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Single-Molecule Detection in Temperature-Controlled Microchannels

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***Abstract*—This paper reports a temperature-controlled microfluidic platform for single-molecule studies. Parallel microchannels are formed between glass and quartz, and are integrated with on-chip heaters and temperature sensors. Combined with surface derivatization and fluorescent labeling of biomolecules, temperature-dependent changes in the rates of fluorescence photobleaching are measured. The results demonstrate the potential of the device to provide an ideal environment for temperature-dependent single-molecule studies.**

Keywords-microfluidics; microchannel; single-molecule detection; temperature control

I. INTRODUCTION

Manipulation of matter on an atomic and molecular scale has critically benefited from single-molecule detection. Using highly sensitive optical probes and microscopy, single-molecule behaviors are detected and studied [1]. For example, laser-induced fluorescent spectroscopy [2] is an effective method for studies on individual macromolecules, providing molecular structure and function information that is difficult to obtain by conventional techniques. Much research on molecular dynamics [3] or kinetics [4] employing single-molecule fluorescence spectroscopy has also been developed. With its significant impact on biomolecular studies, single-molecule detection has become a major topic of biologic research.

MEMS technology is uniquely positioned to facilitate single-molecule studies by providing a well-controlled micro- or nanoscale environment in which biomolecules are effectively manipulated and detected. For example, DNA can be separated using electrophoresis and detected using single-molecule fluorescence burst counting techniques on a microchip [5]. In addition, using quantum dots, both DNA and proteins have been detected at the single-molecule level on a microchip, whereby the use of a microchannel leads to improved resolution [6]. Single-molecule DNA amplification and analysis have been demonstrated in a microfluidic device, with detection for electrophoretic separations accomplished by confocal fluorescence microscopy [7]. Using ac and dc fields in a microreactor, individual molecules have also been precisely transported to a minuscule laser-focused detection volume, allowing quantitative detection of attomoles of DNA targets [8].

Temperature is an important parameter in single molecule studies. However, conventional temperature control methods are cumbersome and inflexible, and are therefore not well suited to single molecules. In contrast, MEMS has the potential to enable effective and accurate temperature control in a micro environment tailored to the needs of single-molecule studies. To demonstrate this potential, we present initial results on single-molecule in microfluidic channels with real-time temperature control using MEMS heaters and temperature sensors. As a model single-molecule system, single fluorescent dyes are detected via single-step photobleaching under a series of controlled temperatures. The temperature-dependent rates of photobleaching are also determined; verifying that photobleaching of single fluophores intensifies with increasing temperature. This provides a proof-of-concept example for MEMS temperature-control in single-molecule studies.

II. EXPERIMENTAL SECTION

A. Design

The device consists of microchannels sandwiched between a quartz slide with immobilized biomolecules and a microfabricated coverslip incorporating thermal-control elements (Fig. 1a). It is fitted onto the stage of an inverted fluorescent microscope configured for prism-based total internal reflection fluorescence (TIRF) microscopy. The evanescent field generated by TIRF penetrates ~100-200 nm into the aqueous sample, leading to excitation of fluorescent dyes in a femtoliter volume (5-15 fl). Optical detection of fluorescence is achieved by collecting the fluorescence through a high numerical aperture objective and focusing it onto a charge-coupled device (CCD) camera (Fig. 1b). The single-molecule attachment is realized on the top quartz slide. After the surface aminosilanization, a NHS-PEG-biotin-streptavidin-

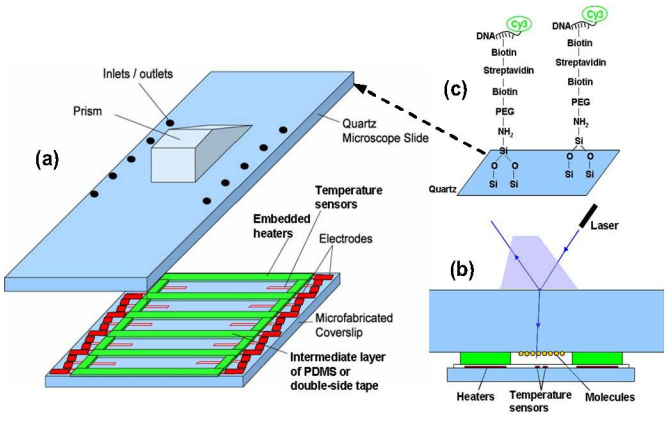

Fig.1. (a) Schematic of temperature-controlled microfluidic channels, (b) Single-molecule detection scheme, and (c) quartz surface coating chemistry.

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biotin coupling of the molecule to the quartz surface [3] is employed (Fig. 1c).

Due to the sensitivity of molecular behavior to temperature, it is important for the temperature control of the microchannels to be accurate and uniform. We have performed a preliminary simulation of the temperature distribution in the channel under the heating of the microchannels. The simulation is based on a two-dimensional heat conduction model considering a cross section of the channel as well as the associated substrate and PDMS channel wall. The heater is represented as a line heat source through which a constant current is passed. Convection in the liquid is ignored for simplicity. Using FEMLAB to solve the problem, we find that with 5 mA of current applied to the heater, the temperature non-uniformity over a channel cross section 200 × 200 μm2 in dimension, as is chosen in our experiment, is smaller than 0.02 ℃. This estimate suggests that the temperature uniformity in the channel is adequate for temperature-dependent single-molecule studies.

B. Fabrication

To fabricate the coverslip (Fig. 2), a 15-nm Cr film and 100-nm Au film are deposited via electron beam evaporation and patterned by a subsequent lift-off process. Since both heaters and sensors are designed with identical Au thicknesses, this step of metal film deposition and patterning yields both resistive heaters (serpentine wires 100 μm wide, in a 20 mm× 3 mm square) and temperature sensors (serpentine wires 50μm wide over an area ranging from 0.5-4.5 mm^2). Then 20 nm of PECVD silicon nitride is deposited as a passivation layer for the functional units. Contact pads for the sensors are then exposed by reactive ion etching (RIE).

Preparation of the quartz surface starts with a cleaning and surface activation using KOH (VMR, Inc.). Then Vectabond (Vecta Labs, Inc.) is flushed to aminosilanize the surface group. Next, the quartz is derivatized with a mixture of *N*-hydroxysuccimidyl ester polyethylene glycol (NHS-PEG) and biotin-derivatized NHS-PEG (both Fisher Scientific, Inc.) through a two-step treatment. The end functionalized group is

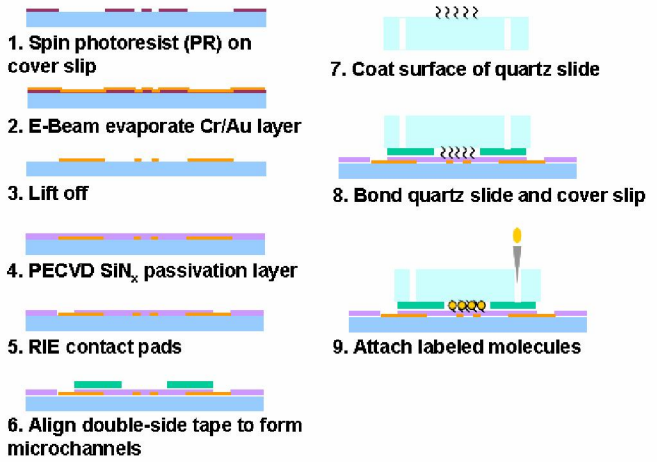

Fig. 2. Fabrication and bonding of the heater and sensor chip and surface coating of the quartz slide

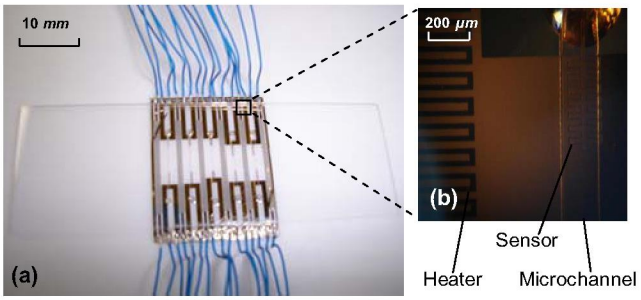

Fig. 3. (a) A Packaged device. (b) Heater and sensor near the inlet.

biotin, which can serve to anchor biotin-derivatized molecules by biotin-streptavidin-biotin bonding.

The heater and sensor chip is bonded to the functionalized quartz slide using an intermediate layer of PDMS or double sided tape (approximately 80 μm thick), which also defines the microchannels. Five parallel channels are designed to incorporate multiple biochemical experiments with controls. A photograph of the packaged device appears in Fig. 3a, and the structure near an inlet or outlet can be seen in Fig. 3b. The heaters are distributed evenly across the entire coverslip in order to provide uniform heating, whereas the sensors are located inside the channels in order to probe the real-time temperature.

Single molecules are anchored on the quartz surface inside the channel immediately prior to detection. Streptavidin is injected and incubated (10 min), and then followed by injection and incubation (10 min) of DNA section (INTDNA, Inc.) The DNA section has two end groups, one of which is labeled with Cy3 fluorophore whereas the other is labeled with biotin such that it can bind to the quartz surface. For measurement of photobleaching of single fluorophores, the DNA section here serves as a fluorophore carrier and does not participate in any biochemical reaction.

C. Single-molecule detection

After the device is fabricated and packaged, a calibration on each temperature sensor is performed by using an environmental chamber (Delta 9023, Delta Design) and tracking its electric resistance at different ambient temperatures. Resistance measurements are made with a digital multimeter (HP 3478A, Agilent). A linear fit to the sampled resistance data characterizes the resistance-temperature relationship of each sensor, which is used to determine the temperature in the channel during experiments.

After molecular immobilization, the device is mounted onto a TIRF microscopy system (TE2000-U, Nikon). The on-chip heaters and sensors are connected to the servo circuit. Only the two heaters near the current observation channel are activated to efficiently provide a uniform temperature field inside the channel under examination. Current is supplied by a general DC power supply (E3631, Agilent) but adjusted according to the feedback from the readout of temperature sensor. The load current (0-10 mA) is optimized to reach steady state for any particular sensor by comparison with the corresponding temperature-dependent relationship. Single-

molecule detection is then performed and the fluorescent intensity is tracked (Power Meter, Thorlabs) over an adequate time for photobleaching to occur. Photobleaching rates are calculated by statistical analysis of 100 samples at temperatures ranging from 25-50 ℃. This temperature range is relevant to many biochemical processes of interest to single-molecule studies.

III. Results and Discussion

A. Calibration of temperature sensors

The temperature sensors are resistors with temperature-dependant resistances [9]. The relationship between the resistance and temperature is assumed linear such that

$$R = R_0[1+\alpha(T-T_0)]$$

where R is the sensor resistance (Ω) at temperature T (℃), R_0 is the sensor resistance (Ω) at a reference temperature T_0, and α is the temperature coefficient of resistance (TCR) of the sensor. Measurement of sensor resistance at a number of temperatures in the range of interest allows determination of these parameters. These calibration results allow us to use this equation to determine the microchannel temperature from the measured sensor resistance during single molecule studies.

In order to attain accurate temperature control, we can use accurate measurement of the sensor resistance. For example, with a resistance measurement resolution of 0.01 Ω as allowed by a 5.5-digit digital multimeter, we can achieve a temperature measurement resolution of better than 0.1 ℃ with a temperature sensor about 416.7 Ω in resistance. Such a sensor, with a serpentine shape, can be fitted within an area of 4 mm^2, which is sufficiently small to allow localized temperature control for single-molecule measurements.

For this device, each temperature sensor is calibrated to attain its TCR curve before it is used for temperature control. The resistance measurement of a typical temperature sensor is shown in Fig. 4. The resistance of the sensor ranges from 493.2-495.5 Ω within temperature variations of 30-50 ℃, with a TCR of 2.4×10^{-4} 1/℃. Thus, using a 5.5-digit multimeter, temperature control at a resolution of 0.1 ℃ is achieved, which exceeds the normal requirement for temperature-dependent single molecule detection.

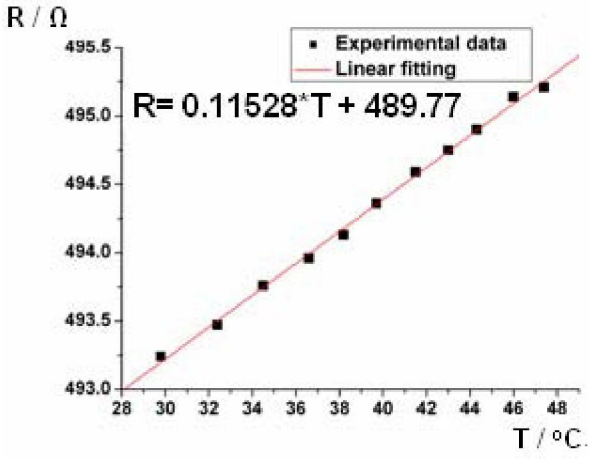

Fig. 4. Calibration of a typical temperature sensor.

B. Relationship of photobleaching versus temperature

One fundamental property of fluorescence that is expected to be temperature-dependent is the rate of photobleaching [10]. Photobleaching is caused when a fluorophore in an excited electronic state chemically reacts with oxygen, irreversibly generating a new, non-fluorescing chemical species.

In single-molecule detection, photobleaching of a single fluorophore is very easily visualized as an irreversible, single-step drop from a highly- fluorescent state to a non-fluorescent state. Population histograms of the photobleaching times of a large number of single fluorophores at a given temperature can be fitted to a single exponential decay in order to determine the photobleaching rate at the given temperature. Because photobleaching is a binary interaction between the excited-state fluorophore and oxygen, an increase in temperature should increase the number of effective collisions between the excited-state fluorophore and oxygen and hence, increase the rate of photobleaching. This expectation has been experimentally confirmed and the temperature-dependence of the photobleaching rate is well established [10]. The fluorescent dye is shown at two temperatures (25℃ and 32℃) at time intervals of 0 and 2 seconds after initial exposure to inducing laser light (Fig.5). The fluorescence micrographs indicate that fluorescence intensity decays more rapidly with increasing temperature.

Concerning the sensitivity and accuracy of on-chip temperature sensors, a series of different channel temperatures (25, 30, 35, 40, 45 and 50℃) are achieved and the fluorescent decay times are obtained from single exponential fits of

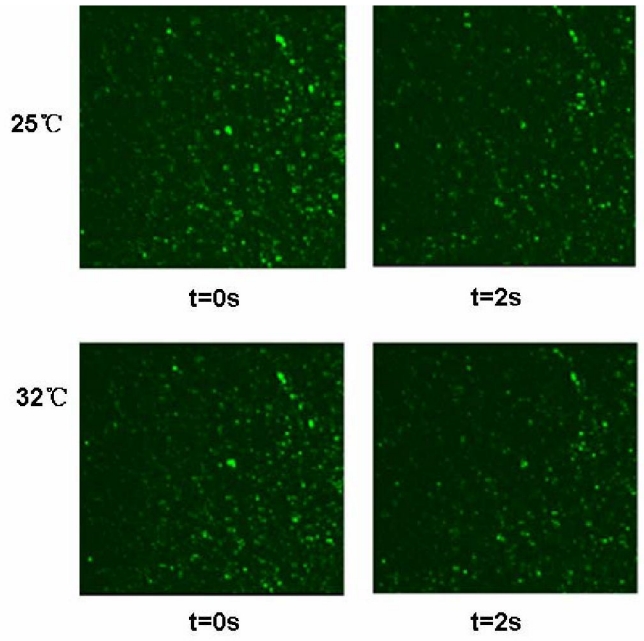

Fig. 5. Fluorescence images at 25℃ and 32℃, and 0 and 2 s after initial exposure.

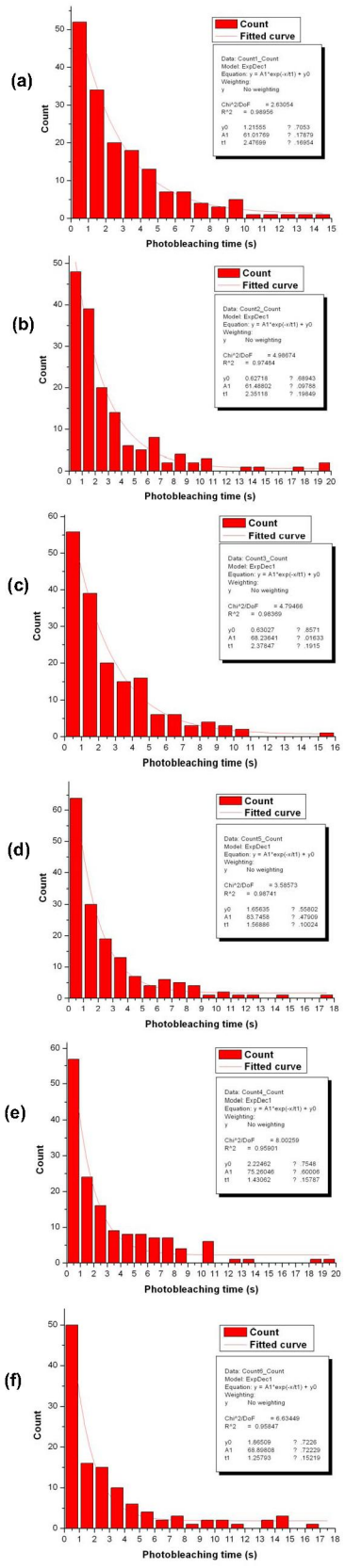

Fig. 6. Population histogram of photobleaching times and their exponential fits at (a) 25℃, (b) 30℃, (c) 35℃, (d) 40℃, (e) 45℃, and (f) 50℃.

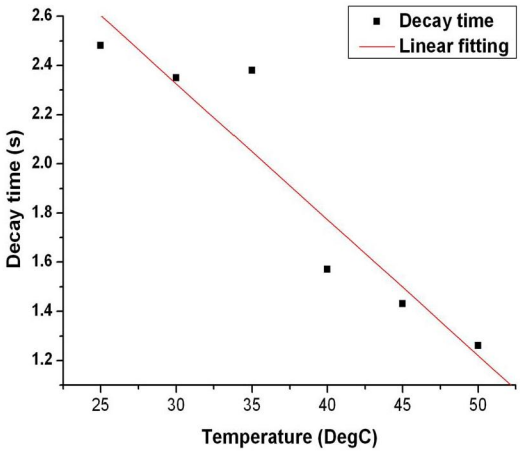

Fig.7. Dependence of fluorescence decay time on temperature

population histograms of the photobleaching times. At each temperature setpoint, a sampling of fluorescence intensity as a function of time from 100 single molecules is measured and the photobleaching time is recorded. A population histogram of photobleaching times is created using this data and fitted to an exponential decay curve (Fig.6). Decay times under different temperatures are acquired from the exponential fitting of the data. The decay times show a clear trend to decrease as the temperature increases (Fig. 7), which can be well fitted to a linear curve. This observed temperature dependency is in accordance with previous reports [10] and confirms our ability to accurately control and measure temperature in our single-molecule detection microfluidic device.

IV. CONCLUSION

An integrated temperature-controlled device for single-molecule detection has been presented. On-chip heaters and temperature sensors are fabricated on a coverslip to sandwich microchannels with a quartz slide coated to immobilize single molecules. After proper characterization of temperature-dependent relationship, the device is used for single-molecule detection at a series of controlled temperatures. By recording and analyzing photobleaching rates as a function of temperature, the application of temperature-controlled microchannels to single-molecule studies is demonstrated.

The results reported here set the stage for temperature-dependent kinetic and thermodynamic studies of biochemical reactions at the single-molecule level. The device described here will allow these measurements to be made with a range, accuracy and sensitivity not currently possible with present single-molecule detection technology. In addition, such temperature-dependent measurements are critical for mechanistic studies of enzyme activity, thus our approach is expected to be a valuable tool in single-molecule studies of biochemical reactions.

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