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Controlling the geometry and the coupling strength of the oscillator system in plasmodium of *Physarum polycephalum* by microfabricated structure

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Summary. The plasmodium of the true slime mold *Physarum polycephalum*, which shows various oscillatory phenomena, can be regarded as a collective of nonlinear oscillators. Partial bodies in the plasmodium, which are assumed to be nonlinear oscillators, are mutually connected by microscale tubes named plasmodial strand. The interactions among the oscillators can be strongly affected by the geometry and the dimension of the tube network. Investigation of the collective behavior under the condition that the configuration of the network can be manipulated gives significant information on the characteristics of the plasmodium from the viewpoint of nonlinear dynamics. In this study, we have developed a new method to control the geometry and the tube dimension of the plasmodium with a microfabricated structure. It is shown that the geometry of the plasmodium can be manipulated with a microstructure which is fabricated of ultrathick photoresist resin by photolithographic processes. In order to confirm that not only the geometry but also the dimension of the tubes can be controlled with the microstructure, we observed the cross section of the patterned plasmodium with a three-dimensional internal-structure microscope. By observing the oscillatory behavior of the partial bodies of the patterned plasmodium, it was confirmed that the coupling strength between two oscillators, which corresponds to the dimension of the plasmodial strand, can be controlled by the microstructure. It is concluded that the present method is suitable for further studies of the network of Physarum plasmodium as a collective nonlinear oscillator system.

Keywords: Cell patterning; Coupled oscillators; Microfabrication; *Physarum polycephalum*; Plasmodium; Three-dimensional internal-structure microscope.

Introduction

Nonlinear dynamics becomes important for the studies of collective cells, for example, cardiac cells, neurons, and other oscillatory or excitable cells (Winfree 1980, Hoyer et al. 1998). In these biological systems, the connections between the elements are not highly developed individually. Nevertheless, the collectives of such elements self-organize to show various biological functions, such as information processing in nervous systems and cooperative beating in the cardiac system. In order to understand the mechanism of biological self-organization, it is important to investigate the behavior of the systems under the condition that the connections of the elements can be manually controlled.

The plasmodium of the true slime mold *Physarum polycephalum* is a huge multinucleated unicellular organism whose size is up to 1 m. The plasmodium is not subdivided into cells and is an aggregate of endoplasm without any highly differentiated structure like nervous systems. Despite its simple structure, the plasmodium demonstrates sophisticated biological functions. Namely, a large cluster of the plasmodium is able to behave as a single entity based on the information from its surroundings. The information acquired by a local partial body drives the cooperative behavior by transmitting the information throughout the whole

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cluster. The information is considered to be processed through the interaction among the partial bodies. This leads to, e.g., the attracting (escaping) behavior of the plasmodium to (from) attractants (repellents) (Knowles and Carlile 1978, Miyake et al. 1996).

These functions, information transmission and processing, are said to be realized through the intrinsic oscillatory phenomena in the plasmodium, such as thickness oscillation (Matsumoto 1986, 1988), which synchronizes with the shuttle streaming of endoplasm (Kamiya 1950), and oscillation in concentrations of ATP (Yoshimoto et al. 1981) and Ca^{2+} (Natsume 1992). These phenomena are supposed to be generated by mechanochemical reactions caused by complicated interactions among intracellular chemicals, proteins, organelles, etc. (Ueda et al. 1986). From the viewpoint of nonlinear dynamics, the plasmodium can, therefore, be modeled as a collective system that consists of nonlinear oscillators interconnected with certain coupling strength (Miyake et al. 1992, Takahashi et al. 1997, Takamatsu et al. 1997). In this model, an oscillator can be defined for each partial body in the plasmodium and the coupling strength among the oscillators would correspond to the amount of the shuttle streaming of endoplasm inside the tube structures which interconnect the partial bodies. It is, therefore, expected that by controlling the growth pattern and the dimension of the tube structure, i.e., the geometry and the coupling strength of the oscillator network, we could observe various types of collective behavior which could help us to understand the characteristics of the plasmodium as a nonlinear dynamical system.

Several methods for controlling the growth pattern of the plasmodium have been reported so far. In one method, pure endoplasm once sucked from the plasmodium into a glass pipette was extruded into water (Baranowski and Wohlfarth-Bottermann 1982). The diameter of the tube structure is controlled by the inner shape of glass pipette, and endoplasmic streaming is regenerated after the extrusion. It is, however, difficult to maintain the tube dimension throughout long-time experiments without the support of the glass pipette. In another method, the plasmodium was grown on a nonuniform substrate, where drops of agar medium containing nutrients were discretely spotted (Halvorsrud and Wagner 1998). The network of plasmodium was formed on this substrate so that tube structures interconnect the drops; but there is no restriction for the dimension of the tube itself.

In this study, we fabricate a microstructure in which the plasmodium can be cultivated to spontaneously follow the geometry of the channel structure keeping the dimension of the internal tube structure by the wall of the channel. In order to confirm that the dimension of the tube can be controlled by the present method, the internal structures of the plasmodia grown on the microstructure are observed with the three-dimensional internal-structure microscope (3-D ISM). The dimension of the tube structure is measured by analyzing the image taken by the 3-D ISM. Furthermore, the thickness oscillations of the plasmodia with different tube dimensions are measured and analyzed to discuss the relation between the tube dimension and the oscillatory behaviors.

Material and methods

Fabrication of the microstructure

The microstructures were fabricated of a negative photoresist resin (NANO SU-8 50; Microlithography Chemical Corp.), which are thick sheets (ca.100 μm) having variously designed openings. An example of the structure consists of two circular wells connected by a microchannel as shown in Fig. 1.

The fabrication of the microstructure was performed by photolithographic processes as follows. An aluminum layer was deposited on a silicon wafer by vacuum evaporation as a sacrificial layer before the spin coating of the photoresist. Then the photoresist was patterned through a mask with UV light and developed.

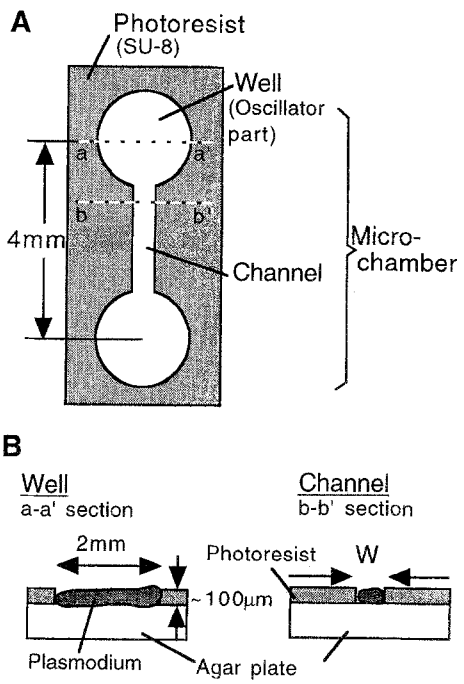

Fig. 1A, B. Design of the microstructure for the two coupled oscillators

Finally, the aluminum layer was removed by 35% HCl solution to release the photoresist structure.

Preparation of the plasmodium

The plasmodium of *Physarum polycephalum* was cultured on a 1.5% agar plate and was fed with oatmeal. The microstructure was put on another 1.5% agar plate. The tip portions of the plasmodium were transferred to the openings on the microstructure, for example, the two wells in Fig. 1. The samples were cultured in the condition of 25 °C and relative humidity of 60–80% for 5–15 h until the plasmodium followed the pattern of the microstructure as shown in Figs. 2 and 3 (the details are explained in Results).

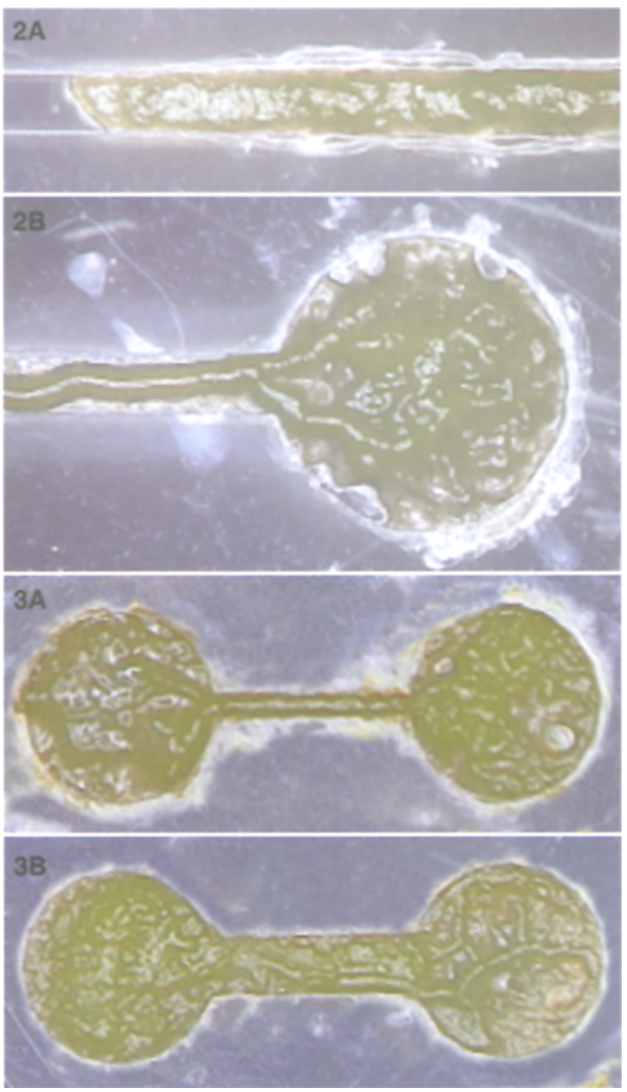

Fig. 2 A, B. Growing process of the plasmodium in the microstructure. Channel width, 300 μm. **A** Tip portion. **B** Root portion

Fig. 3. Plasmodium grown in the microstructure; channel width, 200 μm (**A**), 600 μm (**B**)

Three-dimensional internal-structure microscope

The idea of the 3-D ISM system was firstly proposed by Higuchi (Kobayashi et al. 1995) and the system was developed by a research group of the Higuchi "Ultimate Mechatronics" project at Kanagawa Academy of Science and Technology (Yokota et al. 1996, 1998). The system enables us to observe not only the surface of an opaque sample such as the plasmodium but also its arbitrary cross sections and three-dimensional internal structure with real color and high spatial resolution. The system comprises a microslicer with a controller (PC98; NEC), observation units (a microscope, BX40, Olympus; a color CCD camera, DXC-930, Sony), a laser disc recorder (LVR-3000AN; Sony) and an image processor (a graphic work station, TITAN2-900; Kubota computer) as shown in Fig. 4 Samples that are kept frozen by a cooler are successively sliced at every 0.1–5 μm in thickness by the microslicer. Then thousands of images of the cross sections through the microscope are taken by the CCD camera and the data are recorded by the laser disc recorder. Subsequently, the images are converted into digital form and are stored in the image processor as the two-dimensional images. These data are processed by an image processing software (Application Visualization System, AVS) to be reconstructed into a full color three-dimensional image for further analysis.

Pretreatment of the plasmodium for 3-D ISM

There is less contrast of color image between inside (endoplasm) and outside (ectoplasm) of the tube structure of the plasmodium. Thereupon, the dye solution (5 mg of Neutral red per mol, 25 mg KCl, 1 mM NaCl, 10 mM morpholine propane sulfonic acid, pH 7.1) was injected into the tube so that only the tube structure was stained. Within 20 min after the injection, the samples were frozen with liquid nitrogen and then embedded into embedding compounds (OCT-compound) in a vessel made by paraffin. In order to slice thinly such soft organism, the samples were kept at –15 °C for 10 min until the embedding compounds were frozen.

Observation and measurement of the tube dimension

The samples were set into the microslicer and successively sliced at every 3 μm in thickness while the temperature of the sample holder was maintained at –10 to –20 °C by the cooler. Concurrently, the two-dimensional images of the cross sections of the plasmodia were

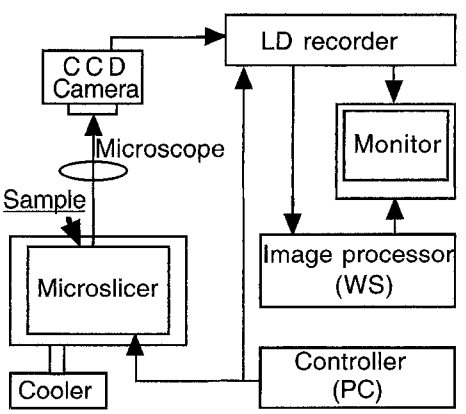

Fig. 4. Schematic diagram of the three-dimensional internal-structure microscope system (3-D ISM)

captured (Fig. 5 A, B). From these images, only the color stained by the red dye solution was extracted by the image processing software (Fig. 5 C, D), and then area size (S_t), width (W_t), and height (H_t) of the tube were measured (Fig. 7 A). Assuming the extracted area as a round shape, the tube diameter was calculated from the area size by the relation:

$$\text{tube diameter} = 2\sqrt{S_t/\pi}.$$

Furthermore, ellipticity was calculated by the relation:

$$\text{ellipticity} = 1 - H_t/W_t.$$

Observation of the thickness oscillation

The plasmodia grown in the microstructure as shown in Fig. 3 were set under a microscope (SMZ-2T; Nikon). The transmitted light through the plasmodium was detected by the CCD camera (C2400; Hamamatsu). The image data converted by the flame grabber (LG-3; Scion Corp.) were sequentially stored in the PC (Power Macintosh G3; Apple) every 4 s. We have confirmed that the change in the transmitted-light intensity is inversely proportional to the change in thickness within the range of thickness oscillation (data not shown). The experiments were performed at room temperature (25 ± 1 °C).

Analysis of the thickness oscillation

In order to enhance the change of thickness in images obtained by the method mentioned above, we calculated differential images between the two images at time t and $t + \Delta t$ ($\Delta t = 4$ s) according to the ordinary method (Matsumoto et al. 1988).

The spatial wavelength of oscillation in the natural plasmodium ranges from 5 mm to several centimeters. The size of the wells (oscillator part) was determined as 2 mm, which is equal to or less than a half of the spatial wave length, and within this area the plasmodium shows almost synchronized oscillation in the natural plasmodium. We averaged the original images over the well parts in the microstructures to obtain the time courses of the change in thickness of individual plasmodia, which could be an indicator for the behavior of each plasmodium as an oscillator.

In order to analyze the time course of the phase relation between two oscillators, we calculated the cross correlation coefficient $C(t)$ from the time courses of the change in thickness according to the following equation:

$$C(t) = \frac{1}{N} \frac{\sum_{k=-N/2}^{N/2} \left(T_1(t+k\Delta t) - \overline{T_1}\right) \cdot \left(T_2(t+k\Delta t) - \overline{T_2}\right)}{\sigma_1 \cdot \sigma_2}.$$

Here, $T_i(t)$ is the thickness of the oscillator i at time t. $\overline{T_i}$ is the time average of $T_i(t)$, and σ_i is standard deviation of $T_i(t)$ from the time $t - (N\Delta t)/2$ to $t + (N\Delta t)/2$. The time period $N\Delta t$ for summation was set about 100 s in this calculation, which is comparable to one period of the oscillation. The coefficient $C(t)$ being equal to 1 or −1 means that the relation between two oscillators is in-phase or anti-phase, respectively.

In the calculation of the proportion of anti-phase oscillations in duration, the phase relation is regarded as in anti-phase when $C(t)$ remains below −0.75.

Results

Geometry patterning of the plasmodium by the microstructure

Figure 2 shows the growing process of the plasmodium in the microstructure. The plasmodia locally put in the wells grew along the channel pattern during the culture because plasmodia prefer the moist medium to the hydrophobic one covered with the microstructure made of the photoresist resin. In the early stage of the growing process, the shape of the plasmodium tip portion was flat and not structured (Fig. 2 A), but at the root portion connecting to the well the tube structure was formed (Fig. 2 B). The plasmodia from the

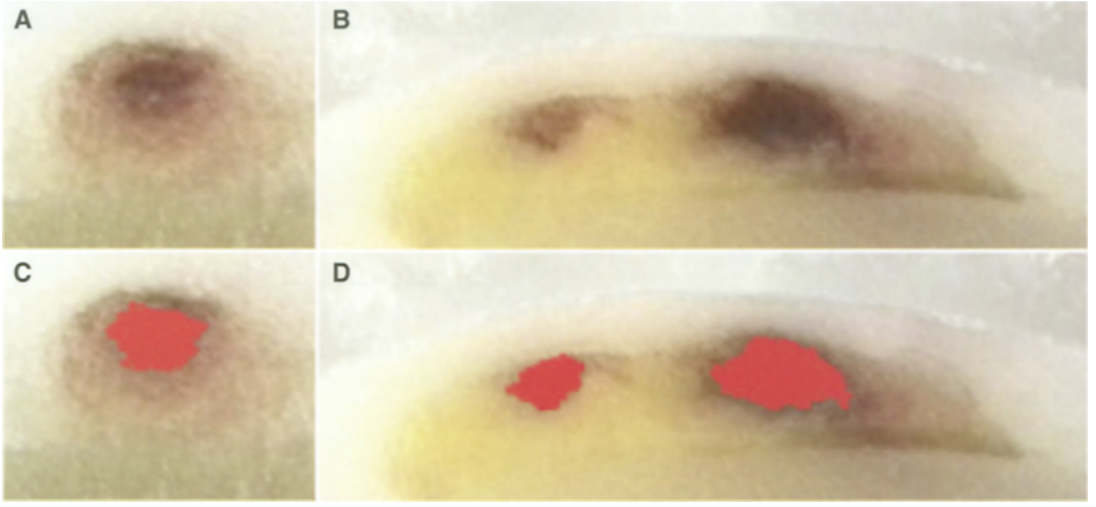

Fig. 5 A–D. Two-dimensional images of the tube structure. The yellow parts are the ectoplasm of plasmodia. The red parts are the endoplasm. The white parts are the embedding compounds. Channel width, 200 μm (**A** and **C**), 800 μm (**B** and **D**). **C** and **D** Areas stained by the red dye are extracted

both wells contacted around the center of the channel to be physically connected through the tube structure, where the endoplasmic streaming were observed. This method can be widely used for the patterning of the plasmodia not only by microstructures with different channel widths (Fig. 3) but also by differently shaped microstructures, e.g., round or rectangular (data not shown).

Dimensional control of tube structure by the microstructure

The tube structures of the plasmodia grown in the microstructure were observed with 3-D ISM, where the microchannel width (W) ranges from 100 to 800 μm. Figure 5 shows the cross sections of the plasmodia. In the narrow channel, the plasmodium has a single tube (Fig. 5A). In the wide channel, the plasmodium has several tubes (Fig. 5B). The bifurcation of the tube occurs when the channel width is around 500 μm.

Figure 6A shows the reconstructed three-dimensional image of the plasmodium and Fig. 6B shows the extracted image of the tube structure. The extracted image provides the detailed feature of the dendritic tube structure of the plasmodium in the wide channel.

Figure 7B shows the relation between channel width and tube diameter. The tube diameter becomes larger depending on the channel width but is saturated when the channel width is beyond 400 μm. At this point, the single-tube structure becomes a multitube structure, namely the dendritic one. Figure 7C shows the dependence of ellipticity on the channel width. The ellipticities are all about 0.3, which means the cross section shapes of tubes are slightly elliptic but do not depend on the channel width.

Thickness oscillation in two coupled oscillators

The thickness oscillation of the plasmodium in the microstructure for two coupled oscillators were observed with various microchannel widths (W) ranging from 50 to 800 μm as shown in Fig. 8.

With the narrow channel ($W = 50$ μm), coherent oscillation was not observed in each oscillator (Fig. 8Aa) and the amplitude of oscillations is very small (Fig. 8Ab). Although the endoplasmic streaming was certainly observed inside the tube structure, there was weak interaction between the two oscillators (Fig. 8Ac).

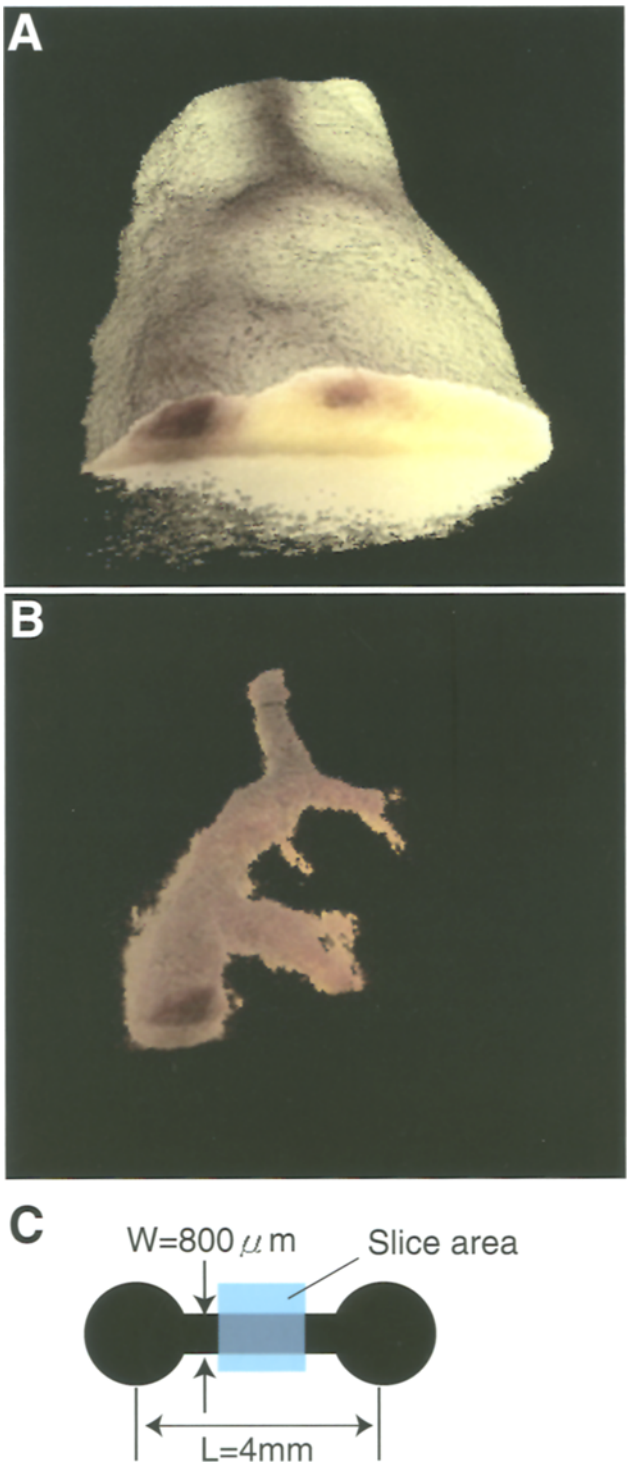

Fig. 6. **A** Reconstructed three-dimensional image of plasmodium. **B** Extracted image of the tube structure. **C** Diagram of the measured area

With the relatively wide channel ($W = 400$ μm), anti-phase oscillation were often observed as shown in Fig. 8Bb. The alternating wave propagation from one

oscillator to another was observed in its spatio-temporal pattern (Fig. 8Ba). The anti-phase oscillation is, however, not always stable and sometimes perturbed (Fig. 8Bc). The stability of anti-phase oscillation, namely, the proportion of duration at which the state remains in anti-phase increases depending

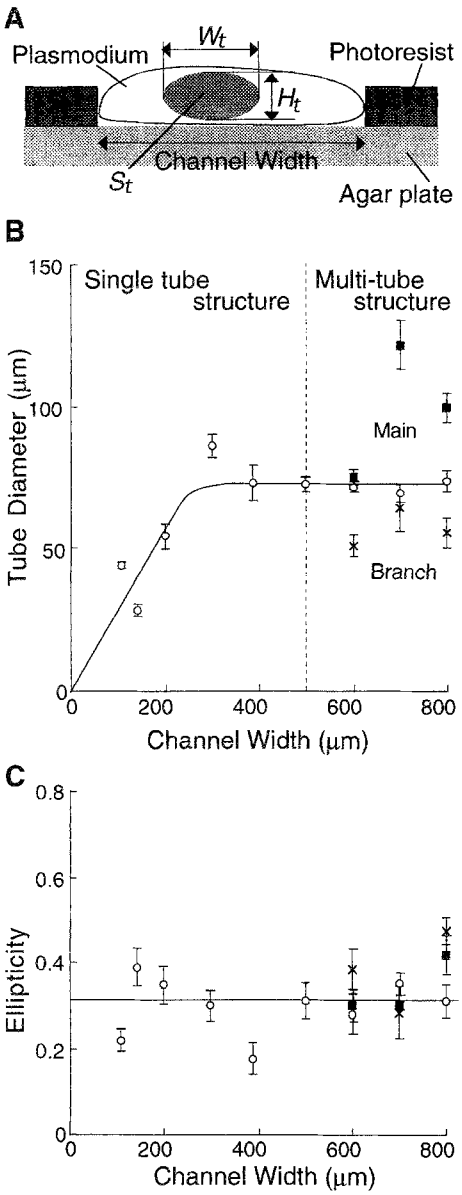

Fig. 7A–C. Relation between the channel width and the tube's feature. **A** Sketch of parameter measurement. **B** Relation between channel width and tube diameter. **C** Relation between channel width and ellipticity of the tube. For **B** and **C**, the data were calculated from more than 10 points on the tube structure. Circles are for single-tube structures. Black squares and crosses designate main and branching tubes in multitube structures, respectively

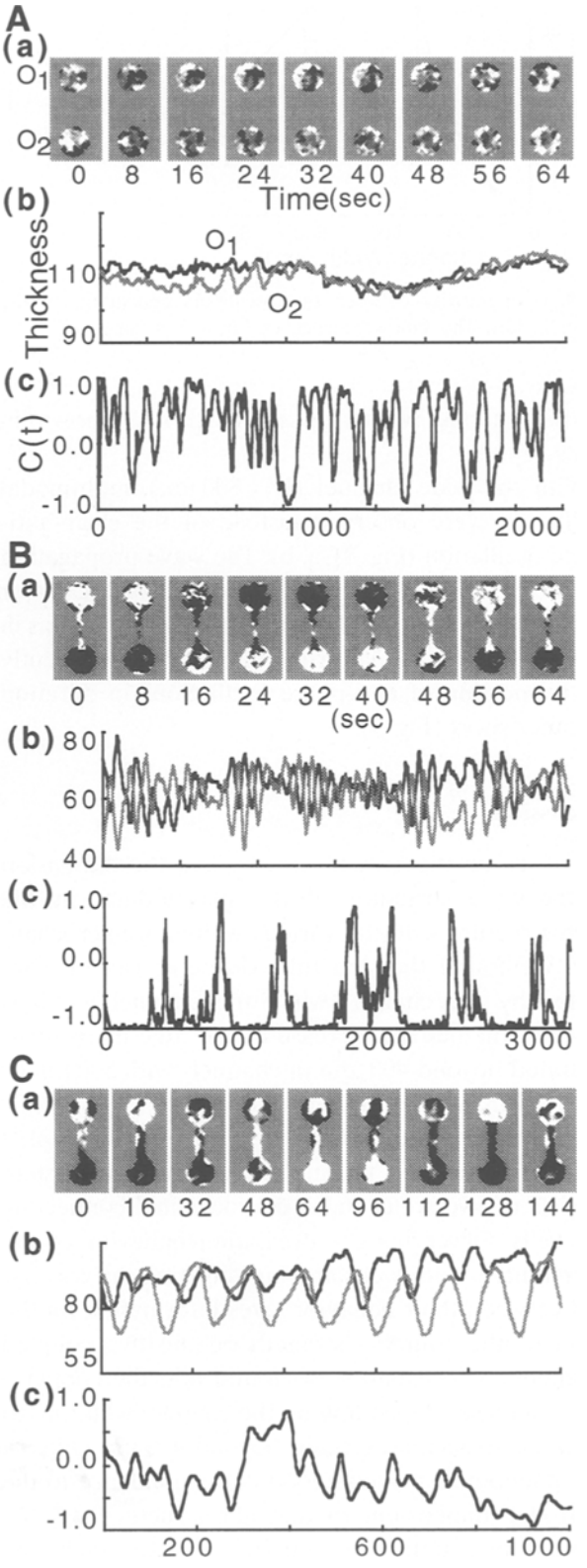

Fig. 8. Oscillations in two coupled plasmodia; channel width, 50 μm (**A**), 400 μm (**B**), 800 μm (**C**). **a** Spatio-temporal change in thickness, where black and white colors indicate increase and decrease in thickness, respectively. O_1 and O_2 designate the two oscillators. **b** Time course of the change in thickness of each oscillator. **c** Time course of cross correlation coefficients between two oscillators

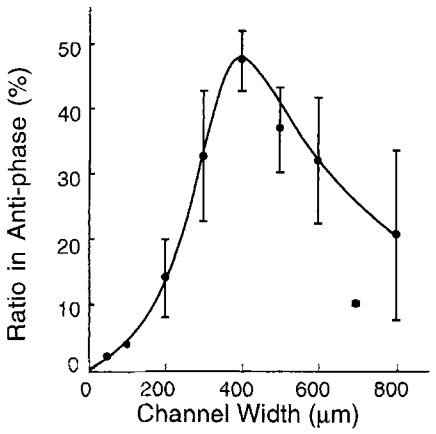

Fig. 9. Cooperativity between two oscillators depending on the channel width. The data were calculated from 3–4 samples

on the channel width up to 400 μm as shown in Fig. 9.

With the wide channel ($W = 800$ μm), multimodal oscillation were observed instead of the clear anti-phase oscillation (Fig. 8Ca, b). The wave propagation was observed not only in the direction of the major axis of the microchannel but also in other directions in its spatio-temporal pattern (Fig. 8Ca). Consequently the proportion of anti-phase oscillations in duration becomes short (Fig. 9).

Discussion

We observed the growth pattern and the dimension of the tube structure of the plasmodium in the microstructure with the various widths of microchannels. We found that the tube diameter can be controlled by the channel when the channel width is 100–400 μm, but the increase of the tube diameter is saturated beyond 400 μm. In channels with 500 μm or more width, the tube branches (Fig. 7).

We analyzed the correlation between two coupled plasmodia grown in the microstructures. This system consists of two wells and a microchannel connecting the wells. Observing the oscillation behavior of the plasmodia in both wells and investigating the correlation between their behavior gives information on the effect of the coupling strength on the two coupled plasmodia as oscillators. We found that the cooperativity increases depending on the channel width up to 400 μm but decreases again beyond 400 μm (Fig. 9). This phenomenon shows good correspondence to the controlled dimensions of the tube structure (Fig. 7). Namely, the coupling strength between two oscillators can be controlled by the microchannel as long as a single-tube structure grows in the microchannel. However, when a dendritic tube structure is formed, it is difficult to control the coupling strength in a straight forward manner, because the effect of the endoplasmic streaming becomes complicated according to the geometry of tubes in this situation.

The tube dimension is important for the survival of the plasmodium, because the endoplasmic streaming observed in the tube structure transports nutrients over the whole body of the plasmodium. The high cooperativity between two coupled plasmodia in the relatively wide channel ($W = 400$ μm) would suggest that the larger diameter of tube is the more advantageous to the transportation of endoplasm in the single-tube structure (Figs. 8B and 9). The increase in tube diameter, however, will be limited (Fig. 7B) by the properties of the tube materials, such as viscoelasticity and surface tension, and gravity. The dendritic structure of the tube can be profitable for the transportation of much more nutrients and the wide coverage in a two-dimensional space such as the wide channel.

In conclusion, we succeeded in flexibly controlling not only the geometry of the Physarum plasmodium but also the dimension of its tube structure by the microfabricated structure. Using the microstructure for the two coupled plasmodia, namely, the coupled two-oscillator system, we elucidated that the coupling strength among the oscillators depends on the diameter of the tube structure of the plasmodium. The present method can be widely applied to the analysis of the oscillator network in the Physarum plasmodia with various configurations.

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