

## Electronic Supplementary Information

### A Microfluidic Approach for Investigating the Temperature Dependence of Biomolecular Activity with Single-Molecule Resolution

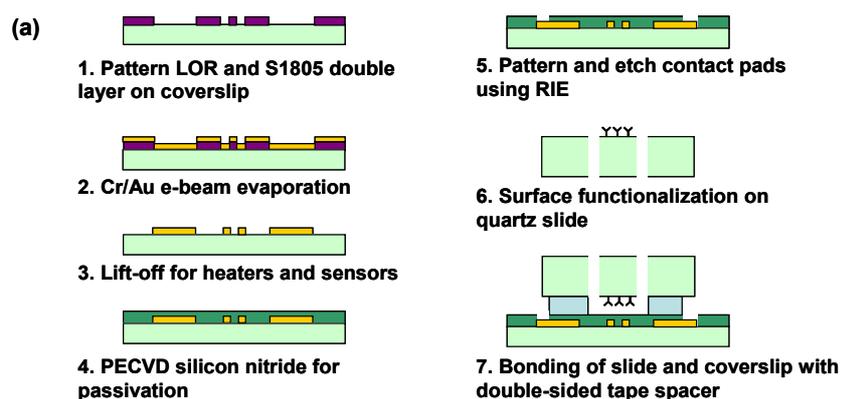
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#### Fabrication and surface functionalization

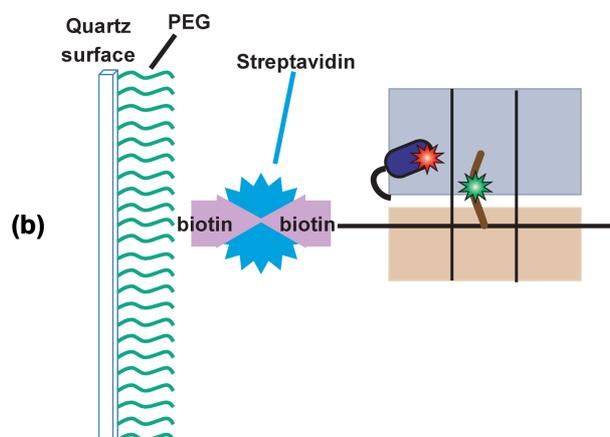
The fabrication of the microfluidic chip (Figure S1a) began on a glass coverslip with patterning a double layer of LOR 3A and Shipley S1805 photoresists (both from MicroChem, Inc.) to define the heaters and temperature sensors. A 10-nm chromium film and a 100-nm gold film were then deposited via electron-beam evaporation, and patterned with a lift-off process to form the heaters and temperature sensors. A 200-nm silicon nitride passivation layer was next deposited using plasma enhanced chemical vapor deposition (PECVD). Contact pads for the heaters and temperature sensors were defined by photolithography and exposed by reactive ion etching (RIE).

A quartz microscope slide containing drilled inlet and outlet ports was cleaned, aminosilanized, and subsequently derivatized with a mixture of *N*-hydroxysuccinimidyl (NHS) ester-derivatized polyethylene glycol (PEG) and a bifunctionalized NHS ester-PEG-biotin (Laysan Bio, Inc.) [1-3]. This multi-step treatment creates an optically clean, low background fluorescence, PEG- and PEG-biotin-derivatized quartz surface that is randomly and sparsely populated with biotin groups. Biotinylated biomolecules can then be immobilized and spatially localized using a biotin-streptavidin-biotin bridge (Figure S1b).



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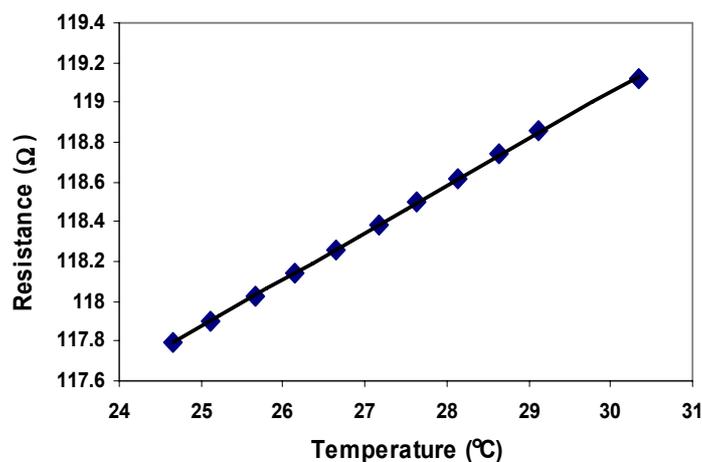
**Figure S1. (a)** Fabrication and surface functionalization processes. **(b)** Surface immobilization strategy: quartz flow cell is first passivated with a mixture of PEG and PEG-biotin and then incubated with streptavidin prior to use. PRE<sup>A</sup> ribosomal complexes carrying biotinylated-mRNA are then immobilized onto the surface of the streptavidin-derivatized flow cell via a biotin-streptavidin-biotin bridge.

### Calibration of temperature sensors

Resistive temperature sensors can be represented in terms of a linear relationship between the sensor resistance and temperature:

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

where  $R$  is the sensor resistance at temperature  $T$  and  $R_0$  the sensor resistance at a reference temperature  $T_0$ , with  $\alpha$  the temperature coefficient of resistivity (TCR) of the sensor. To determine the parameters in this relationship, we measured the sensor resistance at a series of temperatures. This is accomplished by placing the microchip in a thermal environmental chamber (Delta 9023, Delta Design) maintained at a known temperature. Typical measurement results are shown in Figure S2, from which we can observe a highly linear relationship between resistance and temperature as expected from Eq. (1). A least-squares linear fit of Eq. (1) to the measurement data allowed for the determination of  $R_0$ ,  $T_0$ , and  $\alpha$ . For example, with data shown in Figure S2, the parameters were determined to be  $R_0 = 117.795 \, \Omega$  at  $T_0 = 24.7 \, ^\circ\text{C}$ , with  $\alpha = 2.01 \times 10^{-3} \, 1/^\circ\text{C}$ .



**Figure S2.** Measured electric resistance of a temperature sensor, showing highly linear dependence on temperature.

### Preparation of pre-translocation complex analogs (PRE<sup>-A</sup>)

A single-cysteine variant of *Escherichia coli* ribosomal protein L1 was fluorescently labeled with a Cy5 acceptor fluorophore ((Cy5)L1) and reconstituted into mutant *E. coli* ribosomes lacking ribosomal protein L1 as previously described [1, 4, 5]. *E. coli* tRNA<sup>Phe</sup> was labeled with a Cy3 donor fluorophore ((Cy3)tRNA<sup>Phe</sup>) at the naturally occurring 3-(3-amino-3-carboxy-propyl) uridine residue at position 47 within the central fold, or elbow, domain of the tRNA body and aminoacylated with phenylalanine as previously described [1, 4, 5]. Ribosomes with (Cy5)L1 were enzymatically initiated onto a 5'-biotinlated mRNA and enzymatically elongated by one amino acid such that they carried fMet-Phe-(Cy3)tRNA<sup>Phe</sup> at the P site [1]. Ribosomal elongation complexes prepared in this manner were purified by sucrose density gradient ultracentrifugation [1, 4, 5] and were subsequently immobilized onto the surface of the streptavidin-derivatized microchannel.

Surface-immobilized ribosomal elongation complexes were incubated in 1 mM puromycin in Tris-Polymix buffer (50mM Tris-OAc, 100mM KCl, 15mM Mg(OAc)<sub>2</sub>, 5mM NH<sub>4</sub>OAc, 0.5mM Ca(OAc)<sub>2</sub>, 10mM 2-mercaptoethanol, 5mM putrescine, and 1mM spermidine) [1] supplemented with an oxygen scavenging system (9300 µg/mL glucose oxidase, 40 µg/mL catalase and 1% β-D-glucose)[1] and a triplet state quencher cocktail (1mM 1,3,5,7-cyclooctatetraene (Aldrich) and 1mM *p*-nitrobenzyl alcohol (Fluka)) [1, 4] for 5 min at room temperature. Puromycin is a ribosome-targeting antibiotic that mimics the 3'-terminal residue of an aminoacyl-tRNA, binds to the ribosomal A site, participates as the acceptor in the peptidyl transfer reaction, and subsequently dissociates from the ribosome, carrying the nascent polypeptide with it [1]. The resulting puromycin-reacted ribosomal complex contains a deacylated P-site tRNA<sup>Phe</sup> and serves as an analog of an authentic PRE complex which we designate as PRE<sup>-A</sup> [1].

## TIRF microscope and smFRET measurements

smFRET measurements were performed on a laboratory-built, prism-based TIRF microscope based on an inverted fluorescence microscope (Nikon TE2000-U) and using a diode-pumped solid-state 532-nm laser (CrystaLaser, Inc.) as an excitation source and a Cascade II:512B electron multiplying charge-coupled device (EMCCD) camera (Photometrics, Inc.) as a detector. Simultaneous detection of the fluorescence emission of both Cy3 and Cy5 fluorophores for 100-200 individual, spatially-resolved PRE<sup>-A</sup> complexes over an observation area of 60×120 μm<sup>2</sup> was achieved using a PlanApo 1.2 numerical aperture 60× water immersion objective (Nikon, Inc.), optically separating the Cy3 and Cy5 emission signals using a Dual-View image splitting device (Photometrics, Inc.), and simultaneously imaging the Cy3 and Cy5 emission signals on the two halves of the 512×512 pixel CCD chip within the Cascade II:512B EMCCD camera. Cy3 and Cy5 emission signals arising from single PRE<sup>-A</sup> complexes were collected at a time resolution of 50 ms/frame.

## Imaging processing and data analysis

Single PRE<sup>-A</sup> complexes were identified and Cy3 and Cy5 emission intensity *versus* time trajectories were extracted using the MetaMorph software suite (Molecular Devices) as previously described [1]. Using MATLAB, raw Cy3 and Cy5 emission intensity *versus* time trajectories were baseline corrected, corrected for bleed-through of the donor signal into the acceptor channel (typically ~7%), and used to calculate smFRET *versus* time trajectories using  $I_{Cy5}/(I_{Cy3}+I_{Cy5})$ , where  $I_{Cy3}$  and  $I_{Cy5}$  are the fluorescence intensities of Cy3 and Cy5, respectively, as previously described [1] (Figure 7a).

Dwell-time histograms for GS1→GS2 and GS2→GS1 transitions at the various temperatures were plotted as previously described [1]. Specifically, each smFRET *versus* time trajectory was first idealized using a hidden Markov model using the HaMMY software suite [6]. We then used the idealized trajectories to plot a histogram of the idealized FRET values. The data points within the first second of each smFRET trajectories were included to avoid the accumulation of FRET value at 0 due to photobleaching of the fluorophores. The two peaks apparent in the resulting histograms were fitted with Gaussian distributions using user-specified initial values of 0.1 and 0.7 (Figure 7b), corresponding to the approximate FRET values of GS1 and GS2, respectively. The center and the full width at half height of the resulting Gaussian distributions were used to determine the thresholds for each FRET state. Using these thresholds to define the GS1 and GS2 states in the idealized trajectories, transition events were identified and dwell time spent in GS1 or GS2 before transitioning into GS2 or GS1 were extract, respectively. Population histograms of the dwell times spent in GS1 or GS2 were then plotted as a function of the dwell time [1] (Figures 7c and 7d).

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