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Control of Flagellar Motor Using a Real-time Local Environment Chemical Stimulation System

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***Abstract*—Single cell analysis has attracted much attention to reveal the localized biological information in detail. Local environmental control technique is developed to analyze the localized detail properties of single cells. In this paper, we propose the local environmental chemical stimulation system with micro dual pipettes to stimuli the local reagent concentration dynamically and automatically. Local environmental chemical stimulation by dual pipettes is applied to the rotational speed control of bacterial flagellar motor, which is a rotary molecular machine. Here, we show quick response and rotational speed control of Na^+-driven flagellar motor in both accelerating and relaxing directions was demonstrated by automatic switching the local spout between Na^+-containing and Na^+-free solutions with dual pipettes. It was shown that the rotational speed could be maintained by automatic controlling the spouting velocity of Na^+-containing and Na^+-free solution with multiplying the applied DC voltage. Furthermore, it was confirmed that by applying the P-control, rotational speed of flagellar motor could be controlled more stably.**

I. Introduction

Single cell analysis has attracted much attention to reveal the detailed and localized biological information, which is not obtained by the conventional group cell analysis due to the statistical assay [1], [2], on individual cells [3], [4]. To analyze the detailed and localized properties of single cells, local environmental control technique, which is the technique to change and sense the local environment around single cells, is effective. The probe-type devices, such as pipettes, can be arbitrarily positioned by micro/nano manipulators [5]. And there is a possibility that the local environment can be controlled and measured with submicrometer-scale spatial resolution by the use of micro/nano-probes [6], [7]. Therefore, local environmental control technique with micropipettes, which are pipettes with a micrometer scale ejection/injection hole, is expected to get more detailed and localized information on single cells [8].

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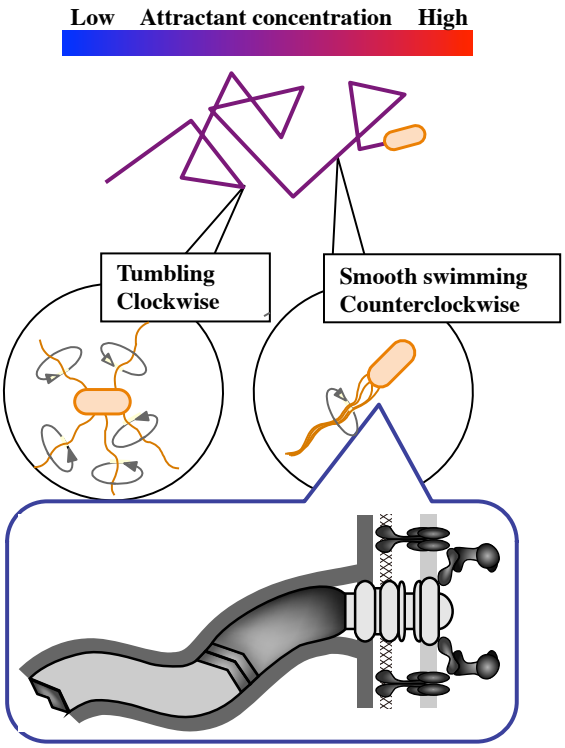

Fig. 1. Schematic of the swimming bacterial behavior and flagellar motor.

Many bacteria can swim in liquid by rotating their flagella and migrate toward favorable directions by using different two swimming mode (Fig. 1). The bacterial flagellum has a helical filament that works as a propeller. Each flagellum consists of the helical filament extending from the cell body, the basal body embedded in the cell surface, and the flexible hook that connects them [9]–[12]. More than 20 structural proteins are required for this motor (see Fig. 1 underlying schematics). The bacterial flagellar motor is a molecular machine that converts ion-motive force (IMF) into mechanical force; the energy source of the rotation is the electrochemical gradient of H^+ or Na^+ ion across the cytoplasmic membrane. The stator of the flagellar motor consists of PomA and PomB in the Na^+-driven motors of *Vibrio alginolyticus*, (*V. alginolyticus*) or MotA and MotB in the H^+-driven motors of *Escherichia coli* (*E. coli*) [13]–[15]. These stator complexes are thought to function as

specific ion channels. Furthermore, it is already revealed that chimeric protein PotB (the C-terminal domain of B subunit of *V. alginolyticus* was exchanged by *E. coli*) act as the Na^+-driven type. Detailed single cell analysis revealed the step size of the flagellar motor rotation [16]. However, the mechanism of the flagellar motor rotation is still largely unknown. Therefore, new approach or method has been desired, and the local environmental stimulation with micropipettes can be a new approach for this issue.

In fact, Piper *et al.* used a glass nanopipette for local and repeatable delivery of water-soluble reagent in ionic solution to make localized controlled changes in reagent concentration on a surface [17]. Then they applied their method to control the Na^+-driven flagellar motor in single *E. coli* cell by dosing Na^+ ions. Observing the dynamic response of flagellar motor like this will make a great contribution to clarify the mechanism of the flagellar motor rotation. However, their system is not enough to make an intended dynamic change in reagent concentration. When they dose Na^+ ions to accelerate the rotational speed of Na^+-driven flagellar motor, they can only wait for diffusion of Na^+ ions to relax the rotational speed. If both drastic increase and decrease in Na^+ ion concentration can be generated, it will be possible to observe the flagellar dynamic response independent from the influence of the Na^+ ion diffusion. Therefore, the method to stimuli the local reagent concentration more dynamically is desired for more detailed analysis of the mechanism of the flagellar motor rotation. From this viewpoint, Nogawa *et al.* suggested dual pipettes system, which realize dynamic and arbitrary changes in local reagent concentration [18]. However this suggested system was regulated by manual control and time resolution is video rate (30 fps). Furthermore, analysis has done with offline calculation. Therefore this system could not achieve online control. These specifications are not enough to analyze detail phenomenon of biology.

In this paper, we propose the improved local environmental chemical stimulation system with micro dual pipettes to produce the dynamically and automatically change in local reagent concentration. We developed new system that integrates dual pipettes system and high speed camera system as shown Fig. 2. In this system, the spouting amount from the pipette is regulated by the rotation measurement information from high speed camera and automatically controlled by computer with D/A board. The rotational speed of the Na^+-driven flagellar motor in single *E. coli* cell is automatically controlled in both accelerating and relaxing directions by switching the local spout between Na^+-containing and Na^+-free solutions with dual pipettes.

II. Materials and Methods

A. Fabrication of Glass Micropipette

Glass micropipettes are fabricated by heating and pulling the borosilicate glass tubes (outer/inner diameters are each 1 mm/0.6 mm; GD-1, Narishige, Japan) with the puller (PC-10, Narishige, Japan). Its diameter of the tip can be controlled by the heating power, load, pulling velocity, etc. In this paper, glass micropipettes with a ~1 μm inner diameter were used.

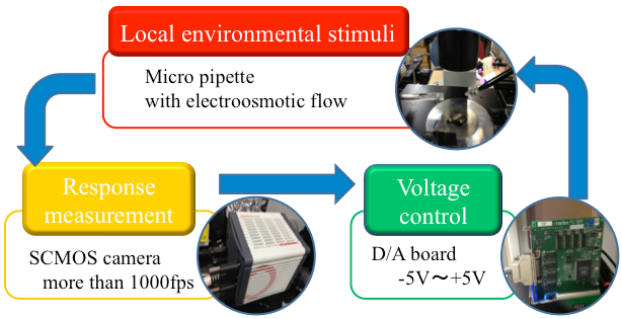

Fig. 2. Schematic of the real-time local environment stimulation System.

B. Bacterial Strains

In this paper, *E. coli* mutant strain, which has Na^+-driven chimeric flagellar motor, was used for the experiment of flagellar rotational speed control. *E. coli* mutant strain YS34 (ΔcheY, fliC::Tn10, ΔpilA, ΔmotAmotB) was transformed with plasmids pYS11 (fliC sticky filaments, ampicillin (Amp) resistance) and pYS13 (pomApotB, isopropyl-β-Dthiogalactoside (IPTG) inducible, chloramphenicol (Cm) resistance) [16]. Cell samples were prepared in the following procedure and cultured for experiment. First, cells were grown overnight at 30 ˚C with shaking in LB medium (1% (in weight/volume) bacto tryptone, 0.5% (in weight/volume) yeast extract, and 0.5% (in weight/volume) NaCl) containing the appropriate antibiotics (Cm and Amp). The overnight culture in LB medium was inoculated into TG medium (1% (in weight/volume) bacto tryptone, 0.5% (in weight/volume) NaCl, and 0.5% (in weight/volume) Grycerol) containing the appropriate antibiotics and the inducer (Cm, Amp, and IPTG) at a tenfold dilution and grown at 30 ˚C for 3 h. Then, cells were centrifuged by centrifuge (1130, Kubota, Japan) in a centrifuging tube at 6000 rpm/min for 5 min. The sedimented cells were suspended in 1.5 mL Na+-motility medium (10 mM potassium phosphate (pH 7.0), 0.1 mM ethylenediaminetetraacetic acid (EDTA), and 85 mM NaCl), Na+-containing solution.

C. Experimental Set Up for Rotational Speed Measurement of Flagellar Motor

In this paper, tethered cells were used for the experiment of the rotational speed control of the Na^+-driven flagellar motor. Generally, it is difficult to directly observe and measure the rotational speed of the flagellum under the optical microscope because of its ultrathin filament and high-speed rotation. “Tethered cells” mean the cells whose flagella are attached onto a substrate [19]. When the flagella of a cell are attached onto a substrate, cell bodies rotate as a result. Thus, in these experiments, tethered cells are used to observe rotational speed of flagellum by observing rotational speed of the cell body. YS34/pYS11/pYS13 used in this paper is not only Na^+-driven type, but it is suitable for making tethered cells because of the special flagellar filament called “sticky filament,” which easily sticks to a substrate. Its flagella rotate only in the counterclockwise direction by

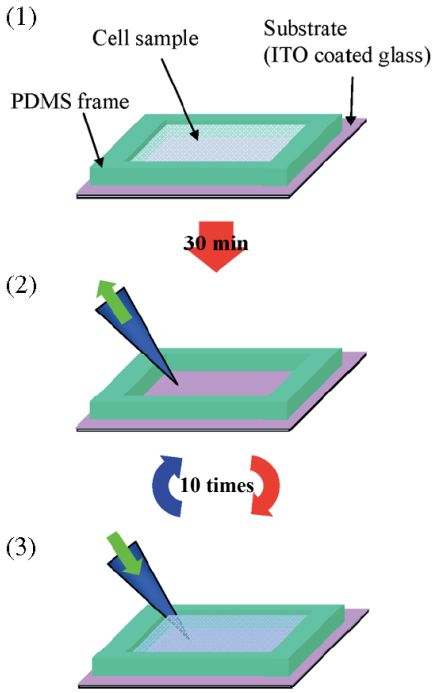

Fig. 3. Schematic of the preparation of the tethered cells. (1) Dropping the solution containing cells onto indium tin oxide (ITO) coated glass. (2) Pumping out the solution to remove extra cells and Na^+ . (3) Replacing the solution to Na^+ -free solution.

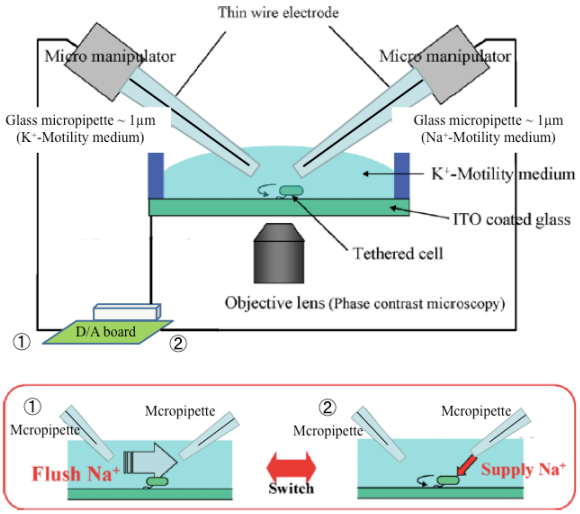

Fig. 4. Schematic of local environmental stimulation system with micro dual pipettes for rotational speed control of Na^+ -driven flagellar motor.

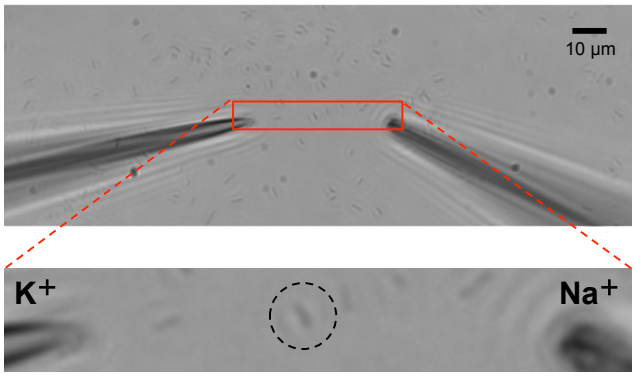

Fig.5. Optical microscopy image of actual experiment for rotation measurement

deletion of cheY gene, though flagella generally change the rotational direction between clockwise and counterclockwise under CheY protein regulation in response to the surroundings. And the plane of rotation with its tethered cell is approximately parallel to the substrate. Therefore, YS34/pYS11/pYS13 is suitable for the measurement of the rotational speed. The tethered cells were prepared in the following procedure, as shown in Fig. 3.

1) Polydimethylsiloxane (PDMS) frame was set onto the substrate. Cell sample was poured into the bath. While it was left for 30 min, cells attached their flagella onto the substrate.

2) The solution was pumped out to remove the swimming cells, which did not attach their flagella onto the substrate.

3) The solution was replaced by K^+-motility medium (10 mM potassium phosphate (pH 7.0), 0.1 mM EDTA, and 85 mM KCl), Na^+-free solution, to remove the Na^+ ions in the bath.

4) After waiting for 1 min to stabilize the cells and the solution, steps 2) and 3) are repeated (total of ten times). (Finally, to distinguish the rotatable cells from the cells attached to the substrate, Na+ ions were added into the bath solution in extremely small amount.)

In this paper, cell bodies of *E. coli* were observed by phase contrast microscopy with the inverted optical microscope IX73 (Olympus, Japan) and the objective lens LUCPLFLN 40×PH (Olympus, Japan). By phase contrast microscopy, which applies the phase difference, the living cells can be observed with high contrast, and then cell bodies of *E. coli* look like the small black ellipses.

D. Real-time Rotational Speed Measurement by High speed camera

To obtain high time resolution data of the rotational speed, we placed SCMOS camera, which can be acquired image at more than 1000 fps (ORCA-Flash4.0, Hamamatsu photonics, japan), on microscope. Then, by creating the image acquisition program, we constructed a high-speed image acquisition environment. We performed the construction of the observation program for flagellar motor rotation that can measure rotational speed of tethered cell in real time. The living cells can be observed with high contrast by phase contrast microscopy, and then cell bodies of *E. coli* look like the small black ellipses. Therefore, after making the

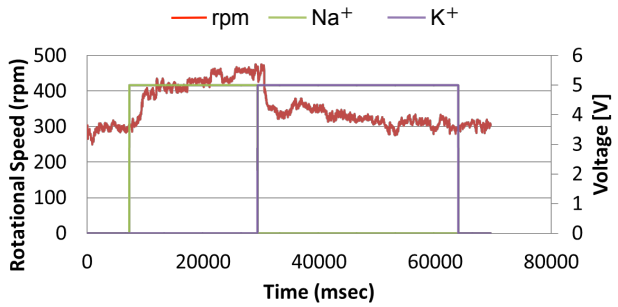

Fig. 6. Experimental results of step stimulation

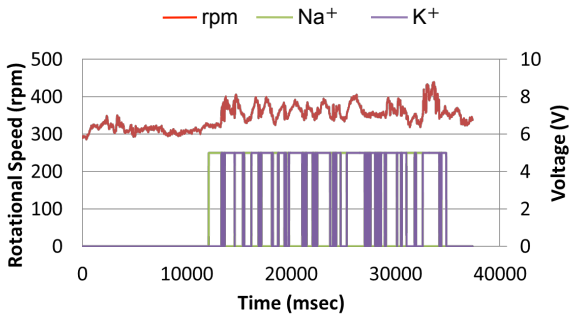

Fig. 7. Control of the rotation speed of flagellar motor by the local environmental stimulation system (target value: 350 rpm, on/off control)

acquisition of images at 100 fps, we measured the rotational speed by binarizing the image and track the position of the center of gravity. After that, in order to make clear the effect of environmental change, the experimental data were processed by the Chung-Kennedy filter [20] to filter out the fluctuation of the rotational speed in the 360° rotation. These calculation was achieved within 10 msec including image acquisition.

E. Configuration of Dual Pipettes System

When the spout of Na+-containing solution is conducted with only single pipette as Piper *et al.* did, diffusion of Na^+ ions is the only way to reduce the Na^+ ion concentration. In addition, even if the solution is not being spouted, Na^+ ions diffuse through the pipette–bath interface and are unintentionally supplied to the flagellar motor. In this case, the response of the Na^+-driven flagellar motor in the rotational speed is affected by the diffusion rate. In fact, we confirmed that the flagellar motor rotation was affected by diffusion of Na^+ ions in the rotational speed control experiment with a single pipette (data not shown).

Therefore, for more detailed analysis of the response of the Na^+-driven flagellar motor by intentional and rapid reduction of the Na+ ion concentration, we constructed the local environmental chemical stimulation system with micro dual pipettes, as same as Nogawa *et al.* As shown Fig. 4, this system has two pipettes. The glass micropipettes with a ~1 μm inner diameter were used for the spout of Na^+-motility medium, the Na^+-containing solution, and for the spout of K^+-motility medium, the Na^+-free solution. The bath was filled with the K^+-motility medium. The DC voltage was applied between indium tin oxide (ITO; it is a transparent conductive material.) coated glass at the bottom of the bath and the thin wire electrodes inserted into the pipettes.

We can control the spout both Na^+-containing and Na^+-free solutions independently by regulating the applied voltage from D/A board (PCI-3329, Interface Japan, japan) in computer. Through this dual pipettes system, Na^+ ions are supplied to the flagellar motor on the arbitrary single cell by spout from the micropipette. Then the Na^+ ions diffusing from the Na^+-micropipette and Na^+ ions remaining around the cell can be flushed to immediately stop the feeding of Na^+ ions to the flagellar motor by spout from the K^+-micropipette.

These pipettes are fixed on the two micromanipulators, respectively. So, it is possible to approach the pipettes to the arbitrary single cell, and the cell–pipette distances can be controlled independently.

F. Spout From Micropipette

Generally, with a small-scale channel, it is difficult to pass and spurt the fluid by pressure because the influence of frictional force is significant [21]. To overcome such a difficulty, we have spouted the fluids from the pipettes with electric migration forces by applying DC voltage between the solution in the pipette and the solution in the bath [22]. This method is suitable for the spout from small-scale channel in which the surface force is dominant, because the electric migration forces affect individual charged particle. With this method, the spouting volume from the pipettes increases with multiplying the applied DC voltage. Furthermore, the response of spout is very fast because of direct manipulation by an electric field. For all these reasons, in this paper, we also employed the method of spouting the fluids by applying the DC voltage.

G. Integration of Dual Pipettes System and High speed camera system

We developed a system that integrates dual pipettes system and high speed camera system. In this system, the spouting amount from the pipette is regulated by the rotation measurement information from high speed camera. Therefore, rotational speeds of *E.coli* cell can be controlled depend on feedback information from high speed camera. It is possible to perform a 10 msec cycle (including image acquisition and calculation) stimulation control by using this system.

III. Rotational Speed Control of Flagellar Motor by Real-time Local Environment Stimulation System

A. Experimental Results of Step Stimulation

First, we conducted the control experiment, which was done with filling Na^+-free solution into the both pipettes. It was confirmed that there was no change on the rotational speed by applying the DC voltage at 5 V (data not shown).

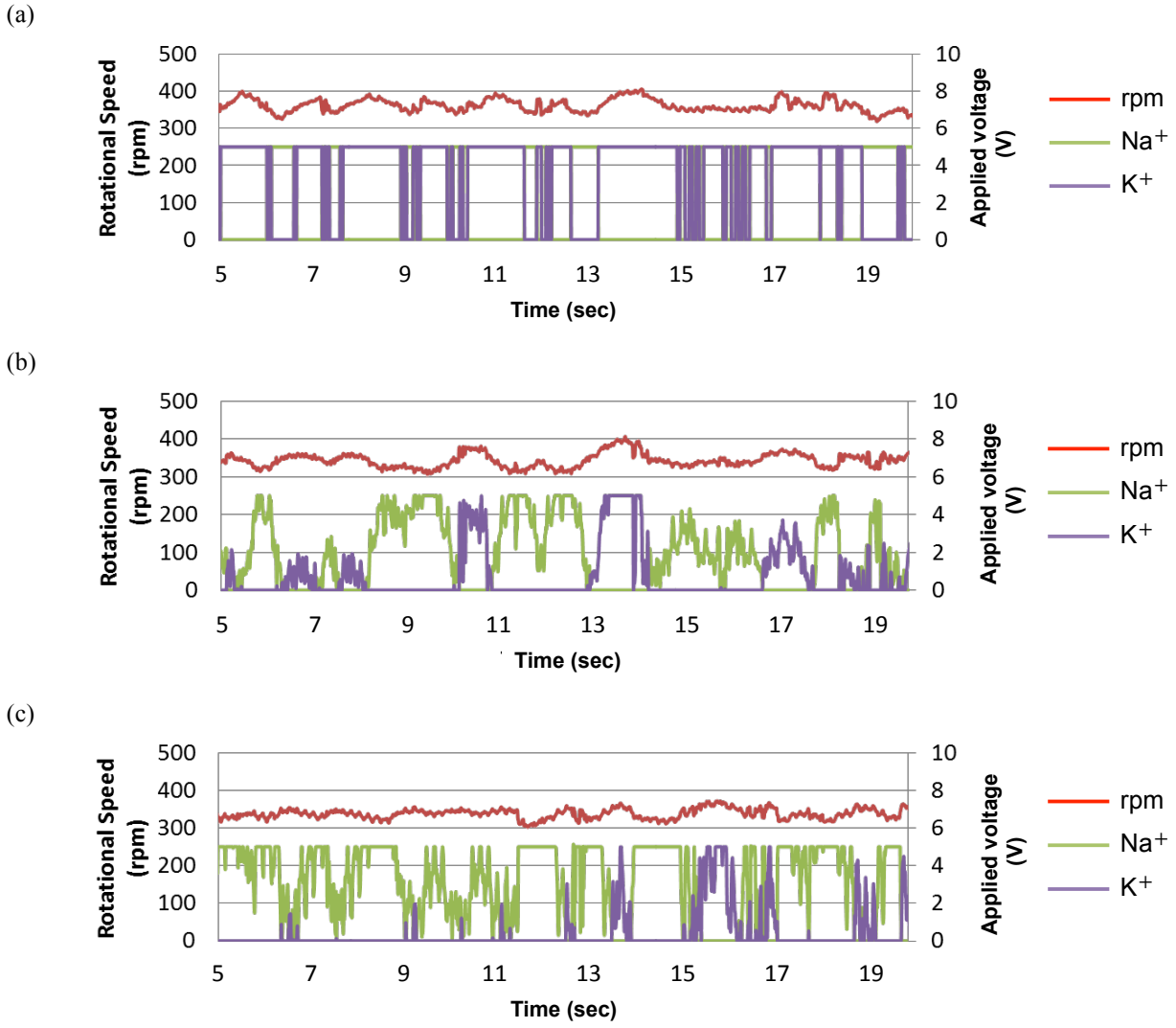

Fig. 8 Control of the rotation speed of flagellar motor by the local environmental stimulation system (target value: 350 rpm). (a) on/off control, (b) P control [Kp: 0.16], (c) P control [Kp: 0.32]

Fig. 5 shows actual experimental picture by optical microscopy. As shown this figure, so many tethered *E. coli* cell were found and we set dual pipette around target cell. Fig. 6 shows the experimental result of the rotational speed control of Na^+-driven flagellar motor in single *E. coli* cell by dual pipettes at 5 V. In order to make clear the effect of switching the solutions, the experimental data were processed by filter to filter out the fluctuation of the rotational speed in the 360° rotation.

Fig. 6 is the time-series data of the rotational speed of single cell body with switching the spout between dual pipettes. The green line in Fig. 6 represents the terms of applied voltage for which the Na^+-motility medium was spouted from the micropipette. The purple line represents the terms of applied voltage for K^+-motility medium. From this result, it was shown that the rotational speed of Na^+-driven flagellar motor was iteratively increased/decreased in a near-pulse shape, by switching the local spout between Na^+-containing and Na^+-free solutions with dual pipettes. By flushing, the rotational speeds of Na^+-driven flagellar motor were immediately relaxed after 3 s of switching. Furthermore, the rotational speeds of Na^+-driven flagellar motor were completely relaxed (as same as base speed) after 20 sec of flushing by the applied DC voltages to Na^+-free solutions. This result shows that the automated local environmental chemical stimulation system with dual pipettes considerably improved the controlling performance in local reagent concentration as same as previous manual system [18]. This local environmental chemical stimulation system with dual pipettes might be effective to clarify the mechanism of the flagellar motor rotation, which is largely unknown.

B. Experimental Results of Auto speed control with feedback data

Figs. 7 and 8 show the experimental results of the rotational speed control of Na^+-driven flagellar motor in single *E. coli* cell. We measured the same cell in Fig. 6. In this experiment, we tried to maintain rotational speed at 350

rpm. Fig. 7 shows the time-series data of the rotational speed of the single cell body controlled by on/off regulation. In this control, controller switch the out put voltage Na+-containing to Na+-free solution, when rotational speed exceeds 350 rpm. On the other hand, controller switch the out put voltage Na+-free to Na+-containing solution, when the rotational speed become less than 350 rpm. From this result, we confirm that rotational speed was kept around 350 rpm automatically. However, these values are fluctuated. To improve these fluctuations, we applied P-control for controlling spout amount from micropipette.

Fig. 8 shows comparison data of (a) on/off control, (b) P control [Kp: 0.16], and (c) P control [Kp: 0.32]. These data are the results of 5 sec after the stimulus. We calculate parameter value of P-control from step stimulation result and typical data of P control are shown in Fig. 8. Compare with on/off control and P control [Kp: 0.32], fluctuation was improved. Average of on/off control is 362 rpm and dispersion is 301 rpm, on the other hand, average of P control [Kp: 0.32] is 338 rpm and dispersion is 174 rpm. These results show that the rotational speed of Na^+-driven flagellar motor was controllable by controlling the spouting volume or velocity of Na^+-containing solution with changing the applied DC voltage.

IV. Conclusion

In this paper, we proposed the improved local environmental chemical stimulation system with micro dual pipettes and high speed camera, and realized to control the local reagent concentration dynamically and automatically. Then, local environmental chemical stimulation by dual pipettes was applied to the rotational speed control of bacterial flagellar motor, which is a rotary molecular machine. Quick response and rotational speed control of Na^+-driven flagellar motor in both accelerating and relaxing directions was demonstrated by switching the local spout between Na^+-containing and Na^+-free solutions with dual pipettes. Then it was shown that the rotational speed could be maintained automatically by controlling the spouting velocity of Na^+-containing and Na^+-free solution with multiplying the applied DC voltage. Furthermore, it was confirmed that by applying the P-control, rotational speed of flagellar motor could be controlled more stably. This local environmental chemical stimulation system with dual pipettes will make a great contribution to clarify the mechanism of the flagellar motor rotation through getting the information on the dynamic response. Therefore, we will improve time resolution of our system as future work.

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