A ciliate memorizes the geometry of a swimming arena

Itsuki Kunita1, Tatsuya Yamaguchi2, Atsushi Tero2,3, Masakazu Akiyama1, Shigeru Kuroda1 and Toshiyuki Nakagaki1

1Research Institute for Electronic Science, Hokkaido University, N20W10, Kita-Ward Sapporo 001-0020, Japan
2Graduate School of Mathematics, and 3Institute of Mathematics for Industry, Kyushu University, 744 Motooka, Nishi-ku, Fukuoka 819-0395, Japan

Previous studies on adaptive behaviour in single-celled organisms have given hints to the origin of their memorizing capacity. Here we report evidence that a protozoan ciliate Tetrahymena has the capacity to learn the shape and size of its swimming space. Cells confined in a small water droplet for a short period were found to recapitulate circular swimming trajectories upon release. The diameter of the circular trajectories and their duration reflected the size of the droplet and the period of confinement. We suggest a possible mechanism for this adaptive behaviour based on a Ca\textsuperscript{2+} channel. In our model, repeated collisions with the walls of a confining droplet result in a slow rise in intracellular calcium that leads to a long-term increase in the reversal frequency of the ciliary beat.

1. Introduction

The behaviour of protozoa has been examined for over a hundred years under various external conditions and has been compared with intelligent behaviour in higher animals [1–8]. It has long been observed that even protozoa behave in a highly adaptive way towards complicated environmental conditions [9–15]. These reports have repeatedly stimulated discussion on the possibility of something like primitive intelligence. The adaptability of protozoa and its ethological implications has become a classical topic, but much still remains to be understood. Because of the relative simplicity of unicellular systems compared with multicellular organisms, the physical mechanism of adaptation may be easier to clarify. Insight has been provided by a number of studies such as solving a maze [16] and anticipating periodic environmental events by the slime mould Physarum [15]. These observations encourage us to rethink how this capacity for adaptation develops from the physical nature of the organism.

Concerning the capacity to learn the geometry of a space, impressive pioneering work on a protozoan ciliate Paramaecium was performed by Bramstedt in 1935 [17]: after the organism was transferred from a tiny container to a large container, it swam freely but followed a trajectory that was similar to the shape of its previous container. Although this finding was striking, some other research groups carefully re-examined it and drew negative conclusions [18,19]. Here we attempt to throw more light on memory capacity of this type.

In this report, we design a new quantitative experiment and confirm that another species of ciliate Tetrahymena shows the capacity to learn spatial configurations. We first carry out a qualitative characterization of the adaptive behaviour. We then propose a possible physical mechanism for this type of memory capacity in ciliates, based on the regulation of electric phenomena in the membrane that are closely coupled with ciliary motion. Finally, we discuss the implications of this memorizing capacity from the perspective of comparative ethology.

2. Organism and methods

Protozoan ciliates Tetrahymena were cultured in a liquid medium (KCl 8 mg l\textsuperscript{-1}, MgSO\textsubscript{4} 8 mg l\textsuperscript{-1}, CaCl\textsubscript{2} 8 mg l\textsuperscript{-1}) and incubated in a dark room at room...
temperature (23–25°C). After being allowed to swim in a wide space freely for several tens of minutes, specimens of *Tetrahymena* were confined in a spherical droplet of culture medium (0.3–0.6 mm in diameter (φ), approximately 0.014–0.11 μl) embedded in mineral oil for 15 min. Next, the specimens were transferred to the much larger space of a Petri dish (35 mm in diameter, 3 ml of medium).

To minimize any space-dependent effects of unknown chemicals that may be released from the organism during swimming in the droplet, the liquid medium was thoroughly mixed just after the organism was transferred from the droplet to the Petri dish. The specimens then began to swim freely.

The swimming motion of *Tetrahymena* was monitored under dark-field illumination using a stereoscopic microscope (Olympus, SXZ16). Microscopic images were captured using a CCD camera and recorded on video. The recorded images were transferred and saved on a personal computer. Using customized software, we analysed the swimming trajectories of *Tetrahymena* by the conventional method of video image analysis.

To characterize a swimming trajectory, the maximum distance (MD) was defined as MD(t) = \max \left\{ \sqrt{(x(t) - x(t + h))^2 + (y(t) - y(t + h))^2} \mid -τ ≤ h ≤ τ \right\}, where (x(t), y(t)) is the position of an organism at time t. MD was plotted as a function of τ and could saturate at some value of diameter when the swimming trajectory was circular. At last, statistical occurrence of MD was plotted in the function of τ. Numbers of cells were 34 (φ = 0.3 mm), 31 (0.4 mm), 30 (0.5 mm) and 22 (0.6 mm).

For statistical confirmation of spatial extent of swimming trajectory after the confinement, the width of the circular shape of trajectory (approximate diameter) was measured for each circular shape on the whole trajectory of swimming, and the normalized frequency was plotted. The numbers of circles were 757 (φ = 0.3 mm), 292 (0.4 mm), 375 (0.5 mm) and 76 (0.6 mm).

3. Adaptive behaviour towards the shape and size of a swimming space in *Tetrahymena*

In an open space, *Tetrahymena* usually swim in a straight line at a velocity of 0.81 ± 0.27 mm s⁻¹ (mean ± s.d., N = 30), and changed the direction of swimming at a frequency of 0.01–0.1 Hz, as shown in figure 1a1.

Figure 1a2–a4 shows the swimming trajectories observed in a tiny spherical droplet (the diameter, φ = 0.3 mm): *Tetrahy-
mena* repeatedly turned at the droplet wall and moved closely (figure 1a2) or approximately (figure 1a3) along the wall, and sometimes in a different manner (figure 1a4). The swimming patterns (figure 1a2 and figure 1a3) were often observed in this confined space over a few minutes. The pattern of swimming changed on a longer time scale.

After the organism was transferred from the confined space to a wide, open space, the swimming trajectory repeatedly traced a circular shape that was similar to the previous confined space (figure 1a5,a6). Sometimes the organism swam for a period of time almost in a straight line or in a large arc of much lower curvature; occurrences of these modes of swimming are apparent in the trajectories in figure 1a5,a6. The adaptive trajectory lasted from a few minutes to an hour, and the duration time differed from one individual to the next. The statistical occurrence of this type of trajectory was 45%, and the occurrence of swimming similar to figure 1a1, in which there was no adaptive change in the trajectory, was 53%, in a total of 117 specimens sum-
mapping the difference ϕ (0.3, 0.4, 0.5, 0.6 mm). The remaining of cases were the other types. The duration of con-
finement was too short at 5 min to observe the type in figure 1a5,a6, and too long at 30 min (70% of organisms tended to stop moving).

Figure 1b1–b3 shows the characterization of swimming type. In a segment of swimming trajectory extracted in the time interval of 2τ, the statistical occurrence of MD of any two positions along the segment was measured with respect to τ. In the type in figure 1a5, MD was saturated at 0.4 mm as the trajectory is approximately circular at a diameter of 0.4 mm (figure 1b2). Such saturation was not observed in figure 1b1 when the trajectory was almost straight like figure 1a1. The trajectory was somewhere in between, like figure 1a6, as shown in figure 1b3.

For modes of swimming shown in figure 1a5 and a6, nor-
malized frequencies of the diameters of the circular trajectories (excluding periods of time in which the organism swam in a straight line or wide arc) were determined and are shown in figure 1c. The diameter of the swimming trajectory increased with the diameter of the confined space experi-
enced, which varied from 0.3 to 0.6 mm. The diameter of the swimming trajectory increased with φ and was approxi-
mately 1.3 times larger than φ (figure 1d). We are at present unable to explain this difference, and it is unclear whether the difference is physiologically significant.

In the control group that did not experience the confined space, only fewer than 10 times of the circular trajectories were counted when 80 individuals were traced for 1 min, and their diameters ranged from 1 to 2 mm. So the circular trajectory in the diameter of less than 1 mm was not observed at all for the accumulated 80 min of observation time. By con-
trast, after the experience of confined space, the numbers of circular trajectory (the diameter was mainly less than 1 mm) were 757, 292, 375 and 76 for the accumulated total observation time of 38 min, 15 min, 26 min and 23 min for droplet diameters of 0.3 mm, 0.4 mm, 0.5 mm and 0.6 mm, respectively. Therefore, we concluded that a circular trajec-
tory less than 1 mm did not result from some stochastic fluctuations of swimming trajectory but was clearly induced by the confinement.

For the data of diameter distribution shown in figure 1c, the statistical significance tests of non-parametric type were done. It is because a pre-condition of equal variance for parametric ANOVA and multiple comparison tests was not satisfied: the variance of each group (different size of confined space) was not equal using Bartlett’s test for equal variance. By means of non-parametric Kruskal–Wallis test, the medians varied signifi-
cantly among the groups (p < 0.0001), and the rank sum differed significantly between any pair of groups (p < 0.05) by Dunn’s multiple comparison test. Therefore, we concluded that the diameter of trajectory induced by the confinement differed significantly in the groups.

The swimming speed also changed while the swimming tra-
jecotory was circular: for instance, when φ = 0.3 mm, 0.39 ± 0.12 (mean ± s.d., N = 14) in the droplet and 0.56 ± 0.16 after the confinement. In the other diameters, the speeds after the confinement were 0.51 ± 0.09 (φ = 0.4 mm), 0.61 ± 0.13 (φ = 0.5 mm) and 0.49 ± 0.09 (φ = 0.6 mm).
4. A mathematical model for adaptive behaviour to a confined space

The swimming of a ciliate is driven by the collective motion of many cilia, which is controlled by the membrane potential and the Ca$^{2+}$ current [6,20–22]. When the anterior part of a ciliate collides with an obstacle, Ca$^{2+}$ ions flow into the cell and lead to reversal of the ciliary beat. The ciliate then changes direction and the turning angle depends on the period over which Ca$^{2+}$ exceeds the threshold concentration [23]. A spontaneous turn sometimes occurs without collision with the wall due to excitation of the membrane evoked by internal and external fluctuations [24–28].

Based on the biochemical process that controls swimming, we propose a simple mathematical model. Let a ciliate be represented by a point particle with position $(x(t), y(t))$ at time $t$. The swimming motion can then be described as $(\dot{x}, \dot{y}) = (\bar{v} \cos \theta(t), \bar{v} \sin \theta(t))$, where $\bar{v}$ is the speed (constant) and $\theta(t) \in [-\pi, \pi]$ is the measured angle of the swimming direction with respect to the x-axis, as shown in figure 2a.

Next, we describe the motion when the cell comes into contact with the vessel wall: the ciliate moves along the wall without any frictional resistance. That is, it slips along the wall. As shown in figure 2a, the swimming trajectory is then given by the projection of the hypothetical free motion (in the case of no wall) onto the vessel wall, which is

$$
\begin{align*}
\dot{x} &= \bar{v} \cos \theta(t) \\
\dot{y} &= \bar{v} \sin \theta(t)
\end{align*}
$$

where $\theta(x, y) \in [-\pi, \pi]$ is the angle of the vessel wall at the contact point with the cell and $\theta(t) \in [0, \pi/2]$ is the contact angle between the vessel wall and the direction of hypothetical free motion of the cell.
5. Numerical simulation of the model

Figure 2b shows the simulation of normal free swimming without any collision with the wall when $u_1$ is in an excitable regime. Typical trajectories of swimming look like a combination of straight motion and stochastic turning (figure 2b1). This turning results from a fluctuation-induced spike of $u_1$. The spikes $P_1$, $P_2$ and $P_3$ in the time course of $u_1$ (figure 2b2) correspond to the three turning events $P_1$, $P_2$ and $P_3$, respectively, in figure 2b1. A typical trajectory of a spike in the state space of $u_1$ and $v$ is shown in figure 2b3.

Figure 3 shows the results of simulation ($\theta_0 = 40^\circ$) for two different sets of parameters: an excitable $u_1$ (figure 3a1–a3) and an oscillatory $u_1$ (figure 3b1–b3). The simulated trajectories of swimming are similar to the real ones (figure 1) during (figure 3a1,b1) and after (figure 3a2, b2) confinement in the small space.

At the initial condition (time 0), the organism contacts the wall at an angle of $90^\circ$, and the next time-step changes its direction of movement a little because $\varphi > \varphi_0$. This slight change in direction continues with further collisions as long as $\varphi > \varphi_0$ and the contact angle $\varphi$ decreases to $\varphi_0$ (figure 3e3).

While the contact angle gradually decreases, $u_0$ increases by $s (=0.003)$ at each change of direction and continues to increase over a series of frequent turning events as the decay rate of $u_0$ is slow. The increase in $u_0$ implies that the swimming trajectory becomes ever more curved.

Around the saturation level of $u_0$ at $\varphi_0$, the curvature of swimming becomes constant while the frequency of active turning and the decay speed of $u_0$ become balanced. This simulated trajectory (figure 3e1) is similar to that observed experimentally (figure 1a2). This saturated state of $u_0$ is maintained for some time after the vessel wall is removed at time 900 in the simulation, and the simulated (figure 3e2) and experimental (figure 1a5) trajectories remain similar.

Surprisingly, the diameter of circular motion after confinement is 1.3 times larger than that of the confined circular space, as also observed for the real organism. This relationship was reproduced for confined spaces with a range of sizes (figure 3d), provided that $\psi_0 = 40^\circ$. This adaptive circular motion slowly disappears (much later than time 1500) and straight motion is recovered ($u_0 = 0$, data not shown).

The simulation described above was performed in the excitable regime of $u_1$ and noise-induced spikes of $u_1$ were observed. These spikes result in a transient modulation of the curvature of swimming trajectory.

In the alternative regime of oscillatory $u_1$, the basic behaviour of $u_0$ observed for excitable $u_1$ was reproduced, even though regular oscillations of $\varphi$ and $u_1$ are involved. The simulated trajectory during (figure 3f1) and after (figure 3f2) confinement are similar to the experimental trajectories in figure 1a3 and ab with respect to the circular motion and its modulation.

As shown in figure 3c, the simulated diameters of free motion were approximately 1.3 times larger than the
In the case of oscillatory u, I = 0.15(a1–a3) and in the case of excitatory u, I = 0.0((a1–a3). Trajectories during (a1,b1) and after (a2,b2) the confinement. Time courses of key variables (a3,b3) and the confinement ended at time 900. The parameters for both cases were as follows: vessel size = 0.2 mm, v = 0.3 mm s⁻¹, ɛ = 0.0001, s = 0.003, τ₀ = 0.0025, τ₁ = 20, τ₂ = -0.3, τ₃ = -1, τ₄ = 4, τ₅ = 1, b₁ = 5, φ₀ = 40° and ζ₁, ζ₂ ∈ [-0.25, 0.25]. (a1,a2,b1,b2) Drawn to the same scale. (c) Simulated relationship between the diameter of the confined space and that of the circular trajectory just after confinement. The dashed line indicates where the two diameters are equal. All parameters were the same as (c). (d) Schematic of geometrical analysis of the relationship between the two diameters. See the main text for details.

Figure 3. Mathematical modelling for swimming behaviour during and after confinement. Numerical simulations in the case of excitatory u, I = 0.0((a1–a3) and in the case of oscillatory u, I = 0.15(a1–b3). Trajectories during (a1,b1) and after (a2,b2) the confinement. Time courses of key variables (a3,b3) and the confinement ended at time 900. The parameters for both cases were as follows: vessel size = 0.2 mm, v = 0.3 mm s⁻¹, ɛ = 0.0001, s = 0.003, τ₀ = 0.0025, τ₁ = 20, τ₂ = -0.3, τ₃ = -1, τ₄ = 4, τ₅ = 1, b₁ = 5, φ₀ = 40° and ζ₁, ζ₂ ∈ [-0.25, 0.25]. (a1,a2,b1,b2) Drawn to the same scale. (c) Simulated relationship between the diameter of the confined space and that of the circular trajectory just after confinement. The dashed line indicates where the two diameters are equal. All parameters were the same as (c). (d) Schematic of geometrical analysis of the relationship between the two diameters. See the main text for details.

6. Discussion

As the confinement in a tiny droplet is an artificial condition, one may wonder if there indeed is such a space in the wild and if the circular swimming pattern observed is actually physiologically meaningful. Careful discussion on this matter is needed.

In stagnant waters like shallow rice paddy fields and ponds which are typical habitats for ciliates, there may be dense aggregates of fibrous algae like *Spirogyra* that form three-dimensional meshes with many small cavities. Or there may be aggregates of debris at the bottom of the paddy field or pond that look like media with many small-scale pores. Although the exactly spherical shape examined in this report is hardly possible under such natural conditions, cavities whose shape can be approximated by deformed spheres are to be expected. In this sense, a capacity to adapt to a confined spaces is not meaningless.

On the other hand, let us assume that there is no such cavity in the wild. Organism possess a high-enough capacity to adapt to a shape never experienced before. This, in turn, infers that...
Tetrahymena has generalized capacity for spatial memory. Whether or not spherical cavities occur naturally, the adaptive capacity reported here is of potential physiological usefulness. When we confined them for more than 30 min, we observed that 70% of the individuals after exhibiting a circular trajectory stopped all movement. One may argue that the experimental conditions were stressful to the organisms. A different interpretation, however, is possible. In order to avoid frequent contacts with the wall, it may be favourable to cease movement, which would constitute an adaptive behaviour. As the organisms started to swim again sometime after the confinement, we can exclude a weakening of its physical condition by the confinement.

The adaptive behaviour reported here is of time scale shorter than a life span, and much shorter than multiple generations. It may be expected that complicated geometries of real micro-habitats are exerted in the wild. Possible advantages of this capacity will be examined in the future.

In the mathematical modelling performed in this paper, we assumed that Ca2+ and their Ca2+ channels played the key role, as many previous papers on the electrophysiological regulation of swimming behaviour in ciliates strongly suggested their importance [6,20,22,23]. This does not mean that Ca2+ and Ca2+ channels indeed contribute to the development of the adaptive behaviour reported in this paper. Therefore, this point awaits experimental verification.

The mathematical model proposed here, however, will still hold in a mathematical sense even if the key molecules are not Ca2+ and Ca2+ channels, because we assumed only two things (i) slower elicitation of a response that can accumulate their repetitive stimulation and (ii) dependency of the response sensitivity on the contact angle. The first assumption needs to be examined biologically and mathematically.

The key assumption in our model is the slow regulation of the Ca2+ channel, which is supported by reports that the shutdown of the Ca2+ current in Paramecium involves fast (10 ms range) and slow (10–100 s range) kinetics [29], and that such slow dynamics of the Ca2+ current is also widely found in both invertebrate and vertebrate neurons. It is thus plausible that adaptation and memorizing at the level of the cell might be embedded in the slow dynamics of channel motion. The adaptive capacity observed in this report is maintained for some time after the experience of confinement, and this might result in some benefit to the organism.

The swimming speed decreased down to 0.39 ± 0.12 in the droplet (the diameter is 0.3 mm) in comparison with 0.81 ± 0.27 in the wide-open space before being confined, and increased up a little to 0.56 ± 0.16 in the wide-open space after being confined. This change in the swimming speed is consistent with the well-known correlation between the swimming speed and curvature of swimming trajectory in Paramecium [21]; the swimming speed slows down while the curvature of swimming trajectory is larger, due to the increase in intracellular Ca2+ concentration which is induced by change in K+-Ca2+ composition of external medium. Although the species of organism is different from Tetrahymena, it is known that the swimming trajectory is circular in the diameter of 0.2–0.8 mm when the swimming speed is one-half or more slower than the normal speed (0.8–1.2 mm s⁻¹), and that the diameter is smaller at the slower swimming speed. This relationship of diameter and speed was very similar to the results observed in our experiment.

It is noted that a ciliate Paramecium is capable of swimming in a circular trajectory in a wide-open space under the specific conditions of external medium. This capacity can contribute to the space memory of spherical shape. Then a question arises: what kinds of shape other than spheres does the ciliate memorize? The original research done by F. Bramstedt showed a triangle was a possibility [17]. To test various shapes of arena is very interesting.

The capacities of adaptation and memory are of interest in basic biology and comparative ethology. In the field of neuroscience of higher animals, molecular events involved in the long- and short-term potentiation of synaptic connections in neural circuits have been studied in relation to the involvement of the N-methyl-d-aspartate receptor (NMDA receptor). Even in protozoa, like Paramecium and Tetrahymena, the NMDA receptor plays a key role in the regulation of swimming behaviour by modulating the membrane potential and the Ca2+ influx/efflux across the membrane [30,31]. This similarity implies a common evolutionally origin of capacity of memory and adaptation between ciliates and higher animals.

The range of time periods over which the real organisms exhibited the circular trajectories varied from minutes to hours. This large variation may be explained through the mathematical model. In the model, the time derivative of the slow variable $u_0$ is just proportional to $-\epsilon u_0$ as this is assumed to be the first order approximation of much more complicated dynamics for real channel molecules. The values of the proportional constant $\epsilon$ might be distributed over a range of 10-fold difference. Or, the complicated dynamics of real-world mechanism might be sensitive to internal and/or external noise through a nonlinear effect of dynamical motion.

Many types of smart behaviour in ciliates have been reported over the past 100 years. The capacity of conditioning (associative learning in response to two different stimulations) is one example. Even this type of higher learning capacity might be explained by a study of channel behaviour. This paper suggests a promising future direction for research on the physical ethology of ciliates.

**Competing interests.** We declare we have no competing interests.

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