha University, Incheon 22212, South Korea
** WCSL of Integrated Human Airway-on-a-Chip, Inha University, Incheon 22212, South Korea
*** Department of Mechanical Engineering, Inha University, Incheon 22212, South Korea

Introduction

*C. elegans* was the first multicellular organism to have all its genomes revealed. In particular, *C. elegans* has genome counterparts with humans, including up to 80% of the neuronal circuit, so *C. elegans* has been used as a model organism for specific human gene research. *C. elegans* also has a simple nervous system consisting of 302 neurons and the network of neurons produces a response to external stimuli. C. elegans shows specific behavior responses to various external physical and chemical stimuli. For example, *C. elegans* stores the cultivation temperature in thermosensory neurons and moves toward the cultivation temperature region in a temperature variation. In this study, we developed a microfluidic system for effective thermotaxis analysis of *C. elegans*. The microfluidic channel was fabricated using polydimethylsiloxane (PDMS) by soft lithography process. The temperature gradient (15 – 20°C) was generated in the microchannel and controlled by Peltier modules attached to the bottom of the channel. The thermotaxis of wild type (N2), *tax-4(p678)* and *tax-7(n30)* mutants were effectively analyzed using this microfluidic system. We believe that this system can be employed as a basic platform for studying the neural circuit of *C. elegans* responding to external stimuli.

Keywords *Caenorhabditis elegans* (*C. elegans*), temperature gradient, thermotaxis, microfluidic system

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generate a temperature gradient. A steep temperature gradient (5°C/cm) was stably generated in the M9 buffer filled-microchannel. The steep temperature gradient can promote the rapid response of thermotaxis. With this system, we can perform the thermotaxis analysis of wild-type worms and mutant worms in a steep temperature gradient for a relatively fast analysis time of 10 min. The differences in behavioral responses of wild type and mutant worms are sufficiently distinguished without any special analysis technique in a relatively short analysis time.

**Experimental**

**Preparation of synchronized C. elegans**

The wild type and tax-4(pk678), ttx-7(nj50) mutant strains were selected as targets for thermotaxis analysis. In previous studies, two mutants, tax-4(pk678) and ttx-7(nj50), were reported to represent abnormal thermotaxis. Briefly, tax-4(pk678) is defective in the tax-4 gene that encodes a cyclic nucleotide-gated channel. This channel plays a major role in AFD, the main neuron for thermotaxis. As a result, the tax-4(pk678) moves randomly in a temperature gradient. Meanwhile, the ttx-7(nj50) mutant also represents random movements in a temperature gradient. Thus, these two mutants were selected to compare with wild-type worms, to demonstrate the functionality of our system.

**Temperature gradient generation**

Peltier modules (Laird Technologies, USA, W = 12 × D = 12 × H = 3.6 mm) were used for generating a temperature gradient in the microchannel. Briefly, two Peltier modules were attached to the bottom of both ends of the microchannel and worked as a heater and a cooler, respectively (Fig. 1A). Metal plates were placed under each Peltier module to prevent thermal saturation. Peltier modules were operated with 5 V power and the temperature of the cooler and heater parts were maintained at 15 and 20°C, respectively (Fig. 1A). The temperature was measured using thermocouples (K-type, Fluke Corp., USA).
while the temperature gradient was confirmed using thermochromic pigment (setting temperature 20°C, blue color, CMTECH, Korea). At lower temperatures below 20°C, thermochromic pigment shows a blue color that gets darker with decreasing temperature. The pigment was coated on the bottom side of the channel consisting of slide glass (Fig. S1). As a result, the media solution in the microchannel achieved a steady temperature gradient within 5 min.

Thermotaxis assay
Next, 10 – 20 worms were introduced to the microchannel for analyses. Experiments for each strain were performed at least 10 times. In order to analyze the thermotaxis of each strain in the channel, the channel was divided into four zones with the same length (2.5 mm) (Fig. 1A) and we analyzed the ratio of worms in each zone with an inverted microscope (Eclipse Ti-U, Nikon, Japan) (Fig. 1B).

Results and Discussion
Swimming velocity and bending frequency analysis
First, we analyzed the swimming velocity and bending frequency of *C. elegans*. The swimming velocity was obtained by measuring the time it took the worm to cross the channel from inlet to outlet. Bending frequency was measured by counting the number of body bending moves in a second using live video clips acquired by camera (DS-Qi1, Nikon, Japan) attached to the microscope. These variables of wild type and mutant worms were compared at room temperature (Fig. 2). For the bending frequency, the three strains showed an average value of 1.7/s without a significant difference ($p > 0.05$). While, the swimming velocities of the three strains showed a slight difference, but had no effect ($p > 0.05$). As a result, bending frequency and swimming velocity of the three strains were found to be statistically the same in room temperature.

Determination of cultivation temperature for steep temperature gradient
The cultivation temperature is one of the important factors for thermotaxis of *C. elegans*. Before the thermotaxis assay in the microfluidic system, we tested the wild-type worms with two different cultivation temperatures in order to determine the cultivation temperature. The worms were divided into two groups and cultured at 15°C (low temperature) and 20°C (high temperature), respectively. The growth rate of worms is based on the cultivation temperature, and the growth rate is usually slower at low temperatures as compared to high temperatures. After the egg prep, the worms were cultured for two days at 20°C or three days at 15°C until they matured to the adult stage (body length ~1 mm). When the steep temperature gradient was generated in the microfluidic channel, the two groups showed different responses. The high temperature group showed no response to the temperature gradient (Fig. 3A). On the contrary, the low temperature group detected the temperature gradient and accumulated at the region of their cultivation temperature (Fig. 3B). The results are consistent with a previous study and demonstrate that the thermotaxis in a steep temperature gradient (>1°C/cm) is dependent on the cultivation temperature. As a result, the lower cultivation temperature (15°C) was chosen for thermotaxis assay in the microfluidic system.

Thermotaxis assay in a temperature gradient
Figure 4 shows the distribution of wild-type, *tax-4(p678)*, and *ttx-7(nj50)* worms in the microchannel with a temperature gradient. Without a temperature gradient, all three strains were randomly and evenly distributed in the channel. However, 10 min after the temperature gradient, the wild type worms moved to the left side of the channel, which was close to the cultivation temperature of 15°C (Fig. 4A). However, the *tax-4(p678)* worms were still distributed evenly in the channel (Fig. 4B) and this showed that the *tax-4(p678)* worms do not have a specific temperature preference such as cryophilic or thermophilic. Interestingly, the *ttx-7(nj50)* worms were located on the right side of the channel close to 20°C (opposite to the cultivation temperature) (Fig. 4C), so the *ttx-7(nj50)*
worms showed a thermophilic reaction. However, in the previous study, \textit{ttx-7} (\textit{nj50}) mutants also showed an abnormal thermotaxis rather than a specific temperature like \textit{tax-4} (\textit{p678}) mutants. The \textit{ttx-7} (\textit{nj50}) worms were cultured at 17, 20, and 25°C, and they were exposed to a radial temperature gradient on an agar plate. The temperature at the center of the plate was 17°C and the temperature at the edge was 25°C (temperature gradient <5°C/cm). The \textit{ttx-7} (\textit{nj50}) worms showed random distribution in the temperature gradient regardless of the cultivation temperature. According to this result, the \textit{ttx-7} (\textit{nj50}) should be distributed evenly in the channel, but \textit{ttx-7} (\textit{nj50}) showed a thermophilic response in this study. The main differences in this study are the relatively steeper temperature gradients and the microscale platform. It is possible that a steeper temperature gradient has an effect on the behavior of \textit{ttx-7} (\textit{nj50}) worms, leading to a thermophilic response. To quantify the thermotaxis of worms, we used a ratio of the number of worms in each zone to the total number of worms present in the channel. The ratio of wild-type worms in zone A increases with time (Fig. 5A), which shows the normal thermotaxis of worms. The zone A and D values of \textit{tax-4} (\textit{p678}) worms at 10 min had no statistically significant difference. Finally, the ratio of \textit{ttx-7} (\textit{nj50}) worms had a maximum value at zone D (Fig. 5C), which shows the thermophilic behavior of \textit{ttx-7} (\textit{nj50}) worms. Also, the zone A and D values of \textit{ttx-7} (\textit{nj50}) worms at 10 min had a statistically significant difference ($p<0.05$). Furthermore, we compared the ratio of worms in zone A and D for three strains at 10 min. In zone A, the ratio of N2 worms is the maximum, so this value is set as a basis for the $t$-test. As a result, the difference between each strain was statistically significant (Fig. 5D). In zone D, the basis for the $t$-test is the ratio value of \textit{ttx-7} (\textit{nj50}) (the maximum) and the distribution of worms for each strain was statistically significant (Fig. 5D).

Index analysis

In addition, we employed a thermotaxis index to practically identify the distribution of each strain in the temperature gradient. The index is defined as follows:

$$\text{Thermotaxis index of strain} = \frac{\sum (n \times \text{index value of the zone } X)/N}{N}$$

($n =$ number of worms in each zone; $N =$ total number of worms in the channel)

Index values of +3, +1, −1 and −3 with the same intervals are assigned to each zone from the left (zone A: +3) to right (zone D: −3) side of the channel.
Figure 6 shows the result of index analysis at 10 min after the generation of a temperature gradient. The index value of wild-type worms is 1.27 and higher than the value of other mutants because most of the wild-type worms were gathered in zone A. However, the index value of \textit{tax-4} (\textit{p678}) mutants is close to 0, which means that this mutant does not respond to a temperature gradient and represents significant athermotactic behavior. The \textit{ttx-7} (\textit{nj50}) mutants showed a relatively thermophilic reaction, which is a behavior confirmed by the index value of –1.24. Thus, it was possible to quantitatively compare the different thermotaxis behavior of three strains in a temperature gradient with index analysis.

**Conclusions**

In this study, we developed a microfluidic system that can efficiently analyze the thermotaxis of \textit{C. elegans}. The system was fabricated with PDMS using a soft-lithography process for real time analysis of \textit{C. elegans} movement with a microscope. Thermotaxis of \textit{C. elegans} can be stably identified using this system, even though the temperature gradient was relatively steep (5°C/cm). The thermotaxis of the different strains (N2, \textit{tax-4} (\textit{p678}), and \textit{ttx-7} (\textit{nj50})) can be clearly identified within a relatively fast analysis time (10 min). Wild-type worms preferred the cultivation temperature (15°C), \textit{tax-4} (\textit{p678}) worms were randomly distributed in the temperature gradient without favoring a specific temperature, and \textit{ttx-7} (\textit{nj50}) worms showed a thermophilic response indicated by a preference for a higher temperature than the cultivation temperature. Peltier modules were employed for generating a temperature gradient and these modules can be used to rapidly and steadily change the temperature by adjusting the applied current. Therefore, this system can provide a useful platform for various thermotaxis analysis and for the comparison of temperature-dominated reactions at various temperature gradients, simultaneously. In conclusion, the developed platform in this study is expected to be applied as a basic platform for analyzing the response of a \textit{C. elegans} to complex stimuli including physical and chemical stimuli.
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Supporting Information

The supporting information of this manuscript includes the figure of temperature gradient generation in a microfluidic channel. This material is available free of charge on the Web at http://www.jsac.or.jp/analsci/.

References