

Micro-Particle Transporting System Using Galvanotactically Stimulated Apo-Symbiotic Cells of *Paramecium bursaria*

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It is well known that *Paramecium* species including green paramecia (*Paramecium bursaria*) migrate towards the anode when exposed to an electric field in a medium. This type of a cellular movement is known as galvanotaxis. Our previous study revealed that an electric stimulus given to *P. bursaria* is converted to a galvanotactic cellular movement by involvement of T-type calcium channel on the plasma membrane [Aonuma *et al.* (2007), Z. Naturforsch. **62c**, 93–102]. This phenomenon has attracted the attention of bioengineers in the fields of biorobotics or micro-robotics in order to develop electrically controllable micro-machineries. Here, we demonstrate the galvanotactic controls of the cellular migration of *P. bursaria* in capillary tubes (diameter, 1–2 mm; length, 30–240 mm). Since the *Paramecium* cells take up particles of various sizes, we attempted to use the electrically stimulated cells of *P. bursaria* as the vehicle for transportation of micro-particles in the capillary system. By using apo-symbiotic cells of *P. bursaria* obtained after forced removal of symbiotic algae, the uptake of the particles could be maximized and visualized. Then, electrically controlled transportations of particle-filled apo-symbiotic *P. bursaria* cells were manifested. The particles transported by electrically controlled cells (varying in size from nm to μm levels) included re-introduced green algae, fluorescence-labeled polystyrene beads, magnetic microspheres, emerald green fluorescent protein (EmGFP)-labeled cells of *E. coli*, Indian ink, and crystals of zeolite (hydrated aluminosilicate minerals with a micro-porous structure) and some metal oxides. Since the above demonstrations were successful, we concluded that *P. bursaria* has a potential to be employed as one of the micro-biorobotic devices used in BioMEMS (biological micro-electro-mechanical systems).

Key words: BioMEMS, Green Paramecia, Particle Transport

Introduction

It has long been established that members of *Paramecium* species exhibit galvanotaxis, since paramecia align with an electric field or voltage gradient and swim toward the anode if the electric field is sufficiently strong (Ludloff, 1895; Jennings, 1906; Ogawa *et al.*, 2005). Researchers have predicted that the ciliary reversal required for galvanotactic responses in *Paramecium* species depends on the intracellular increase of the Ca^{2+} concentration (Iwadate, 2003). Recent electrophysiological experiments (with patch-clamping technique) have demonstrated that some *Paramecium* species possess calcium channels which are voltage-dependently gated and involved in ciliary movements (Gonda *et al.*, 2004).

In our previous study (Aonuma *et al.*, 2007), quantification of anodic galvanotaxis performed by green paramecia (*Paramecium bursaria*) was

carried out in the presence and absence of various inhibitors of cell signaling. As expected, the galvanotactic response in *P. bursaria* was completely inhibited by a variety of Ca^{2+} -related inhibitors including calcium chelators. Notably, strong inhibition was observed with inhibitors of T-type calcium channels (Ni^{2+} , 1-octanol, and NNC 55–0396) while none of the L-type calcium channel inhibitors (neither nimodipine, nifedipine, verapamil, diltiazem nor Cd^{2+}) showed inhibitory action. This was the first pharmacological evidence of the involvement of T-type calcium channels in protozoan cellular movements.

Paramecium cells are now considered as model systems for studying the signal transduction mechanisms (Pech, 1995). Since signal perception, processing and reactions are completed within these unicellular organisms, some researchers described the cells of *Paramecium* species as “swim-

ming sensory cells" (Machemer and de Peyer, 1977) or "swimming neurons" (Naitoh, 1982), as summarized by Wilczek (2001).

Taken together, the above studies indicate the possibility for finely geared neuronal controls and engineering of unicellular micro-machineries. In fact, the galvanotactic responsiveness found in *Paramecium* species (especially *P. caudatum*) has attracted the attention of bioengineers in the fields of biorobotics, micro-robotics or BioMEMS (biological micro-electro-mechanical systems) in order to develop electrically controllable micro-machineries (Itoh, 2000; Ogawa *et al.*, 2004, 2005).

In our present study, we demonstrated the galvanotactic controls of cellular migration of *P. bursaria* in capillary tubes (diameter, 1–2 mm; length, 30–240 mm). In addition, we attempted to use electrically stimulated cells of *P. bursaria* as controllable swimming vehicles in the capillary system for transportation of micro-particles of interest diversified in size since *P. bursaria* takes up particles of various sizes (from the size of food bacteria to that of symbiotic algae) through endocytosis. Experimentally, some ex-symbiotic and non-symbiotic *Chlorella* strains have been introduced into apo-symbiotic *P. bursaria* cells as artificial symbionts (Gerashchenko *et al.*, 2000). Here, we demonstrated that the uptake of the particles of interests could be maximized and readily visualized by using apo-symbiotic cells of *P. bursaria* capable of active endocytosis, after forced removal of symbiotic algae according to a reported protocol (Tanaka *et al.*, 2002). Lastly, electrically controlled transportations of particle-packing apo-symbiotic *P. bursaria* cells were manifested in capillary tubes. Furthermore, possible applications of the present study in BioMEMS are discussed.

Material and Methods

Organisms

The green paramecium strain INA-1 (Fig. 1a; syngen 1, mating type I) was originally collected from the Ongagawa River (Kama-city, Fukuoka Prefecture, Japan) at the sampling point INA as described by Michiki *et al.* (2005) and Nishihama *et al.* (2008).

Since the cell line was established after single cell isolation, all the cells in the culture were clones sharing identical genetic background.

Green cells of *P. bursaria* (INA-1 cells) and apo-symbiotic white cells derived from INA-1 cells (INA-1w cells) were cultured as described by Kadono *et al.* (2006). Briefly, the culture medium was prepared with a yeast extract-based nutrition tablet (1 EBIOS tablet/l; Asahi Food & Healthcare, Tokyo, Japan). The culture medium was re-newed every 2 weeks. One nutrition tablet (250 mg) contains 94.2% (w/w) dried yeast homogenates and 5.5% (w/w) carbohydrates. The bacterized nutrition medium was prepared by inoculating the medium with the food bacterium *Klebsiella pneumoniae* 1 d prior to subculturing of the ciliate cells. The ciliate culture was initiated with *ca.* 10–20 cells/ml and propagated to a confluent level (over 1000 cells/ml) under a cycle of 12 h light with *ca.* 3500 lux (30 cm from the light source) of natural-white fluorescent light and 12 h dark at 23 °C.

An emerald green fluorescent protein (EmGFP)-expressing *E. coli* was prepared by transforming DH5 α competent cells (Takara, Tokyo, Japan) with the pRSET/EmGFP vector (Invitrogen, Carlsbad, CA, USA) as described in the user manual provided by the supplier. According to the user manual, the pRSET/EmGFP vector is a bacterial expression vector that contains sequences encoding a fluorescent protein (EmGFP) derived from the green fluorescent protein (GFP), and contains amino acid substitutions that alter the spectral properties of the protein. Upon excitation, this EmGFP emits a fluorescent signal (emerald green).

Particles

Three types of polystyrene microspheres (microbeads) labeled with fluorescent dyes or magnetite, namely carboxylate-modified fluorescent (diameter, 2.28 μ m; colour, Suncoast Yellow) and magnetic (diameter, 2.88 μ m; COMPEL™ Uniform Magnetic Microspheres) microspheres (Bangs Laboratories, Inc., Fishers, IN, USA), and MicroPlex Microspheres 333997-29101 (diameter, 5.6 μ m; Hitachi Software Engineering Co., Ltd., Tokyo, Japan), were directly obtained from the vendors. Crystals of inorganic catalysts (MgO, TiO₂) were gifts from Dr. K. Tanaka (K2R Inc., Kitakyushu, Japan). Zeolite was provided by Prof. K. Yoshizuka (University of Kitakyushu, Japan). Indian ink made of pine soot and glue was obtained from a local market.

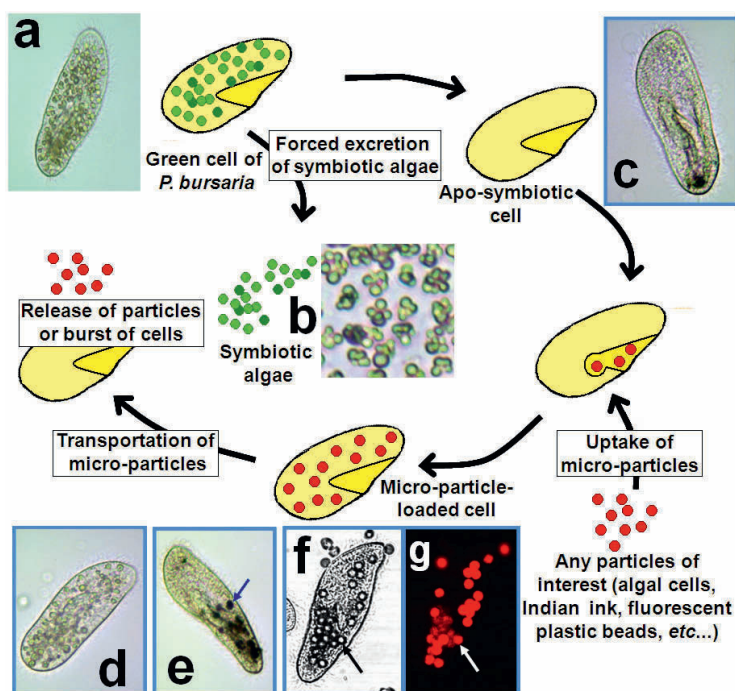


Fig. 1. Schematic diagram showing the excretion and uptake of symbiotic algae or micro-particles by the cells of *P. bursaria*. From green cells of *P. bursaria* (strain INA-1) (a), algal cells (b) can be isolated after homogenization or forced excretion procedures. Alga-free apo-symbiotic ciliates (strain INA-1w) (c) can be prepared after treatment with a herbicide, for example paraquat, or other stressful conditions (forced excretion). Apo-symbiotic ciliate cells (INA-1) can re-take the algal cells to form re-greened ciliates (strain, INA-1r) (d). Uptake of artificial micro-particles by apo-symbiotic INA-1w cells can be visualized by the use of dye (e, Indian ink) or fluorescent labeling of the plastic microspheres (f, g). Arrows in (e) and (f) (also in g) indicate the positions of a digestive vacuole containing ink colloids (e) or the presence of single plastic beads (f, g), respectively. Release of transported particles from the ciliate cells is possible under chemical or electric conditions (*i.e.* pulse of high voltage over 3 V, unpublished results).

Forced removal of green algae and introduction of particles

There are several approaches enabling the forced removal of green algae from green paramecia as discussed later. Here, we employed the protocol of Tanaka *et al.* (2002). Briefly, the green cells were incubated in the presence of a commercial herbicide ($0.1 \mu\text{M}$ paraquat) for over 24 h under light condition (with a fluorescent white lamp, 3000 lux at least). Then, single ciliate lacking algae (Fig. 1c) were separated under a microscope and apo-symbiotic cells derived from a single cell were propagated in the yeast-based medium inoculated with food bacteria as described above. We found that this herbicide treatment merely enhanced the excretion of algae from the ciliate but many portions of resultant ex-symbiotic algae

excreted from the ciliates (Fig. 1b) were still alive and capable of growing *in vitro* (Kadono *et al.*, unpublished results).

Alga-free apo-symbiotic cells, isolated and propagated after forced algal excretion, were used for the introduction of various particles. For re-introduction of green algae (re-greening process), apo-symbiotic *P. bursaria* cells were incubated with ex-symbiotic green algae overnight in an Eppendorf tube at room temperature. The resultant re-greened cells were collected on a nylon mesh (pore size, $10 \mu\text{m}$), and free algae were washed out with 10 ml of fresh medium for three times. In place of algal cells, other particles of interest such as ink colloids and fluorescent and magnetic microspheres, different in size, were applied; as examples typical images are shown in

Figs. 1d–g. In cases of such artificial particles, the time required for successful loading was much shorter (1–2 h only).

Effect of voltage on cell migration

Our preliminary experiments carried out in the open top bath showed that cellular movement leading to anodic accumulation can be observed between 0.5–3.0 V of direct current (DC), while application of higher voltage (over 3 V) was lethal to the cells (Aonuma *et al.*, 2007). Therefore, we applied 1.5 V DC to the cells packed in the capillary tubes by aiming the recovery of intact cells.

Galvanotactic migration assay

As a platform of galvanotactic cell movements, polypropylene tubes (diameter, 1–2 mm) filled with culture medium were connected to 1.5-V battery cells with copper leads (Fig. 2a). No specific metal except for copper was used for preparing a pair of electrodes. For confirming the galvanotactic cell accumulation to the anode, the capillary was simply filled with cell suspension (500 cells/ml), and the galvanotactic condensation of the *P. bursaria* cell population was performed at 1.5 V (Fig. 2b).

For performing the migration of a dense cell population from one end of the tube to the other, galvanotaxis was allowed as follows. Prior to electric charge, 100 cells of *P. bursaria* (50 μ l of dense culture, 2000 cells/ml) were injected to the cathodic end of the medium-filled capillary tube.

In both cases (cell condensation and dense cell migration), the cellular movement was terminated by breaking the current with a switch (Fig. 2a). After exposure to 1.5 V DC, each capillary tube was cut into 4 fractions (Fig. 2d). Then galvanotactically altered localizations of the cells were quantified by counting the cells under a stereomicroscope (SMZ645; Nikon, Tokyo, Japan) after retransferring each fraction onto a depletion slide. All experiments were repeated 4 times, and the mean population in each fraction was expressed as the percentage of the total population (with error bars, S.D.).

Microscopic analysis

Formaldehyde-fixed cells were used for obtaining the microscopic images. The *Paramecium* cells

with and without symbiotic green algae were fixed in 3% (w/v, final content) formaldehyde added to the culture medium at room temperature for 5 min. Microscopic images of *Paramecium* cells were acquired using a Radiance 2100 microscope (Bio-Rad Laboratories, Hercules, CA, USA). The obtained images were processed using Adobe Photoshop software.

Results and Discussion

Effect of cell density, capillary size, and materials

We performed the galvanotaxis of *P. bursaria* using polypropylene tubes as the major platform mostly due to their moderate hardness and flexibility. In addition to polypropylene tubes, we used glass tubes, polycarbonate micro-filtration porous membrane tubes, Tygon tubes, Teflon tubes and other related plastic tubes sized from 1 to 2 mm in diameter for successful galvanotaxis of the *P. bursaria* cells, and could conclude that the impacts of tube materials are limited if the tubes are made of insulators or poor conductors.

The initial cell density optimal for smooth cell gathering of *P. bursaria* (condensation of dispersed cells to anodic fraction) was shown to be 100–300 cells/ml or less. When the initial cell density exceeded over 300 cells/ml, cell gathering was significantly inhibited. In most of the model experiments described below, we employed a polypropylene tube with 2 mm in diameter but finer tubes (diameters of 1.0 mm and 1.2 mm) were also shown to be applicable. All tubes showed successful cell gathering and cell migration of green paramecia (data not shown). However, for easy cell counting and to perform statistic analysis, the number of cells applicable at optimal density in finer tubes must be limited, and for quantitative analysis, the number of cells counted in each replicate must be as great as possible. Therefore, further experiments were performed using polypropylene tubes with 2 mm in diameter.

Quantification of galvanotactic responses

Gathering of cells to the anodic fraction was tested according to the protocol illustrated in Figs. 2b and 2d. With a newly developed method employing polypropylene tubes as the platform for cell movement, galvanotactic cell condensation at the anodic fraction (fraction 4) was successfully

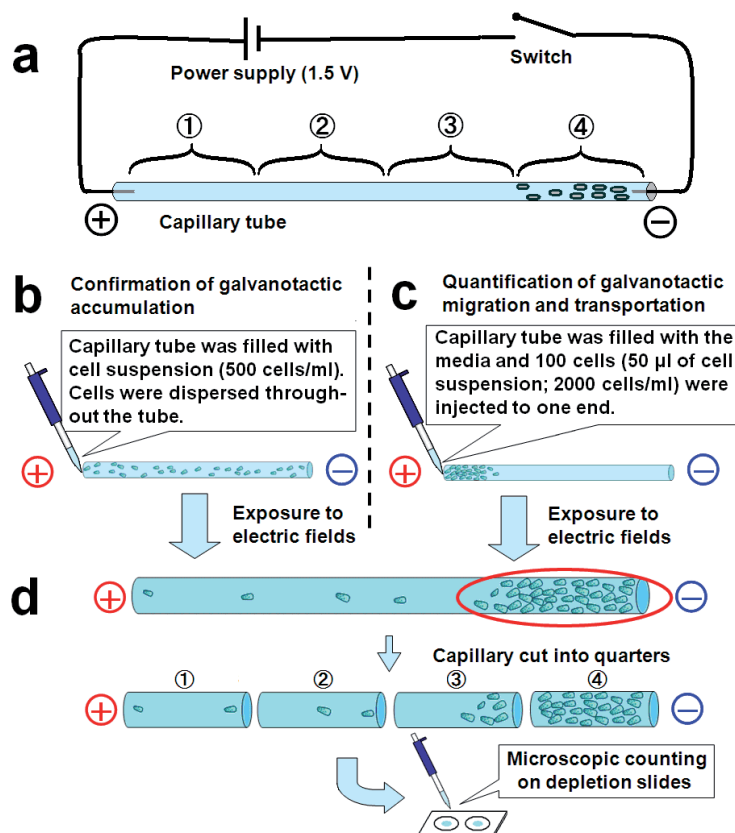


Fig. 2. Galvanotactic cell migration assays in capillary tubes. (a) For allowing galvanotactic migration of *P. bursaria* cells, the ends of the capillary tube filled with the *Paramecium* culture were connected to a 1.5-V battery cell with copper leads. (b) Protocols for the tests confirming the galvanotactic cell condensation at the anode by simply filling the capillary with cell suspension (500 cells/ml) before exposing to the electric field. (c) Tests for confirming the galvanotactic cell migration from the cathodic side to the anodic side within the capillary. Prior to electric charge, 100 cells were injected to the cathodic end of the capillary. (d) After the cells were exposed to 1.5 V, each capillary tube was cut into 4 fractions. Then quantification of cell accumulation or migration was carried out by counting the cells in each fraction.

demonstrated using 60-mm and 120-mm capillary tubes (Fig. 3). While no significant increase in fraction 4 was observed when the electric field was not applied (Fig. 3, left), high accumulation of cells in fraction 4 (anode) was observed by exposing the cells to an electric field (1.5 V) for 10 min (60-mm tubes; Fig. 3a, right) and 20 min (120-mm tubes; Fig. 3b, right). In addition, significant galvanotactic cell condensation was also observed in 30-mm tubes within 5 min of electric treatment (data not shown). In contrast, long-distance cell migration over 240 mm required drastic modifications of the protocols such as alterations of the applied voltage, conductance by supplementation

of ions to the medium, and use of multiple electrodes (Furukawa *et al.*, unpublished results).

Further experiments were performed using 60- and/or 120-mm tubes as indicated.

Quantification of cell migration

In the cell condensation events mentioned above, cells gathered at the anode after swimming for a variety of distances (*i.e.* cells around the anode and cathode had to swim the shortest and longest distances, respectively). To observe the migration of the ready-gathered mass of cells from the cathodic end towards the anodic end, dense cell preparations were injected only to the

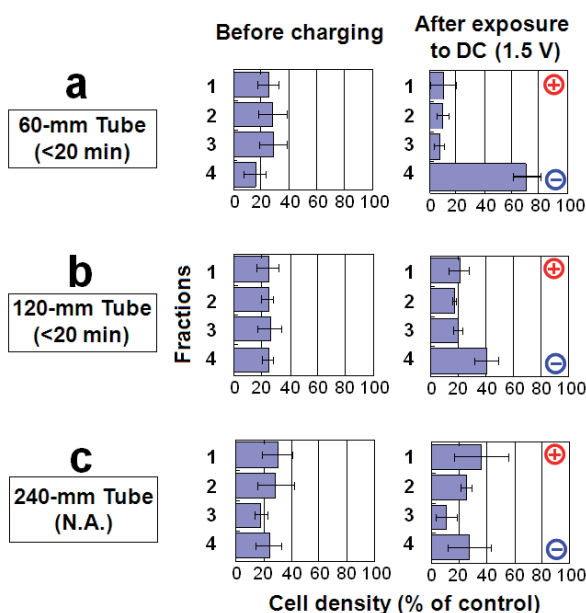


Fig. 3. Effect of electrode distance on the galvanotactic cell accumulation in capillary tubes. Cells evenly dispersed inside the polypropylene tubes (left) were electrically condensed at anodic fractions (right). Experimental results obtained with (a) 60-mm tubes, (b) 120-mm tubes, and (c) 240-mm tubes are compared. Fraction volumes in (a), (b) and (c) were 47.1 μL , 94.2 μL , and 188.4 μL , respectively. Tube diameter, 2 mm. Typical data at (a) 10 min, (b) 20 min, and (c) 40 min of incubation are presented. Times marked in the brackets indicate the approximate time required for manifesting a significant increase in the anodic population. Even over 4 hours of incubation, 240-mm tubes gave no significant accumulation of the cells, thus the time required was simply marked as N.A. (not applicable). Error bars, S.D. ($n = 4$).

cathodic tip of the tubes as illustrated in Figs. 2c and 2d. We observed that application of 1.5 V DC to natural green cells (INA-1) for 10 min resulted in accumulation of 75% of the cells in the anodic fraction (Fig. 4a).

Interestingly, the apo-symbiotic strain (INA-1w) of *P. bursaria* propagated after forced excretion of endo-symbiotic algae in the presence of paraquat, normally responded to electric treatment and almost 80% of the cells reached the anode (Fig. 4b). Similarly, the re-greened cells (INA-1r, Fig. 1d) showed galvanotactic responsiveness leading to massive migration of cells to the anodic fraction (data not shown). Analogously to the re-greening processes, in which the uptake of algae

is followed by the maintenance of algal particles (possibly packed in the membrane structure derived from the digestive vacuole) in the intracellular space, we attempted to introduce a variety of artificial particles of interest into the INA-1w cells. By this way, apo-symbiotic cells of *P. bursaria* could be engineered as swimming vehicles for particle transportation in the micro-combinatorial system.

Speed of cell migration

We have previously examined the time required for completion of anode-directed cell migration of *ca.* 100 *P. bursaria* cells under an 1.5-V electric field applied across 60 mm of distance between two electrodes equipped to an open-top bath containing 2 ml of medium (Aonuma *et al.*, 2007). We have reported that significant accumulation of cells in the anode fraction was observed only after 5 min of continuous application of the DC electric field. In the present system employing capillary tubes, the mean velocity of cell migration was measured by monitoring the migration of the mass of the cells injected at the cathodic end of the tube as illustrated in Fig. 2c. Being located between the anode and cathode producing 1.5 V DC with distances of 30 mm, 60 mm, and 120 mm, the *P. bursaria* cells migrated at 521 mm/h, 330 mm/h and 310 mm/h, respectively.

Transportation of artificial particles by electrically stimulated cells

Cells coloured with Indian ink (colloidal particles consisted of carbon and glue) also showed galvanotactic cell migration (Fig. 4c-1). This indicates that *P. bursaria* cells can be micro-vehicles for the electric control of colloidal particle transport. The apo-symbiotic *P. bursaria* also took up relatively larger particles. When the fluorescent dye-conjugated polystyrene beads, sized 5.6 μm (Fig. 4c-2) and 2.28 μm (Fig. 4c-3), were incubated with the cells of apo-symbiotic *P. bursaria* for 1 h, active endocytosis of these particles was observed. The resultant particle-fed cells were collected and washed prior to galvanotactic experiments. The 5.6- μm beads were shown to be dispersed throughout the intracellular space. In case of the 2.28- μm beads, around 20 beads formed spherical gathering possibly packed in the digestive vacuoles. The number of such aggregates of microbeads exceeded 30 in each cell.

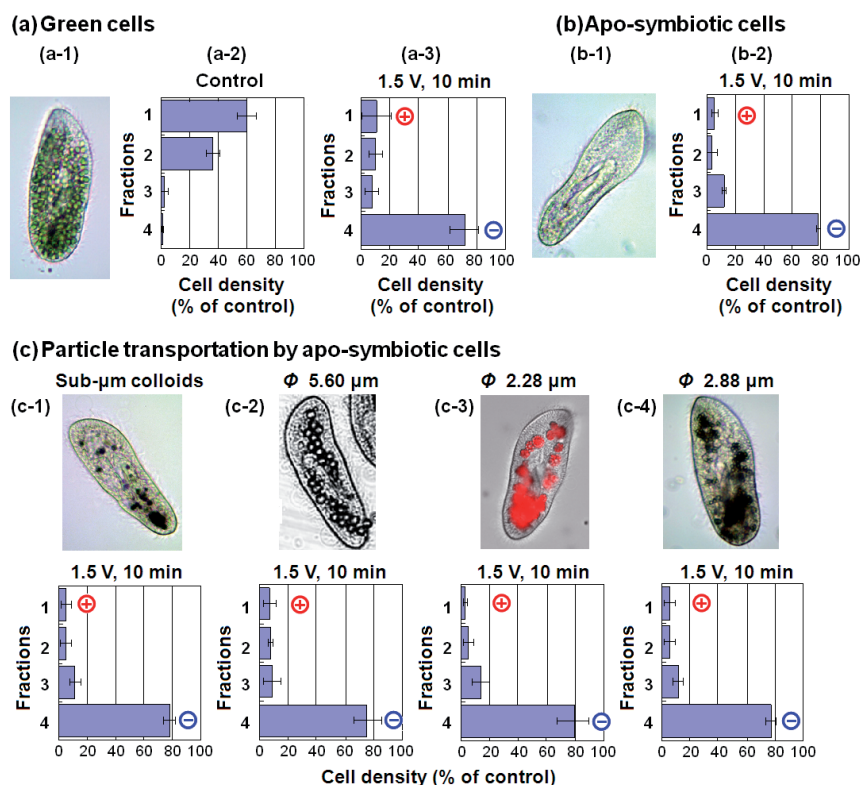


Fig. 4. Particle transportations by particle-fed cells of *P. bursaria* galvanotactically migrating from the cathodic to the anodic end in 60-mm polypropylene tubes. (a) Test with native green cells. (a-1) Microscopic image of a green cell. (a-2) Negative control experiments in which 100 cells were injected at the cathodic end and incubated for 10 min in the absence of an electric stimulus. (a-3) Galvanotactic demonstration under DC at 1.5 V for 10 min. (b) Blank test with particle-free apo-symbiotic cells. (b-1) Microscopic image of an apo-symbiotic cell. (b-2) Galvanotactic demonstration under DC at 1.5 V for 10 min. (c) Test with apo-symbiotic cells fed with various particles. (Top) Visualization of particle uptake enabled in the absence of symbiotic algae. (Bottom) Results of particle transportations by galvanotactically migrating apo-symbiotic cells under DC at 1.5 V for 10 min. The particles introduced into and transported by the apo-symbiotic *P. bursaria* include (c-1) Indian (China) ink which is a mixture of nano-sized colloidal particles, fluorescence-labeled polystyrene beads sized (c-2) 5.60 μ m and (c-3) 2.28 μ m in diameters, and (c-4) magnetically labeled microspheres sized 2.88 μ m. Medium volume in each 1/4 fraction, 47.1 μ l. Error bars, S.D. ($n = 4$).

The INA-1w strain of *P. bursaria* filled up with a massive amount of plastic beads inside its cells also responded to electric stimulation and *ca.* 80% of the cells showed galvanotactic performance (Figs. 4c-2 and c-3), suggesting that around 30 (5.6- μ m beads) or 600 (2.28- μ m beads) particles per cell were transferred upon electric stimulation of the cells. The use of magnetic microspheres (diameter, 2.88 μ m) also resulted in particle aggregation and galvanotactic material transportation. Since magnetic particles can be used to collect magnetic microsphere-fed cells by

specific magnetic devices, recycling of the cells using magnetic microspheres during the designed BioMEMS must be tested in the future research.

Paramecium goes fetching by swimming, eating, and carrying back

The above studies clearly showed that apo-symbiotic cells of *P. bursaria* (INA-1w cells) are capable of particle uptake and cellular locomotion towards the anode, in a controlled traffic platform. However, in the above demonstrations, forced particle uptake and galvanotactic particle

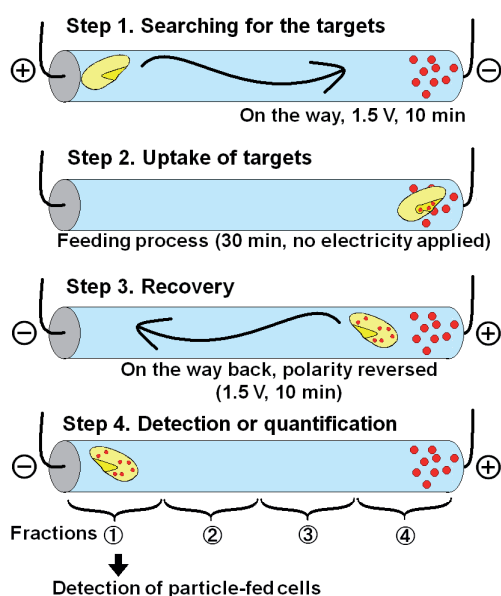
transportation were performed as separate processes. In order to make use of *Paramecium* cells in micro-combinatorial factories as remote-controllable transporters of raw materials or products, the cells have to perform these two different

actions in a sequence according to programmed remote-controlling protocols.

We designed the experiments to prove that cells are capable of sequential actions in the closed system as illustrated in Fig. 5a. To one end of an 120-mm capillary tube filled with *ca.* 340 μ l of fresh medium, 100 cells of apo-symbiotic *P. bursaria* (suspended in 50 μ l of medium) were injected and to the other end a suspension of micro-particles or bacterial culture (10 μ l) was injected. Two electrodes were set at both ends (with the cathode at *P. bursaria* cells and the anode at micro-particles or bacteria) and DC was applied at 1.5 V for 15 min, to allow galvanotactic migration of *Paramecium* cells towards the particles or bacteria present around the anode. After switching off the DC current, uptake of micro-particles or bacteria by paramecia was allowed for 30 min without any application of an electric stimulus. Then the electric polarity was reversed and DC with 1.5 V was applied for another 15 min to allow for *Paramecium*-mediated transportation of micro-particles or bacteria. At the end, the capillary tube was sectioned into 4 fractions and recovery of the *P. bursaria* cells at the original position (fraction 1, Fig. 5a) was assessed by counting the cells under a microscope.

We could observe the high rate of cell recovery at the original position and most of the recovered cells showed uptake of particles (see typical microscopic images, Fig. 5b). The rate of cell recovery is summarized in Table I.

(a) Illustration of fetching processes



(b) A cell carrying magnetic microspheres



(c) A cell showing the presence of EmGFP-expressing *E. coli*

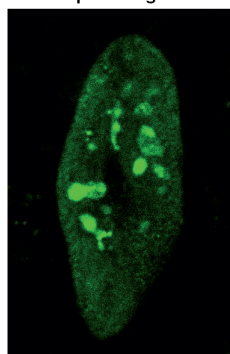


Fig. 5. Sampling of particles of interest by remote-controlled apo-symbiotic *P. bursaria* cells. (a) Design of the experiments. (b, c) Typical images of apo-symbiotic cells showing successful fetching of (b) magnetic microspheres and (c) EmGFP-expressing *E. coli*, after controlled cell migration, active particle (bacterial) uptake, and cell recovery under reversed galvanotactic conditions.

Modification (diversification) of the traffic platform

The above studies showed that cells of *P. bursaria* are capable of migration in horizontally placed straight capillaries (thus, the system was one-dimensional). We further tested the capability of cell locomotion in two- and three-dimensional systems. Figs. 6a–c summarize such attempts.

The route for cell migration inside the 120-mm polypropylene tubes was converted from an one-dimensional (1-D) system to a two-dimensional (2-D) system by gently bending the tubes to 180° to make a round curvature (radius, 20 mm). By this way, the curving route on a horizontal plane was prepared (Fig. 6a). Using this 2-D model, both the cell condensation tests and the cell migration tests were performed. Fig. 6a is showing a typical result of cell condensation test. Through

Table I. Recovery of *P. bursaria* INA-1w cells after galvanotactic migration towards the particles of interest and particle uptake followed by reversal of the electric polarity.

Particles	Cell recovery rate (% of original density)	Confirmation of particle uptake
GFP-labeled <i>E. coli</i>	65	Fluorescence microscope ^a
Magnetic microspheres (diameter, 2.88 μm)	74	Dark spots observed under microscope ^a
Fluorescent beads (diameter, 5.6 μm)	71	Fluorescence microscope
Fluorescent beads (diameter, 2.28 μm)	44	Fluorescence microscope
MgO crystals	61 ^b	See below
TiO ₂ crystals	39 ^b	See below
Zeolite crystals	31 ^b	See below

The cells with particles collected in the anodic fraction (on the way back following switching of the polarity) were counted and expressed as the recovery rates.

^a Typical images are shown in Fig. 5.

^b Since the crystals of catalytic minerals were not labeled with any fluorescent or chromic probe, quantitative analysis of inorganic crystal recovery could hardly be carried out under the microscope. Therefore, the cells recovered in the anodic fraction were counted. However, around one-third of the recovered cells showed the presence of at least one large particle of inorganic crystals suggesting the successful collection of inorganic catalysts.

these attempts, the 2-D capability (on the horizontal plane) of the capillary galvanotactic system was proven.

Then, vertical motions of electrically stimulated *P. bursaria* cells (both upward and downward) within capillaries were tested (Fig. 6b). Moreover, vertical 2-D model with bent tubes (180° curvature with 20 mm radius) placed on the vertical plane was used to prove that electrically stimulated *P. bursaria* cells are capable of continuous actions, namely climbing down followed by climbing up, within the same continuous route (Fig. 6c). By combining the horizontal and vertical models, we could conclude that *P. bursaria* cells are capable of 3-D swimming control by electric stimulation.

In order to design a combinatorial system coupled to cellular action and engineered systems (such as micro-factories), traffics by the electrically stimulated cells must be designed to be controllable. Of course, switching (on, off and reversal) of electricity may be the most likely way of traffic control. However, we must face the situations where diversified pathways are crossing over while electric stimulation is continuously turned on.

We tested the alternative traffic controls of the electrically driving cellular vehicles by designing mechanical channels connecting two to three tubes with stopcocks (Fig. 7). With an I-shaped

stopcock linearly connecting two tubes, galvanotactic cell migration was not blocked when the path was kept open (Fig. 7a, right). As expected, no cells migrated to the anodic fraction when the stopcock was at the close position (Fig. 7a, left). Traffic controls by a T-shaped stopcock allowing connection of three tubes were also performed. This demonstration showed that a right-angled path (90°) branched from a straight route can attract cells when the anode was set at the end of the branching tube (Fig. 7b). Here again, when the stopcock was at stop position, no migration of cells was allowed (data not shown). The data obtained here are all trivial but implying potential uses of tubes and cells connected with such traffic-controlling junctions in diversified sensing and/or transportation systems.

Further traffic controls of electrically stimulated cells

In addition to electric stimuli, light with specific wavelength also attracts or repels the cells of *P. bursaria*. Presently we are studying the traffic controls of the electrically stimulated cell migration by pin-pointed exposure to intense light with specific wavelength (Karaki *et al.*, unpublished results) in addition to mechanical gate-opening controls. Based on our recent study in which the involvement of a T-type calcium channel on the

plasma membrane in *P. bursaria* was revealed (Aonuma *et al.*, 2007), stopped-flow application of calcium chelators which reversibly cease and re-activate the Ca^{2+} -influx-mediated cellular response is now examined in our group (Furukawa

et al., unpublished results). This enables the finely geared neuronal controls of calcium-mediated cellular particle transports through micro-flow controls, even under continuous electric stimulation.

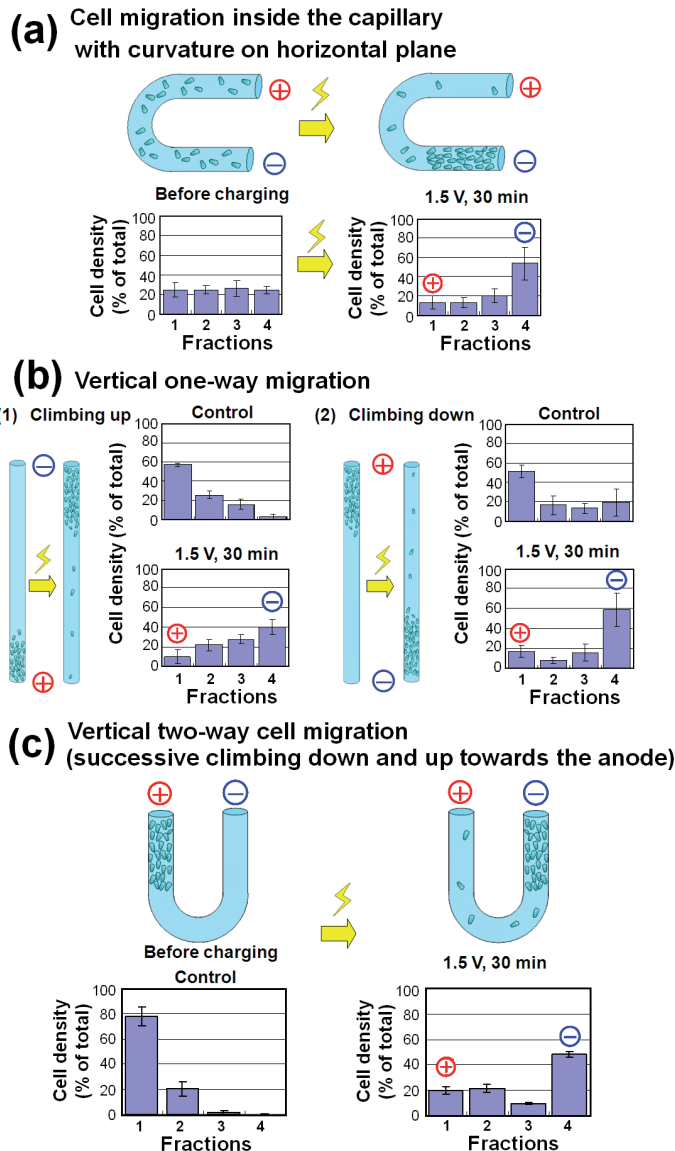


Fig. 6. Demonstration of three-dimensional movements of galvanotactically stimulated *P. bursaria* cells in polypropylene tubes. The route for cell migration inside the 120-mm polypropylene tubes was modified by bending and/or spatially modified orientations (a–c). Medium volume in each 1/4 fraction, $94.2\ \mu\text{l}$ (length, 30 mm; diameter, 2 mm). (a) Effect of 180° curvature, made on a horizontal plane, on galvanotaxis. The radius within the curvature was 20 mm. (b) Effect of vertical orientations on the galvanotactic cell migration. Both upward and downward cell migrations were tested. (c) Effect of 180° curvature, made on a vertical plane, on the galvanotactic cell migration. The radius within the curvature was 20 mm. Error bars, S.D. ($n = 4$).

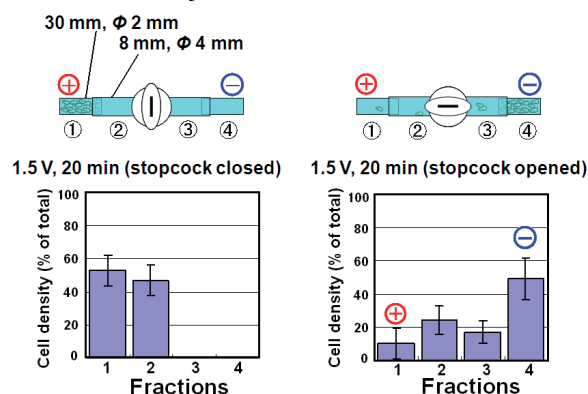
Forced removal of green algae

A single cell of *P. bursaria* naturally harbours several hundreds of endosymbiotic *Chlorella*-like algae in its cytoplasm, and removal of algae from the host organism and reassociation of symbiotic partners can be carried out under laboratory conditions (Kawano *et al.*, 2004). As shown here, the forced removal of green algae is a key step in pre-

paring *P. bursaria* cells with high particle uptake capacity.

To date, several groups have shown that the hosting ciliates and endo-symbiotic algae can be separated from naturally growing *P. bursaria*, and two ex-symbiotic partners can be freely cultured independently (Hosoya *et al.*, 1995; Nishihara *et al.*, 1998; Gerashchenko *et al.*, 2000). The alga-free

(a) Use of I-shaped stopcock for traffic regulation of electrically stimulated cells



(b) Use of T-shaped stopcock for traffic regulation of electrically stimulated cells

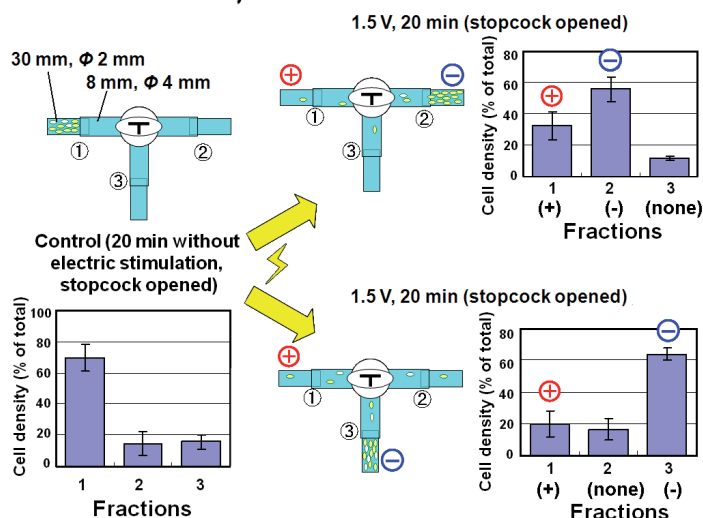


Fig. 7. Traffic controls of the galvanotactically migrating *P. bursaria* cells in polypropylene tubes connected with a stopcock. (a) Traffic control by an I-shaped stopcock. Medium volumes in fractions 1 and 4 were both 94.2 μ l (length, 30 mm; diameter, 2 mm), those in fractions 2 and 3 were both 100.5 μ l (length, 8 mm; diameter, 4 mm). (b) Traffic control by a T-shaped stopcock. Fraction volume, 94.2 μ l (length, 30 mm; diameter, 2 mm) + 100.5 μ l (length, 8 mm; diameter, 4 mm) = 194.7 μ l. Error bars, S.D. ($n = 4$).

cell strains of *P. bursaria* can be readily produced by treating the stocks of green paramecia with photosynthesis-related herbicides such as DCMU (Reisser, 1976) and paraquat (Tanaka *et al.*, 2002). Treatment with the protein synthesis inhibitor cyclohexamide also results in the removal of algae from the host cells (Weis, 1984). The generation of such apo-symbiotic “green paramecium” through natural process (without using any chemical) can be reproducible using a classical protocol in which the green paramecia are maintained and propagated in continuous darkness with excessive supplementation of food bacteria (Siegel, 1960). In addition, isolation of an apo-symbiotic *P. bursaria* strain lacking algae from natural water environments has been reported (Tonooka and Watanabe, 2002).

Although we employed the protocol of Tanaka *et al.* (2002) here, other protocols described above may be available for preparing the high capacity cellular vehicles from *P. bursaria*.

Lastly, we would like to summarize our present work. In the present study, we demonstrated that modified cells of *P. bursaria* can be used as remote-controllable micro-machinery allowing the transport of micro-particles in the complex networks

of capillary tubes. The particles transported by electrically controlled cells include re-introduced green algae (sized *ca.* 3–6 μm), fluorescence-labeled polystyrene microspheres (diameters, 5.6 and 2.28 μm), magnetic microspheres (diameter, 2.88 μm), EmGFP-expressing *E. coli* (*ca.* 2 mm long), and Indian ink (nano-sized or submicro-sized colloidal particles). Presently we are testing the transport of catalytically active particles such as size-controlled zeolite and other crystals of metal oxides using electrically remote-controlled *P. bursaria* cells (preliminary data were presented in Table I). Since the above demonstrations were successful, we conclude that *P. bursaria* has a potential to be used as one of the micro-biorobotic devices in BioMEMS or combinatorial chemistry.

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