2010 IEEE International Conference on Robotics and Automation
Anchorage Convention District
May 3-8, 2010, Anchorage, Alaska, USA

Manipulation of Flagellar Driving Force by Local Environmental Control System with Multiple Nanoprobes

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***Abstract*—Micro/nano robots have been actively studied toward the realization. Now, the micro living organisms have been used as the driving forces for the micro objects. To achieve the fine manipulation of bio driven micro objects, the manipulation methods have to be established. Therefore, the local environmental control technique is desired to manipulate a single cell and to analyze the detailed properties. We have developed the local environmental control system with nano/micro dual pipettes, and achieved the quick-response and iterative rotational speed manipulation of Na^+-driven flagellar motor, which is a rotary molecular machine, by switching the local spout between Na^+-containing and Na^+-free solutions with dual pipettes. In this paper, we upgrade our local environmental control system with nano/micro dual pipettes to achieve the quantitative and long-time-stable manipulation of the rotational speed of the Na^+-driven flagellar motor. We demonstrate fine and long-time-stable rotational speed manipulation of Na^+-driven flagellar motor by simultaneous local spouts of Na^+-containing and Na^+-free solutions using dual pipettes with controlling the spouting velocities independently. And, as the driving force, the rotational torque generated by the flagellar motor is estimated at the range from ~2.3×10^3 to ~2.8×10^3 pN· nm.**

I. Introduction

MICRO/NANO robots have been actively studied toward the realization. With the micro/nano robots, it is expected to lead the developments of novel technologies such as micro/nano surgery inside the human body [1]. The size reduction of whole robotic systems, toward the micro/nano-meter scale, is hindered by a problem of the size reductions of driving force and its energy source. To overcome such a problem, recently, the biomolecular motors and the micro living organisms have been used as the driving forces for the micro objects [1-6]. Their energy sources are chemical phenomena. So, the size of the whole robotic system can be reduced. To utilize these bio driving forces, firstly, it is needed to establish the methods to analyze the detailed properties and manipulate the bio driving forces.

Single cell analysis has attracted much attention to reveal the detailed biological properties, which is not obtained by the conventional group cell analysis due to the statistical assay [7,8], on individual cells [9,10]. To analyze the detailed 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 depending on the operations by use as end-effecters of micro/nano manipulators [11]. And, there is a possibility that the local environment can be controlled and measured with submicro- to nano-meter scale spatial resolution by use of nanoprobes [12,13]. Therefore, local environmental control technique with nanopipettes, which are pipettes with a nanometer-scale ejection/injection hole, is expected to get more detailed properties of single cells [14]. And, it might be useful to manipulate the single cells by controlling the local chemical reagent concentrations.

We have developed the local environmental control system with nano/micro dual pipettes, and achieved to produce more arbitrary and dynamic changes in local reagent concentration (Fig. 1(a)) [15]. In addition, we applied the dual pipettes system to manipulate the rotational speed of Na^+-driven bacterial flagellar motor dynamically and arbitrarily in single *Escherichia coli* cell. Quick-response and iterative rotational speed manipulation of Na^+-driven flagellar motor in both accelerating and relaxing directions was demonstrated by changing the local Na^+ concentration with switching the local spout between Na^+-containing and Na^+-free solutions (Fig. 1(b)). And, it was shown that the rotational speed might be controllable by changing the spouting velocity of Na^+-containing solution with multiplying the applied DC voltage (Fig. 1(c)).

However, when the spout is continued for a long period, the rotational speeds of flagellar motor get into a certain level at any applied DC voltages. It might be caused by the equilibrium between diffusion and supply of Na^+ ions.

Therefore, in this paper, we upgrade our local environmental control system with nano/micro dual pipettes to achieve the quantitative and long-time-stable manipulation of the rotational speed of the Na^+-driven flagellar motor in single *E. coli* cell. Then, it is confirmed by the experiments, and the driving force, the rotational torque, generated by the flagellar motor is estimated.

Manuscript received February 8, 2010. This work was partially supported by MEXT KAKENHI (Grant-in-Aid for Scientific Research on Priority Areas 17076004 and Grant-in-Aid for JSPS Fellows 20056351).

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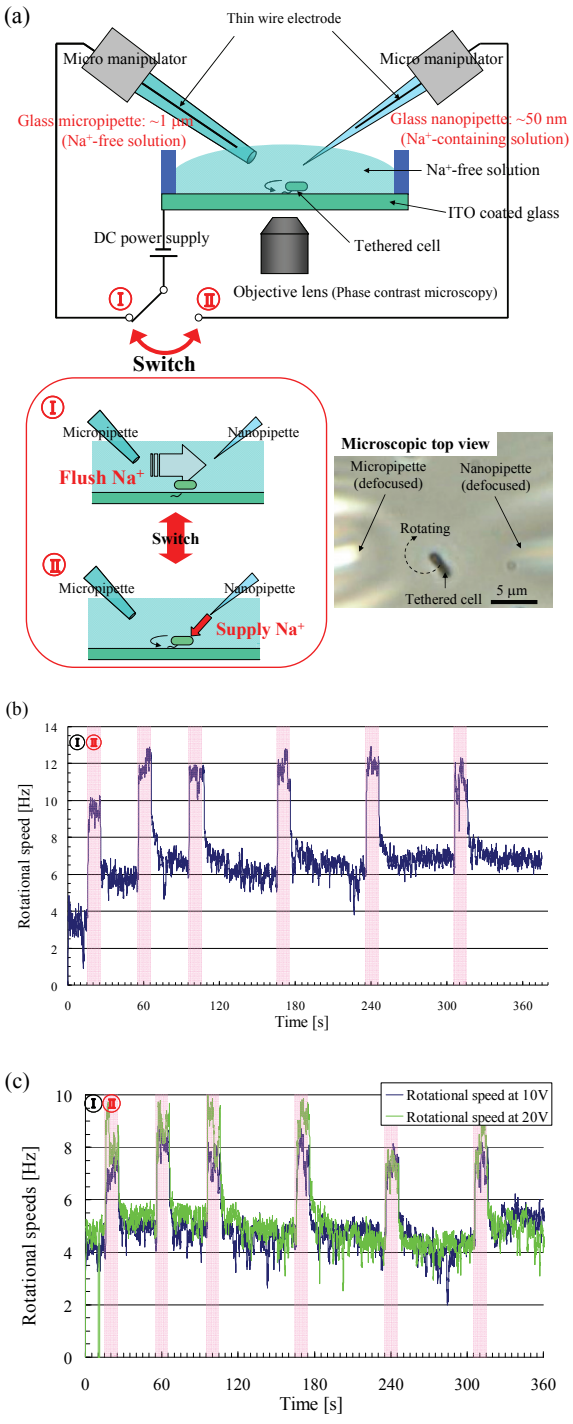

Fig. 1. (a) Schematic and experimental appearance of the local environmental control system with nano/micro dual pipettes for the rotational speed manipulation of Na^+-driven flagellar motor. (b), (c) Experimental result of rotational speed manipulation of Na^+-driven flagellar motor (b) with switching the spout between the Na^+-containing and Na^+-free solutions (c) with multiplying the applied DC voltages, at 10 V and 20 V. I; The terms for which the Na^+-free solution was spouted from the micropipette. II; The terms for which the Na^+-containing solution was spouted from the nanopipette.

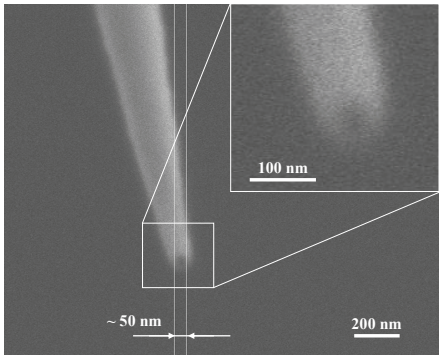

Fig. 2. Fabricated glass nanopipette (SEM image). The upper-right inset is the magnified image of the tip.

II. Materials and Methods

A. Glass Nanopipette

Glass nanopipettes 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 (P-2000, Sutter Instrument, USA). Its diameter of the tip can be controlled by the heating power, load, pulling velocity, etc. Generally, the minimum inner diameter of the tip of the glass nanopipette is ~10 nm [14].

In this paper, glass nanopipettes with a ~50 nm inner diameter (Fig. 2) and glass micropipettes with a ~1 μm inner diameter were used.

B. Spout from Nanopipette

Generally, with a nano-scale channel, it is difficult to pass and spurt the fluid by pressure, because the influence of frictional force is significant [16]. To overcome such a difficulty, we have spouted the fluids from the nanopipettes with electric migration forces by applying DC voltage between the solution in the nanopipette and the solution in the bath [17]. This method is suitable for the spout from nano-meter scale channel in which the surface force is dominant, because the electric migration forces affect individual charged particle. With this method, the spouting velocity/volume from the pipettes increases with multiplying the applied DC voltage. For all of these reasons, in this paper, we also employed this method of spouting the fluids by applying the DC voltage.

C. Bacterial Flagellar motor

Bacterial flagellum is the locomotive organ in liquid. Each flagellum consists of the helical filament that acts as a propeller extending from the cell body, the basal body embedded in the cell surface, and the flexible hook that connects them [18-21]. More than 20 structural proteins are required for this organelle (Fig. 3). The bacterial flagellar motor is a molecular machine that converts ion-motive force into mechanical force; the energy source of the rotation is the electrochemical gradient of H^+ or Na^+ ions across the

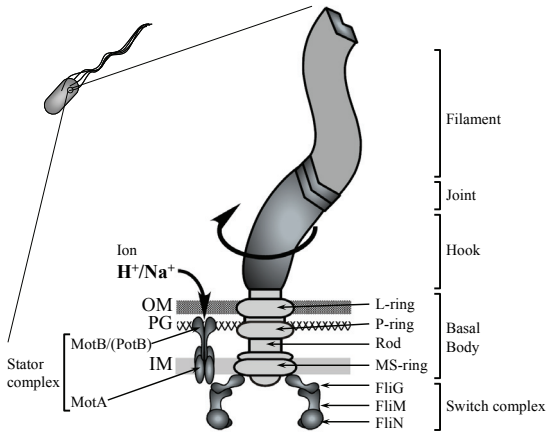

Fig. 3. Schematic of the bacterial flagellum. The substructures and components of the flagellum of *E. coli*. The heavily shaded components are essential components for torque generation and switching (MotA, MotB / (PotB), FliG, FliM and FliN). H^+ / Na^+ influx through the stator complex is believed to generate torque at the interface between the stator component of MotA and the rotor component of FliG. OM; Outer membrane, PG; Peptidoglycan, IM; Inner membrane.

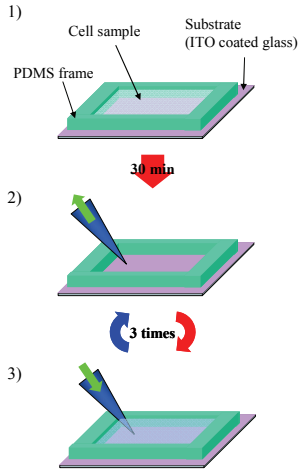

Fig. 4. Schematic of the preparation of the tethered cells. 1) Dropping the solution containing cells onto ITO coated glass. 2) Pumping out the solution to remove extra cells and Na^+. 3) Replacing the solution to Na^+-free solution.

cytoplasmic membrane. The stator of the flagellar motor consists of PomA and PomB in the Na^+-driven motors of *Vibrio alginolyticus*, or MotA and MotB in the H^+-driven motors of *E. coli* [22,23]. These stator complexes are thought to function as specific ion channels. The chimeric protein PotB used in this paper (the C-terminal domain of B subunit of *V. alginolyticus* was exchanged by *E. coli*) acts as the Na^+-driven type [24, 25].

D. Bacterial Strain

In this paper, *E. coli* which has Na^+-driven chimeric flagellar motor was used for the experiment of flagellar rotational speed manipulation. *E. coli* 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) [25].

Cell samples were cultured and prepared in the following procedure. Firstly, cells were grown overnight at 30℃ with shaking in LB medium (1 %(w/v) Bacto tryptone, 0.5 %(w/v) Yeast extract, 0.5 %(w/v) NaCl) containing the appropriate antibiotics (Amp and Cm). The overnight culture in LB medium was inoculated into TG medium (1 %(w/v) Bacto tryptone, 0.5 %(w/v) NaCl, 0.5 %(w/v) Grycerol) containing the appropriate antibiotics and inducer (Amp, Cm and IPTG) at a 10-fold dilution and grown at 30℃ for 3 hours. Then, cells were centrifuged by centrifuge (1130, Kubota, Japan) in a centrifuging tube at 6000 r.p.m. for 5 minutes. The sedimented cells were suspended in 1.5 ml Na^+-motility medium (10 mM Potassium phosphate (pH 7.0), 0.1 mM EDTA, 85 mM NaCl), Na^+-containing solution.

E. Rotational Speed Measurement of Flagellar Motor

In this paper, tethered cells were used for the experiment of the rotational speed manipulation 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” means the cells whose flagella are attached onto a substrate [26]. When cells rotate the flagella with attaching onto a substrate, as a result, cell bodies rotate. That is, tethered cell is the system with which rotational speed of the flagellum can be easily observed as the rotational speed of the cell body.

YS34/pYS11/pYS13 used in this paper is not only Na^+-driven type. 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 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. 4.

1) PDMS (Polydimethylsiloxane) frame was set onto the substrate. Cell sample was poured into the bath. While it was left for 30 minutes, 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 to K^+-motility medium (10 mM Potassium phosphate (pH 7.0), 0.1 mM EDTA, 85

mM KCl), Na^+-free solution, to remove the Na^+ ions in the bath.

4) After waiting for 1 minute to stabilize the cells and the solution, steps 2) and 3) are repeated (total of 3 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 IX71 (Olympus, Japan) and the objective lens LUCPLFLN 60×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.

The rotational speed of the cell body is measured by image processing. Firstly, experimental data was binarized to pick out the cell body as a black ellipse from white background. The centers of the cell body in each frame of the video (29.97 fps) were calculated. The rotational center was determined by circle approximation of the trajectory of the center of the cell body. Then, the angles of the cell body in each frame were determined. Finally, the rotational speeds were calculated frame by frame.

F. Torque Estimation of Flagellar Motor

In this paper, the rotational torque generated by the flagellar motor was estimated from the rotational speed of the cell body. The rotating cell body was simplified as the rotating cylinder. Under this condition, the rotational torque (T) is calculated by the following equations,

$$T = \gamma\omega \tag{1}$$

and

$$\gamma = \frac{\frac{1}{3}\pi\eta L^3}{\ln(L/2r) - 0.66} \tag{2}$$

where γ: rotational drag coefficient, ω: angular velocity, η: viscosity of the environmental solution, L : length of the cylinder, r : radius of the cylinder.

III. Local Environmental Control System with Nano/Micro Dual Pipettes

A. Configuration of Upgraded Dual Pipettes System

When the spout of Na^+ ions is continued for a long period, the rotational speeds of flagellar motor get into a certain level at any applied DC voltages (Fig. 5). It might be caused by the equilibrium between diffusion and supply of Na^+ ions. So, for the quantitative and long-time-stable manipulation of the rotational speed of the Na^+-driven flagellar motor, we upgraded our local environmental control system with nano/micro dual pipettes as shown in Fig. 6.

This system has two pipettes. The glass nanopipette with a

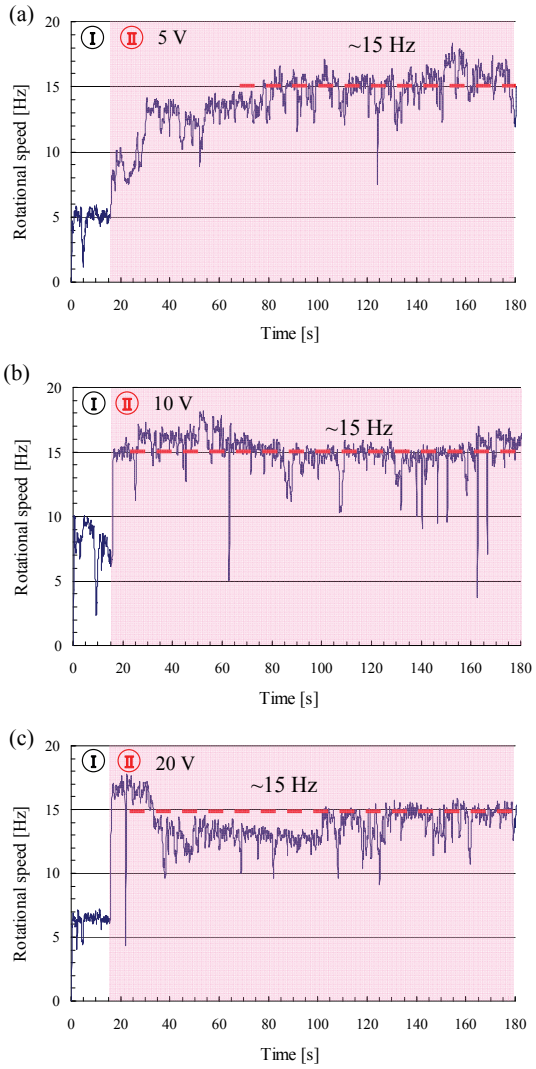

Fig. 5. The rotational speeds of flagellar motor after the long periods of time with applied DC voltages of (a) 5 V, (b) 10 V, (c) 20 V. I; The terms for which the Na^+-free solution was spouted from the micropipette. II; The terms for which the Na^+-containing solution was spouted from the nanopipette.

~50 nm inner diameter was used for the spout of Na^+-motility medium, the Na^+-containing solution. In contrast, the glass micropipette with a ~1 μm inner diameter was used for the spout of K^+-motility medium, the Na^+-free solution. The bath was filled with the Na^+-free solution. The DC voltage was applied between ITO (Indium tin oxide; 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 turn on/off the spout of Na^+-containing and Na^+-free solutions independently, and can change the spouting velocity/volume of the solutions by changing the applied DC voltages independently.

These pipettes are fixed on the two micro manipulators

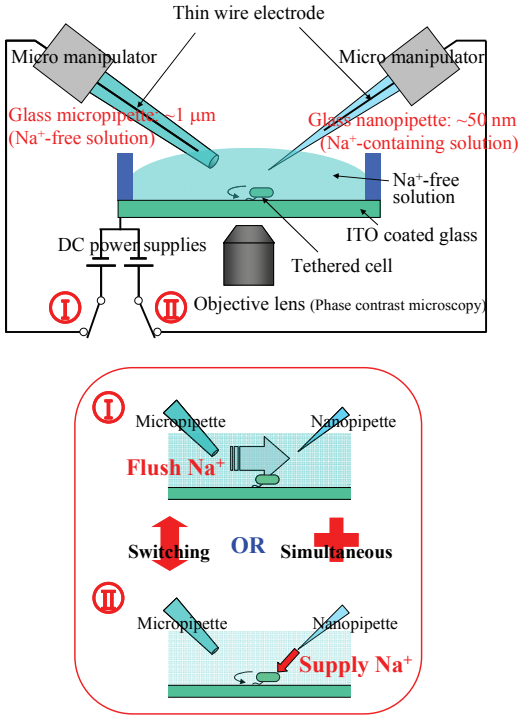

Fig. 6. Schematic of upgraded local environmental control system with nano/micro dual pipettes for the quantitative and long-time-stable rotational speed manipulation of Na^+-driven flagellar motor.

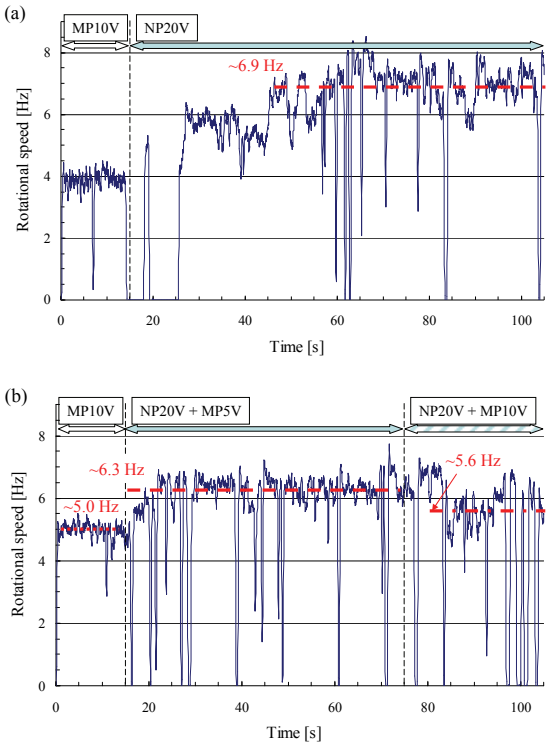

Fig. 7. Experimental results of (a) control experiment, (b) fine and long-time-stable manipulation of the rotational speed of Na^+-driven flagellar motor by simultaneous spouts with changing the applied voltages independently. NP: Nanopipette (Na^+-containing). MP: Micropipette (Na^+-free).

respectively. So, it is possible to approach the pipettes to the arbitrary single cell, and the cell-pipette distances can be controlled independently.

B. Fine Manipulation of Flagellar Rotational Speed by using Difference of Spouts from Nano/Micro Dual Pipettes

Through this dual pipettes system, Na^+ ions are supplied to the flagellar motor on the arbitrary single cell by spout from the nanopipette. And, the Na^+ ions diffusing from the nanopipette and Na^+ ions remaining around the cell can be flushed by spout from the micropipette. So, when the both solutions are spouted simultaneously, it is expected that the local Na^+ concentration is determined by the difference between the spouting velocities of the Na^+-containing and Na^+-free solutions. (And, when the spout is switched between the Na^+-containing and Na^+-free solutions, the feeding of Na^+ ions to the flagellar motor is quickly started/stopped.)

Therefore, the quantitative and long-time-stable manipulation of the rotational speed of the Na^+-driven flagellar motor might be realized without changing the Na^+ concentration of the spouting solution.

IV. Results and Discussions

Firstly, we conducted the two negative control experiments. The first control experiment was done with filling the 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 10 V (data not shown). In the second control experiment, the spout of Na^+ ions was continued for a long period at 20 V (Fig. 7(a)). Figure 7(a) is the time-series data of the rotational speed of single cell body. (The experimental data was processed by the low-pass filter (LPF, threshold frequency; ~0.8 Hz) to filter out the fluctuation of the rotational speed caused by the tethered cell and the analysis error. As mentioned above, with tethered cell, the rotational speed of the flagellum can be easily observed as the rotational speed of the cell body. On the other hand, the rotational speeds of tethered cells often fluctuate because of the high load and the temporal sticking to the substrate.) As a result, the rotational speed got into ~6.9 Hz.

Figure 7(b) shows the experimental result of the rotational speed manipulation of Na^+-driven flagellar motor in single *E. coli* cell. The rotational speed was manipulated by simultaneous local spouts of Na^+-containing and Na^+-free solutions with controlling the spouting velocities independently. Figure 7(b) is the time-series data of the rotational speed of single cell body. From this result, when the Na^+-containing and Na^+-free solutions were spouted simultaneously, the rotational speed was increased from the base level ~5.0 Hz to ~6.3 Hz. It was slightly slower than the

rotational speed in the control experiment (~6.9 Hz, in Fig. 7(a)). After multiplying the applied DC voltage of the micropipette, the rotational speed slightly decreased to ~5.6 Hz. This decreasing of the rotational speed might be caused by the increasing of the spouting velocity of Na^+-free solution. However, because the Na^+-containing solution was still being spouted, it was still faster than the base level (~5.0 Hz).

Therefore, as expected, the quantitative and long-time-stable rotational speed manipulation of Na^+-driven flagellar motor is realized by simultaneous local spouts of the Na^+-containing and Na^+-free solutions with controlling the spouting velocities independently. In addition, when the switching and the simultaneous spouting methods are combined, the local environmental control system with nano/micro dual pipettes will become a powerful tool to manipulate the bacterial flagellar motor, bio driving force.

For the fundamental estimation as the bio driving force, the rotational torque generated by the flagellar motor was estimated. The rotational torque was calculated by (1) and (2) where L : 3 μm and r : 0.5 μm. As a result, the rotational torque were calculated ~2.8×10^3 / ~2.5×10^3 / ~2.3×10^3 pN· nm at ~6.9 Hz / ~6.3 Hz / ~5.6 Hz, respectively.

V. Conclusion

In this paper, we upgraded our local environmental control system with nano/micro dual pipettes to achieve the quantitative and long-time-stable manipulation of the rotational speed of the Na^+-driven flagellar motor in single *E. coli* cell. We demonstrated fine and long-time-stable rotational speed manipulation of Na^+-driven flagellar motor by simultaneous local spouts of Na^+-containing and Na^+-free solutions with controlling the spouting velocities independently. And, the rotational torques generated by the flagellar motor in each condition of spout were estimated ~2.8×10^3 pN· nm (Na^+-containing: 20 V, Na^+-free: off), ~2.5×10^3 pN· nm (Na^+-containing: 20 V, Na^+-free: 5 V), ~2.3×10^3 pN· nm (Na^+-containing: 20 V, Na^+-free: 10 V). By combining the two local environmental control methods (the switching and the simultaneous spouting of the Na^+-containing and Na^+-free solutions) through the local environmental control system with nano/micro dual pipettes, it might be realized to manipulate the bacterial flagellar motor, bio driving force.

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