Miniaturized thermocontrol devices enable analysis of biomolecular behavior on their timescales, second to millisecond

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To establish general-purpose methods and tools for biological experiments on a short time scale is an essential requirement for future research in molecular biology because most of the functions of living organisms at the molecular level take place on a time scale from 1-second to millisecond. Thermal control with on-chip micro-thermodevices is one of the strongest and most useful ways to realize biological experiments at molecular level on these time scales. Novel biological phenomena revealed by the experiments using micro-thermodevices on a 1-second and millisecond time scale will be shown for the proof. Finally, the advantages and impact of this methodology in molecular biology will be discussed.

Introduction

Living organisms have various functions that span a wide range of time scales. When looking down to small functional units of living organisms, cell division takes place during a time scale of a few hours. Going further down into smaller units, most of the functions take place in the 10–10^{-6} seconds time scale at the biomolecular level, and 10^{-6} to 10^{-15} seconds at the atomic level.[1] Folding and conformational change of protein molecules are generally complete in 10–10^{-3} s.[2] To study the molecular functions on this time scale is essential for further biological understanding. Thus, a widely applicable technique to measure the molecular dynamics on a subsecond to millisecond time scale is required.

Measuring response due to external stimulation by changing the environment rapidly has been one of the most common and widely applicable ways to measure the dynamics on a short time scale. To change the chemical environment[3] or temperature[4–6] by laser pulses has been a powerful strategy. However, an observed material often requires biological mutation or chemical treatments beforehand and the laser heating may damage fragile materials not only by the effect of temperature rising but also by exposure to excess light. They also need expensive setups which require high skill to operate. These heating techniques are difficult to couple with thermosensors for simultaneous temperature monitoring.

Especially when measuring at single molecule level, these techniques also involve problems to do with collecting large quantities of data for statistical analysis, because in those experiments the experimental setup must be manually operated from one run to another. This has been a major limiting factor for performing a series of experiments in this field. Therefore, high speed experimental tools must be capable of obtaining large quantities of data. It is preferable that these tools are widely applicable to many kinds of biomaterials and allow experiments with small amounts of samples. The tools must be compact, cheap, and mass producible to allow a series of experiments in typical biological laboratories. For these requirements, temperature transition by a miniaturized thermocontrol device consisting of a microheater and a micro-thermosensor is one of the strongest and most widely applicable strategies. Since thermal mass (heat capacity which is an extrinsic property) is proportional to the third power of the size, the response speed of the thermocontrol device is increased drastically by miniaturization.

Advantages of miniaturized microdevices

Miniaturized microdevices show various advantages in functionality compared to their macroscopic counterparts owing to

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Insight, innovation, integration

Most of the functions of living organisms at molecular level take place on a time scale from 1-second to millisecond, thus this is one of the most important time scales in studying biomolecular functions. Top-down technology enabled on-chip thermocontrol devices of a few hundreds to a few tens of micrometres in size showing the advantages of miniaturization such as fast response, compactness, and mass productivity. As a result, easy access to real-time controlling and probing of biologically important phenomena such as enzymatic activity, mechanical motion of motor proteins, and denaturation dynamics of fluorescent proteins, which happen on a second to millisecond time scale, became possible. The impact and potential of this methodology in molecular biology will also be discussed.

the physical effects when going down in size. In general, a volume effect is proportional to the third power of size while a surface effect is to the second power. Therefore, the former effect is reduced more than the latter in scaling down. Thus, miniaturization achieves advantages such as fast mechanical response owing to the reduced inertial effect and fast diffusion rate owing to the relatively large surface area. Detailed explanations of the effects of scaling down are given elsewhere.[7]

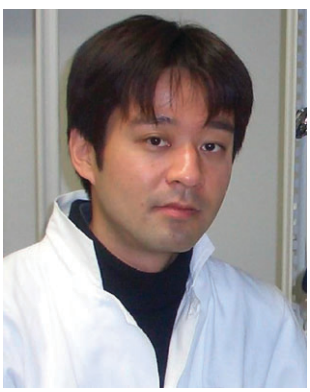
So-called top-down technologies, mainly by the semiconductor microfabrication process, have enabled us to manufacture and fabricate structures of a submicrometre scale.[8] The smallest functional unit in a commercial electronic device has reached the 10 nm scale.[9] MEMS (Micro Electro Mechanical Systems)[10] and μTAS (micro total analysis systems)[11–15] technologies offer the advantages of batch fabrication of numbers of devices as well as an ability to integrate multiple functional units in a small area, which is important for developing smart and sophisticated devices. MEMS technologies have been applied successfully to sensors,[16] actuators,[17] microreactors,[18] radio-frequency devices,[19] and optical devices.[20–24] μTAS enables rapid, highly sensitive and parallel biological/chemical analysis with small amounts of samples thanks to the merits of miniaturization. In handling small amounts of samples in a microscale, it realizes short diffusion distances, high interface-to-volume ratio, and small thermal mass.[25] Material costs and the amount of waste can also be reduced. Miniaturized devices also allow samples to be analyzed in typical biological laboratories. Various biological experiments have been realized by microchip technology[26] frequently by combination with micro-nano manipulation techniques.[27] Taking those advantages, top-down technologies have been applied to thermal devices such as high-speed PCR,[6,28–32] both in flow-through-type[29,30] and in thermal-cycling-type,[6,31,32] or high-speed microcalorimetry.[33–36] Microfabricated fluidic channels, heaters, temperature sensors, and fluorescence detectors were integrated to analyze nanolitre-size DNA samples.[37] Therefore, top-down technologies can meet the demands in molecular biology mentioned above, including fast response, high sensitivity, and obtaining larger quantities of data by establishing systems and tools for biological experiments on a short time scale.

The purpose of this report is to review, discuss and state the importance of the strategy of high speed temperature transition by miniaturized thermocontrol devices which enable real-time biomolecular experiments on a short time scale for microscopic observation. Finally, the impact of this strategy and future prospects will be discussed. For more detailed information, especially on the device fabrication and the experimental conditions, we encourage readers to refer to the original papers.[38–45]

Biomolecule experiments on 1-second time scale

Conventional temperature controlling devices for microscopic observation that control the temperature of a whole sample holder have a number of limitations: (1) response speed is slow, (2) thermal expansion of a sample holder causes defocusing during observation, (3) heat transfers to an objective lens and causes damage. Such limitations can be overcome by controlling the temperature locally.[46,47] Reducing the size of the thermodevice can diminish the total amount of heat for controlling the temperature. Therefore, it offers fast response speed and also prevents disturbance to the microscope such as defocusing while changing the temperature. Controlling the temperature using a microfabricated thermodevice may minimize damage to the materials compared to laser heating, and enable rapid temperature alternation.[48] Furthermore, output of a micro-thermosensor can be easily collected and a microheater can be driven electrically by connecting it to an electronic device.

Fig. 1 shows a micro-thermodevice fabricated by patterning nickel (hereinafter, Ni) on a glass plate.[38] Ni was chosen as the



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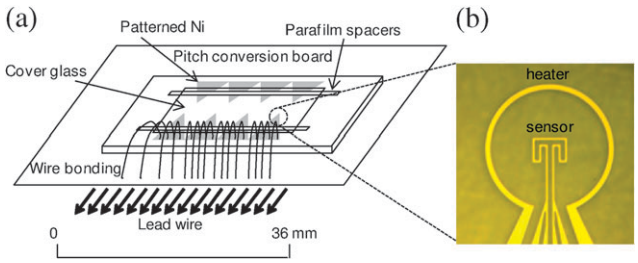

Fig. 1 Ni-patterned glass plate, which is placed on a pitch conversion board to enhance the pitch between the electrodes and connected to lead wires (left). Microscopic view of the integrated microheater and micro-thermosensor (right). H. F. Arata *et al.*, *Appl. Phys. Lett.*, 2006, **88**, 083902, American Institute of Physics. Reproduced by permission.

material for both the heater and the thermosensor to simplify the fabrication process. The diameter of the circular microheater was 400 μm, and the width of the heater wire was 20 μm. The Ni-patterned glass plate was wire bonded to pitch conversion board to enhance the pitch between the electrodes. The heater raises the temperature by joule heating and the thermoresistive-sensor detects the temperature from its resistivity. The input of the microheater was controlled and the output of the thermosensor was calculated into temperature using commercial software. Temperature uniformity and the response speed of the heater were measured by using Rhodamine B as a temperature detector.[39] This was also estimated by conducting numerical simulation by a finite element method using commercial software (FEMLab) the results of which showed excellent agreement with experimental results. Consequently, the temperature accuracy within the circular heater was measured to be 3 °C and the response speeds were 71.5 and 56.9 K s^{-1} for temperature rise and fall, respectively. This response time is comparable with that of a PCR chip, whose volume of reaction chamber is in the same order; about 80 °C s^{-1} for heating and 60 °C s^{-1} for cooling.[32]

This micro-thermodevice of 1-s response time was applied to several biological experiments. Loop-mediated isothermal amplification (LAMP)[49] of a single DNA molecule was achieved in a polyacrylamide gel-based microcontainer coupled with the micro-thermodevice.[40] Hence, total time for reaction including detection was less than 1 hour. This system could also be applied to study single molecule PCR.[41] This micro-thermodevice was successfully adopted to switch the gel–sol transition of thermally responsive supramolecular nanomeshes[50,51] which enabled on/off switching of the rotary motion of F_1.[42] This result suggests that intelligent soft materials such as supramolecular hydrogels give promise of the integration of biomolecules and hard materials for developing novel semi-synthetic nano-biodevices. In the following sections, our major results using this micro-thermodevice will be shown.

Motion control of rotary biomolecular motor F_1-ATPase

Speed controlling assays of F_1-ATPase (hereinafter F_1), a rotary molecular motor, were successfully performed by the micro-thermodevice of 1-s response time.[38] F_1, which acts as

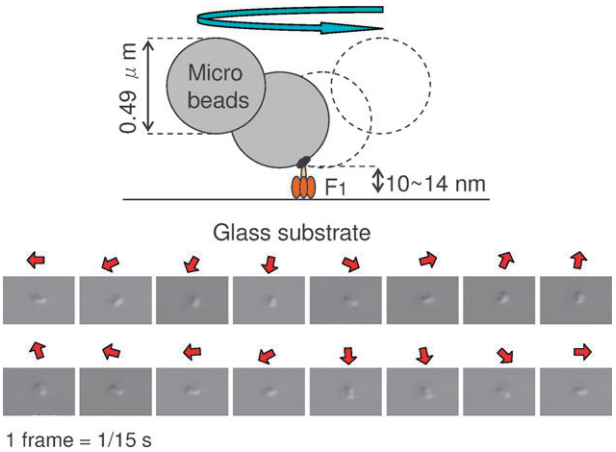

Fig. 2 Schematic of the observation system of a single F_1 molecule. F_1 is attached to the glass plate and microbeads are attached to the rotary axis of F_1 (top). Microscope view of two microbeads being rotated by a single F_1 molecule. The angular velocity is 1.5 rps (bottom).

the major ATP supplier in the cell, is a rotary motor protein found next to the bacterial flagella motor in the living organism.[52–54] Rotation of the F_1 motor driven by ATP hydrolysis can be directly observed with an optical microscope.[52] The protocol of the rotary assay is as shown in a

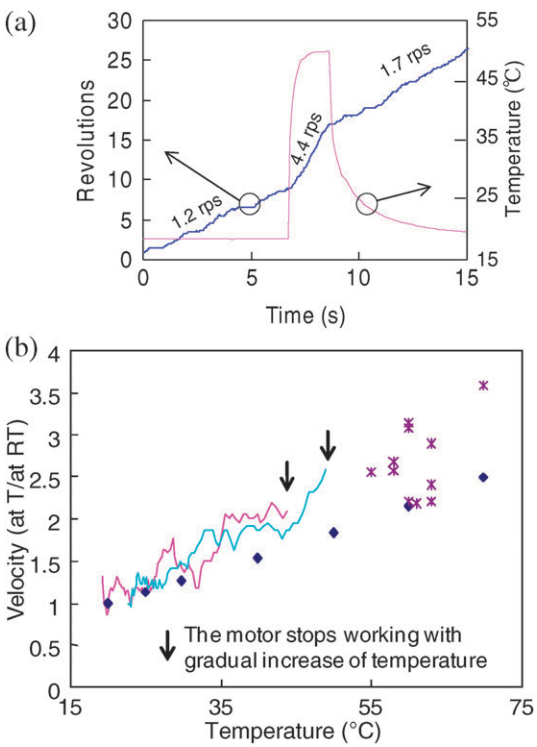

Fig. 3 (a) Time course of the rotation of F_1 while switching the temperature. Angular velocity changed from 1.2 rps to 4.4 rps and back to 1.7 rps. (b) Standardized angular velocity dependent on temperature. Lines represent the data when the temperature was increased gradually. Star-shaped dots represent the data obtained by rapid temperature alternation. H. F. Arata *et al.*, *Appl. Phys. Lett.*, 2006, **88**, 083902, American Institute of Physics. Reproduced by permission.

previous report.[52] Schematics of the rotary assay and a microscopic view of rotating microbeads are shown in Fig. 2. The rotary assay was performed on the micro-thermodevice to alter the temperature in real-time.

Angular velocity control of F_1 rotation was observed by altering the temperature in 1 s. Angular velocity changed from 1.2 to 4.4 rps and came back to 1.7 rps when the temperature was altered from room temperature to 50 °C and back to room temperature (Fig. 3). When the temperature was increased gradually, the velocity of F_1 increased gradually, but stopped at a temperature less than 50 °C since the enzyme cannot survive for long at high temperatures. To measure the velocity at higher temperatures, the temperature should be altered rapidly, as shown in Fig. 3(a), because the enzyme can tolerate higher temperatures for a short period, even at temperatures higher than that which would denature it in a steady state. Acceleration of angular velocity while raising the temperature can be associated with two factors. One is decrease of the viscous drag of water. The other is torque increase in the F_1 molecule. The torque increase depending on the temperature can be calculated by subtracting the effect of the viscous drag of water, which can be numerically estimated. Consequently, it was proved that a rotating F_1 has a tendency to show higher torque at higher temperatures with the estimated increasing rate of 4% per 10 °C as shown in Fig. 3(b). This result shows that the performances, both torque and velocity, of molecular machines can be controlled by this methodology. This may be a useful method when future organic–inorganic hybrid nanosystems are established.[55,56]

Enzymatic assay with PDMS microcontainers

Adopting a poly-dimethylsiloxane (hereinafter PDMS) micro-container on the micro-thermodevice enables such experiments with a small amount of sample in liquid solution.[43] PDMS, a silicone elastomer, has been one the most actively developed and widely used polymers for microfluidics[57,58] because it is transparent to visible light and is adhesive to smooth surfaces. This property is essential to seal the containers against the glass plate for microscopic observation. Additionally, this material is easily patterned through molding techniques.[59] This container prevents the sample materials from diffusing into the solution and enables us to measure the reaction of an extremely small amount of chemical. In a previous report, even the enzymatic activity measurements at the single molecule level could be performed using this PDMS microcontainer.[44,60] Fig. 4 shows a SEM image of microfabricated silicon substrate for use as a mold and a surface of patterned PDMS sheets. The mold of the micro-containers was manufactured by patterning silicon substrate through photolithography and reactive ion etching (RIE). PDMS sheets were patterned by the silicon mold; this process can be repeated many times with the same silicon mold and enables mass production of micropatterned PDMS sheets. The experiment using PDMS microcontainers has the merit that the experiments can be repeated many times simply by changing PDMS sheets through easy bench top preparation. Sample in a solution can be encapsulated in microcontainers

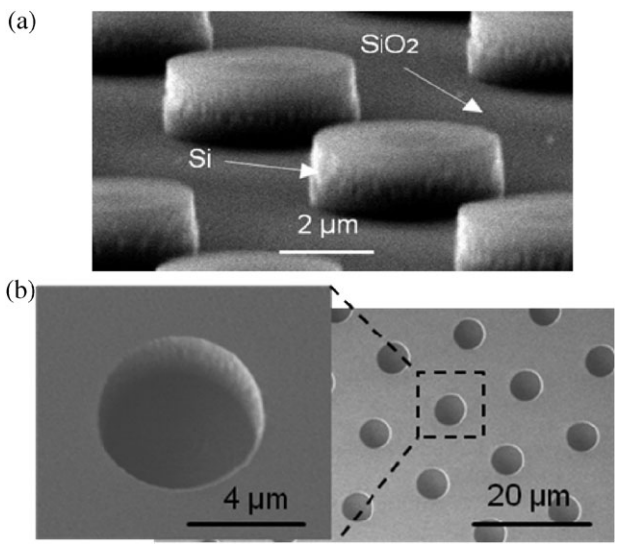

Fig. 4 (a) SEM image of a silicon-patterned cylindrical mold. (b) SEM image of patterned PDMS sheets with volume of 55 fL.

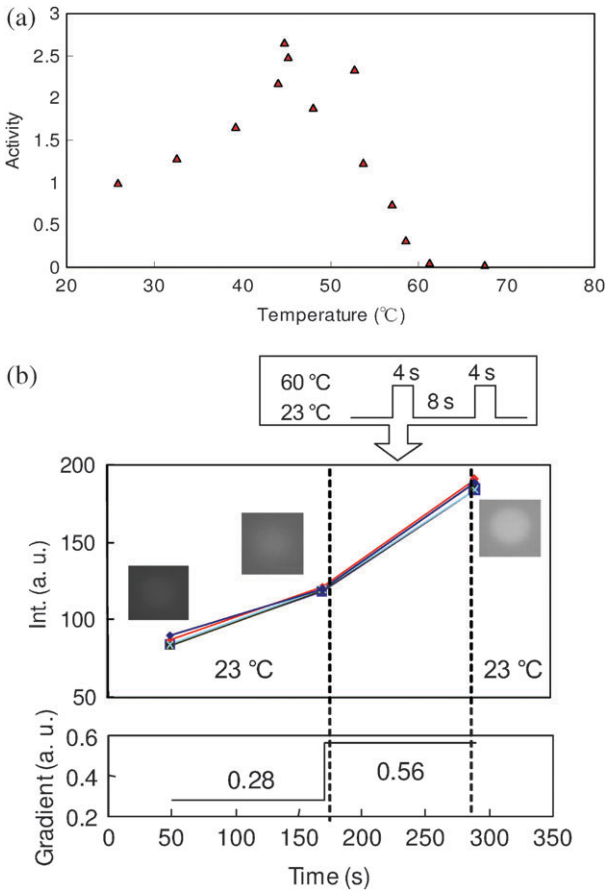

Fig. 5 (a) Activity of β-gal *vs.* the temperature measured by bulk experiment. It shows higher activity at a temperature higher than room temperature and shows a peak at 45–50 °C, a decrease with temperature after the peak and complete cessation of activity over 60 °C. (b) Activity controlled by temperature pulses with a duty ratio of 1/3 (four pulses in 48 s). The increase rate of the fluorescent intensity changed from 0.28 to 0.56. The lines follow the intensity of four individual microcontainers. H. F. Arata *et al.*, *Anal. Chem.*, 2005, **77**, 4810, American Chemical Society. Reproduced by permission.

simply by sandwiching it with a glass substrate and a PDMS sheet. Since air can penetrate into PDMS, typical buffer solution can be encapsulated without any air bubble in a container. PDMS adsorbs protein to some extent, however, this does not affect the outcome of experiments.[44]

The combined system of a micro-thermodevice and micro-containers enabled measurement of the enzymatic activity of β-galactosidase (hereinafter β-gal) at high temperatures by altering the temperature rapidly. β-gal is widely used as a reporter in molecular biology.[61] β-gal is the enzyme that catalyzes hydrolysis of fluorescein di-β-D-galactopyranoside (FDG). A fluorogenic substrate of β-gal, FDG in a buffer solution is hydrolyzed to fluorescein. Fluorescent intensity represents the amount of product produced by β-gal. Therefore, we can measure the activity of β-gal from the time course of the fluorescent intensity. In the steady state, the activity of β-gal *versus* temperature shows a peak at around 45–50 °C. It decreases with temperature after the peak and there is complete cessation above 60 °C (Fig. 5(a)). β-gal was captured in microcontainers mounted on the micro-thermodevice to manage rapid alternation of exposed temperature. In this experiment, the volume of a micro-container was 60 fL; at a β-gal concentration of 37 nM, each container contained around 1300 enzymes. Applying 60 °C heat pulses of 4 s, we successfully increased the activity of the enzyme (Fig. 5(b)). This indicates that the enzymes remained active and increased their activity over a short period, even at temperatures that cause damage after prolonged exposure. This method gives new kinetic information and allows us to measure the temperature stability of biomolecules. It also makes it possible to perform highly sensitive biochemical experiments which can be applied to highly sensitive biosensors.

Millisecond dynamics of fluorescent proteins

Green fluorescent protein (hereinafter GFP) is commonly used as a reporter protein in biological experiments.[62–64] Studying dynamic properties of denaturation of GFP may provide important information for understanding functional mechanisms of protein in general. GFP loses its fluorescence when denatured by temperatures higher than 70 °C,[65,66] pH extremes or guanidinium chloride.[66] To elucidate the mechanism of denaturation at the molecular level, we chose to perform real-time thermal experiments on the millisecond time scale during which these molecular phenomena occur.

To realize those experiments, a thin-film microheater was fabricated by patterning Ni on a glass plate with sputtering and photolithography[45] (Fig. 6). The microheater heats up its internal and external area by joule heating. The heater wire is 200 nm thick and 2 μm wide. The size of the microheater is 20 μm × 20 μm. Such dimensions realize 1 millisecond temperature control. A microfabricated PDMS sheet was mounted onto a glass substrate with thin film microheaters to form microcontainers as described in section 3.2. In this experiment, the cylindrical containers were 4 μm in diameter, 2.2 μm in height, and 28 femtolitres in volume. Samples were captured in microcontainers mounted on the micro-thermodevice to manage rapid alternation of exposed temperature as described in section 3.2. The microcontainers located in the internal area of the microheater were used during the experiments because the temperature of the internal area was proved to be sufficiently uniform by conducting numerical simulation by a finite element method using commercial software (FEMLab).[45]

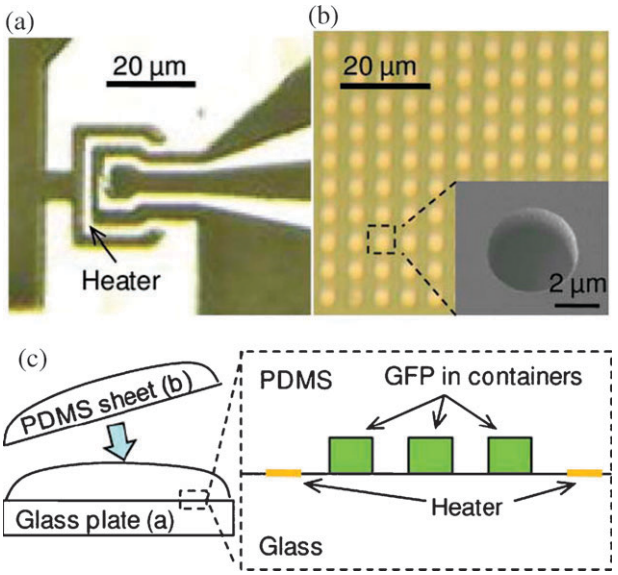

Fig. 6 (a) A Ni microheater fabricated on a glass plate. The bright part is patterned Ni and the dark part is naked glass. (b) A micro-patterned PDMS sheet. Cylindrical holes are patterned on one side of a PDMS sheet. The bottom right is an expanded picture taken by a scanning electron microscope (SEM). (c) A micro-patterned PDMS sheet was mounted on a microheater fabricated glass plate to form microcontainers. Buffers with proteins in microcontainers were heated up by a collocated microheater. H. F. Arata *et al.*, *Lab Chip*, 2008, **8**, 1436, Royal Society of Chemistry. Reproduced by permission.

When the temperature was raised to slightly under 100 °C in 1 ms, the fluorescence intensities of the protein-containing microcontainers were quenched owing to the denaturation of GFPs. The quenching curves of GFPs have a rapid fall at the beginning and a slow decrease afterwards, as shown in Fig. 7(a). This suggests that they have two time constants. The quenching curves of red fluorescent proteins were measured by the same method. Fig. 7(b) shows the quenching curves of red fluorescent proteins. Denaturation was completed in 5 to 10 ms for red fluorescent proteins. The large difference in response time between GFPs and red fluorescent proteins may be due to the strong thermal stability of GFPs[65,67] compared to that of typical proteins. Fitting with bi-exponential decay by the least squares method iteration proved that rAcGFP denatured with time constants of 5 ms and 75 ms, while rGFPuv denatured with time constants of 10 ms and 130 ms. The bi-exponential decay may be caused by the reverse process of two-states renaturation observed by Ueno *et al.*[3] in which a GFP molecule recovered its fluorescence in around 30 s after being denatured by acid. The difference of the parameters of the fitted curve between rAcGFP and rGFP may be explained by the difference in the position of mutated amino acids.

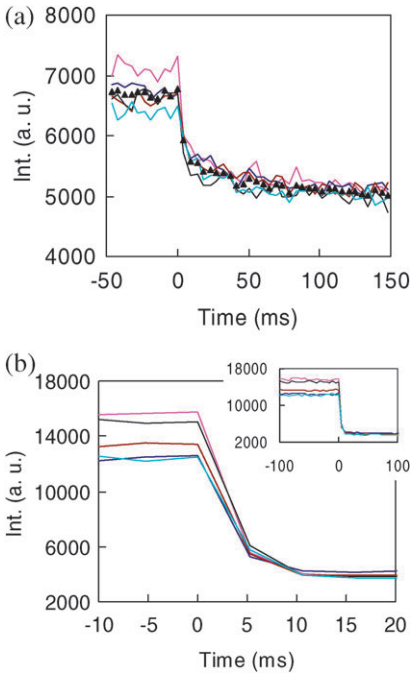

Fig. 7 Time courses of fluorescence intensity of fluorescent proteins in microcontainers. The fluorescent intensity of each microcontainer is plotted in line. The average intensity of all the containers is plotted in triangles. The fluorescent intensity started quenching when the micro-heater was switched on at an elapsed time of 0 s. (a) Group of curves in the graph are from five individual microcontainers containing the same concentration of rAcGFP.[68] The quenching showed bi-exponential decay. (b) The group of curves in the graph is from five individual microcontainers containing the same concentration of rDsRed.[69] Insets show the same data over a longer time range. The quenching was completed within 5–10 ms. H. F. Arata *et al.*, *Lab Chip*, 2008, **8**, 1436, Royal Society of Chemistry. Reproduced by permission.

Conclusion and outlook

As the results shown in each section clearly prove, miniaturization of devices for biological experiments enables experiments on a time scale in which most of the important functions of living organisms happen at molecular level: controlling and measurement of mechanical motion of motor proteins on a 1 second time scale, activity measurement of enzymes at high temperature by altering the temperature rapidly in a 1 second time scale, and dynamics of fluorescent protein denaturation were observed on a millisecond time scale. Thus, it has become possible to probe physical parameters of biomaterials, such as rotary motion and torque of F_1-ATPase or fluorescent dynamics of GFP, easily on a subsecond to millisecond time scale, which is difficult by conventional techniques. To the best of our knowledge, those parameters shown in our work are the first to be measured by those microdevices. Though, at this stage, there exist no comparative data obtained by other techniques, our data showed biological relevance with previous reports using other techniques. Since most of the important functions of a living organism at the molecular level, such as folding/unfolding of proteins, enzymatic activity, and the mechanical motion of motor proteins, happen on a second to millisecond time scale, the proposed methodology using micro-thermodevices promotes experiments on these phenomena at the molecule level. These compact microdevices manufactured by MEMS technologies can be mass produced and allow samples to be analyzed in typical biological laboratories, which cannot be done with sizeable equipment. Furthermore, this device can easily be connected to electronic devices for digital sampling and controlling which may lead to automation of experiments. It also has the potential for on-chip data analysis by integrating an electronic circuit into the same device.

They also have potential applications in industrial research. For example, it might be possible to use a heat sensitive catalytic enzyme at high temperatures by altering temperatures rapidly. It might also be possible to produce heat sensitive products by avoiding prolonged heating which may induce unfavorable sub-reactions. Those applications of microdevices might promote protein engineering such as the development of industrial biocatalysts.[70] Additionally, the micro-thermodevice is applicable to a variety of biomaterials because the device allows us to perform experiments without any mutations or biochemical treatment of the material itself. We observed the thermal quenching dynamics of double stranded DNA with fluorescent intercalator SYBR Green, which completed in 10 ms, by a micro-thermodevice of 1 ms response time.[71] It can also be used as a means of differential calorimetry because the proposed micro-thermodevices can monitor the temperature rise at a definite energy input in real-time on a short time scale as performed by 3D-MEMS devices which are cumbersome to manufacture and use.[33–36] This enables highly sensitive measurements of specific heat capacity, which is an intrinsic material property, change during various biological functions at the molecular level, such as denaturation of nucleic acids, phase transition of lipids or protein denaturation, whose detecting method has hitherto been limited mostly to fluorescent illumination. As another potential application, this micro-thermodevice can be used to study the thermosynthesis concept, biological free energy gain from thermal cycling, whose resulting overall origin of life model may suggest new explanations for the emergence of the genetic code and the ribosome.[72,73]

Theoretically, further miniaturization of thermodevices may achieve response times faster than milliseconds. For example, microsecond to millisecond protein dynamics, which are necessary for catalysis and are an intrinsic property of enzymes as shown by NMR (Nuclear Magnetic Resonance) relaxation dispersion experiments for cyclophilin A,[74,75] can be examined by those devices. However, when the size of the heater gets close to the size of one biomolecule, for example, in the order of a few nanometres to a few tens of nanometres for a typical protein, a single biomolecule must be isolated and put on to a heater. To overcome this alignment problem, improving the spatial resolution of chemical treatment for selective patterning of proteins, such as Ni-NTA surface chemical treatment,[52] will be required. The other way is to manipulate a sample molecule mechanically with the precision of a few nanometres by techniques of nano-isolation and -manipulation of biomolecules using MEMS devices; for example, studies to isolate a single DNA molecule using a 2 μm-wide microchannel[76] or trapping a single DNA molecule using

microtweezers.[77] A micro-thermodevice of this type is highly applicable to microfluidic systems because a thermodevice patterned on a 2D substrate can control the temperatures of any part of 3D structures such as flow channels and reactors mounted on it. Another considerable limitation for this methodology is the fact that a microheater itself is invisible by optical microscope under scale of diffraction limit, 200 nm for a typical optical microscope. Those nanodevices must be designed to facilitate alignment between an invisible heater and protein. Experiments using nanoscale thermodevices are expected to be realized by integration with these MEMS/NEMS (Nano Electro Mechanical Systems)-based manipulation technologies. This may lead to the high-throughput screening of experiments which have so far been performed manually. Hence, these devices are likely to accelerate experimental work and promote understanding of basic phenomena in life science.

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