SINGLE-MOLECULE IMAGING DEVICE USING LOCALIZED EVANESCENT ILLUMINATION IN POLYMERIC NANOHOLES

Takao Ono[1,2,3], Ryo Iizuka[3,4], Takanori Akagi[1,3], Takashi Funatsu[3,4], and Takanori Ichiki[1,3]

[1] School of Engineering, The University of Tokyo, Japan,

[2] Research Fellow, Japan Society for the Promotion of Science, Japan

[3] Core Research of Evolutional Science and Technology, Japan Science and Technology Agency, Japan,

[4] School of Pharmaceutical Science, The University of Tokyo, Japan

ABSTRACT

A single-molecule imaging device using polymeric nanoholes (PNH) was developed. It localizes an excitation volume at the bottom of its nanoholes for the reduction in background noise. Owing to this reduction, the number of types of enzymatic reactions visualized at single-molecule level in PNH was estimated to be approximately 2.5 times greater than that observed by total internal reflection fluorescence microscopy (TIRFM). On the basis of this estimate, PNH were designed and single protein molecule was imaged in PNH of 200 nm diameter and 200 nm depth.

KEYWORDS

Single-molecule imaging, Nano/Microfabricaton, Fluoropolymer, Total internal reflection fluorescence microscopy

INTRODUCTION

Single-molecule imaging is recognized to elucidate mechanisms of biochemical reactions such as enzymatic reactions. To detect weak fluorescence from single molecules, background noise should be markedly reduced. The use of an evanescent field produced by total internal reflection fluorescence microscopy (TIRFM) effectively reduces an illumination volume and is a conventional method for noise reduction in single-molecule imaging. However, in TIRFM, the illumination is only limited in the depth direction and the background noise becomes a serious issue at a high fluorescent molecule concentration. Otherwise, Zero-mode waveguide (ZMW) which further confines the illumination in its metal nanohole is also applied to single-molecule imaging, but there are some concerns about light scattering and metal elusion in ZMW. In this paper, the excitation region was three-dimensionally confined in polymeric nanoholes (PNH) with a diameter of less than the optical diffraction limit (Fig. 1). An amorphous perfluoropolymer, Cytop™ (Asahi Glass Co., Ltd.), which has a refractive index of 1.34, similar to that of water, was used for creating PNH. Total reflection was observed at both the glass/liquid and glass/Cytop interfaces and light scattering is vanishingly low.

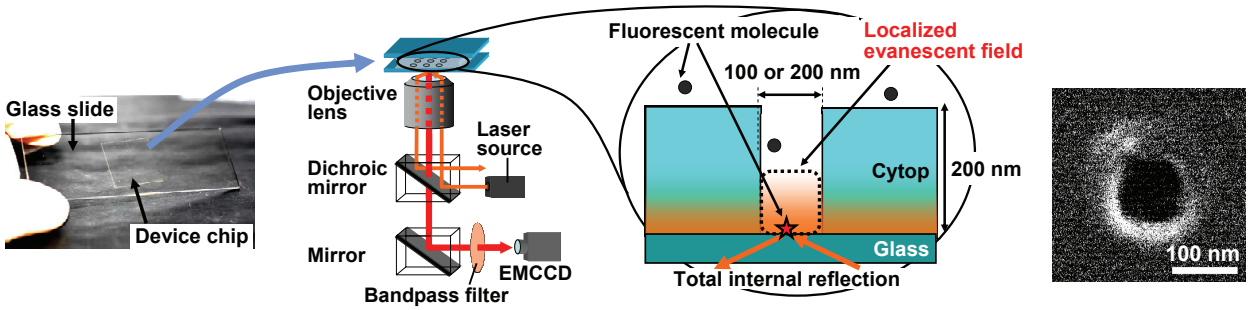

Figure 1. Schematics of PNH. The excitation region is highly confined at the bottom of the PNH. A scanning electron microscope (SEM) image of single nanohole is in the right.

PRINCIPLES AND DESIGN OF THE DEVICE

PNH were designed on the basis of their theoretical performance for noise reduction. The Michaelis constant K_M, an index of the instability of an enzyme-substrate complex, is a useful parameter for evaluating the capability of PNH to visualize enzymatic reactions [1]. A typical enzymatic reaction scheme and K_M as an equilibrium dissociation constant of the enzyme-substrate complex are shown as follows:

$$E+S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E+P \qquad (1)$$

$$K_M = \frac{k_{-1}+k_2}{k_1} \qquad (2)$$

where k_x (x=-1, 1, 2) is the rate constant for the reaction of enzyme (E) and substrate (S) resulting in product (P). To visualize an unstable complex with a high K_M, a high concentration of the fluorescent substrate (Generally, enzyme

molecules are fixed on the device substrate in single-molecule imaging.) and/or prolonged observation are required. In PNH, the three-dimensional confinement of the excitation region in the nanoholes and shielding evanescent light by the polymeric layer were considered to meet these two requirements. Figure 2 shows schematics of performance of PNH.

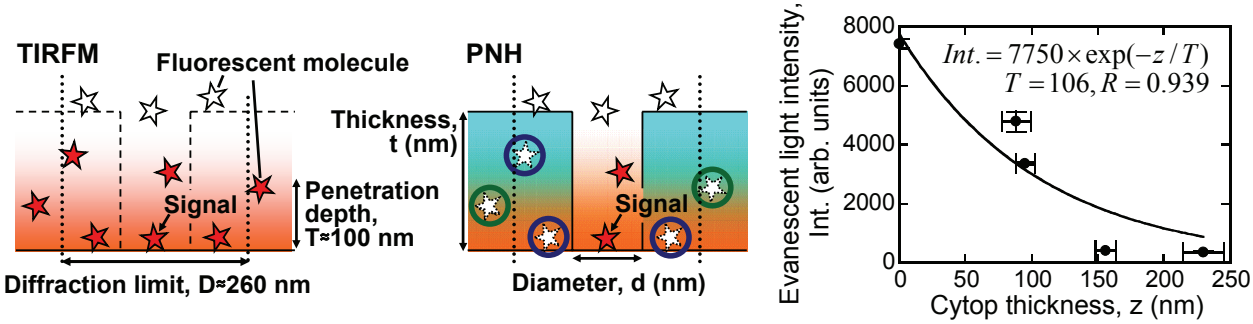

Figure 2. Schematics of the improvement of S/N ratio in PNH in comparison with TIRFM. Red stars, "Signal", and circled stars indicate light-emitting fluorescent molecules, a targeted single molecule (such as a fluorescent substrate binding to enzyme), and fluorescent molecules excluded by the device, respectively. An exponential decay of evanescent light shielded by the polymeric layer is also shown in the right.

Some fluorescent molecules inside the diffraction limit D (≈260 nm) are excluded by PNH of diameter d (Black circles). The excitation volume is reduced to d^2/D^2 and "Signal" can be identified at a D^2/d^2 times higher concentration of fluorescent molecules than that in the case of TIRFM. Some fluorescent molecules outside D are excluded by PNH of depth t (green circles). Evanescent light decays to 1/e at a distance T (≈100 nm) from the glass surface. Hence, the background and its fluctuation decrease to $1/e^{t/T}$ and $1/e^{t/2T}$, respectively, and the S/N ratio of "Signal" to background noise is improved to $e^{t/2T}$ times higher than that in the case of TIRFM. The improved S/N ratio enables the reduction in excitation intensity by $1/e^{t/2T}$ and the extension of fluorescence lifetime by $e^{t/2T}$. As a result, on the value of K_M, the performance of PNH in comparison with that of TIRFM was formulated as Eq. 3.

$$K_{M,PN} / K_{M,TIRFM} = \frac{D^2}{d^2} \exp\left(t / 2T\right) \qquad (3)$$

Here, $K_{M,PN}$ and $K_{M,TIRFM}$ are maximum value of K_M with which reaction can be imaged at the single-molecule level using PNH and TIRFM, respectively. According to Eq. 3, in PNH (d=100 nm, t=200 nm), enzymatic reactions with K_M 20 times higher than that in the case of TIRFM can be visualized, that is, an approximately 2.5-fold greater number of types of enzymatic reactions can be visualized [1].

DEVICE FABRICATION

One million PNH of 100 and 200 nm diameter and 200 nm depth were fabricated on a chip by the thermal nanoimprinting using cryo-etched nanomold (Fig. 3). The nanomold bore more than 100 times of use. After nanoimprinting, thin residual layer was etched using oxygen plasma [2]. The oxygen plasma also hydrophilized the surface of the PNH and reduced the nonspecific adsorption of fluorescent molecules on PNH to 1/5-1/6 for noise reduction (Fig. 4(a)). The nonspecific adsorption was further reduced to approximately 1/4 by surface modification using a polyethylene-glycol-containing surfactant, Pluronic™ F-108 (BASF Corp.) (Fig. 4(b)).

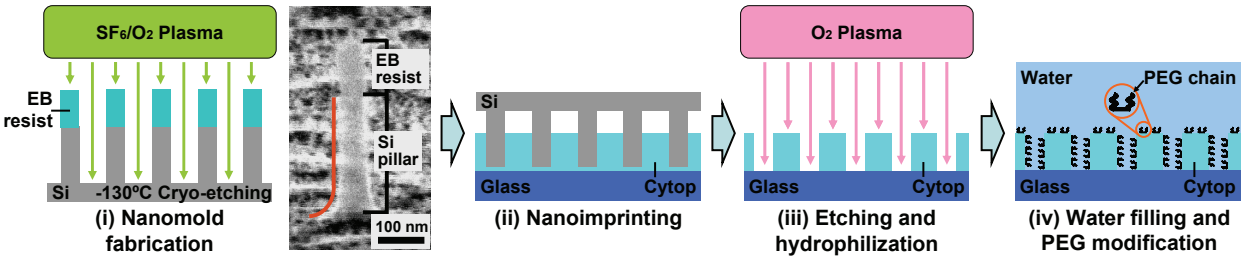

Figure 3. Schematics of the fabrication process.
(i) High-aspect-ratio nanopillars of 100 or 200 nm diameter and 300 nm height was fabricated by electron beam lithography and cryo-etching at -130ºC. SEM image of a nanopillar of 100 nm diameter is also shown.
(ii) The nanomold was pressed against perfluoropolymer-coated (t=200 nm) glass by applying a force of 1.0 kN at 125ºC for 3 min.
(iii) After nanoimprinting, the thin residue layer that formed at the bottom of nanoholes was removed by oxygen plasma treatment, which simultaneously hydrophilized the device's surface.
(iv) A PEG-containing surfactant was introduced into the nanoholes for PEG modification.

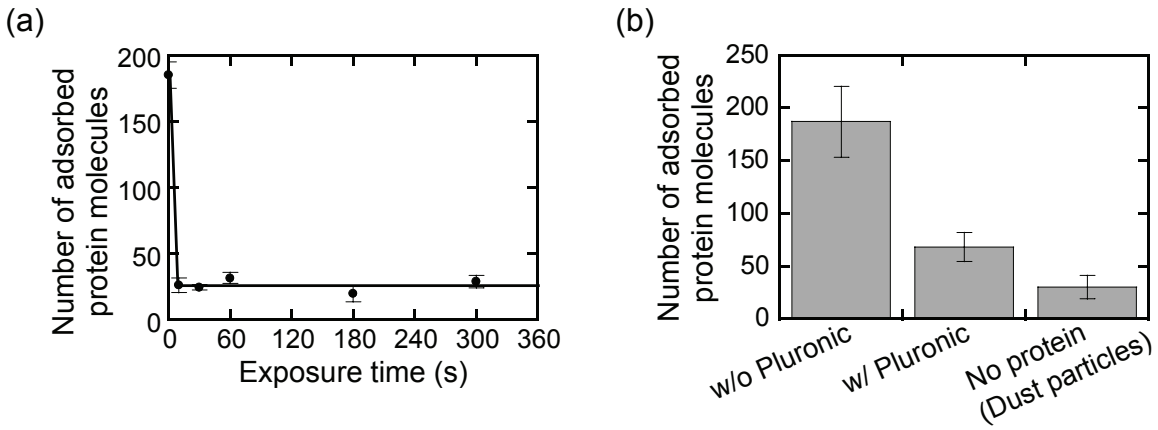

Figure 4. Effects of (a) exposure time of oxygen plasma (150 W RF, 30 mTorr) and (b) surface blocking using PEG-containing surfactant on the number of absorbed Cy5-GroEL molecules in the field of microscopic view. The concentrations of Cy5-GroEL were (a) 20 pM and (b) 30 pM. Results show averages of five points along the y axis. Error bars: one standard deviation.

SINGLE-MOLECULE FLUORESCENCE IMAGING

On the basis of the material, the device design and the fabrication method, PNH were applied to single-molecule imaging (Fig. 5). Single Cy5-modified protein molecule was imaged in PNH of 200 nm depth and 200 nm diameter. Consequently, PNH are promising tool for single-molecule imaging.

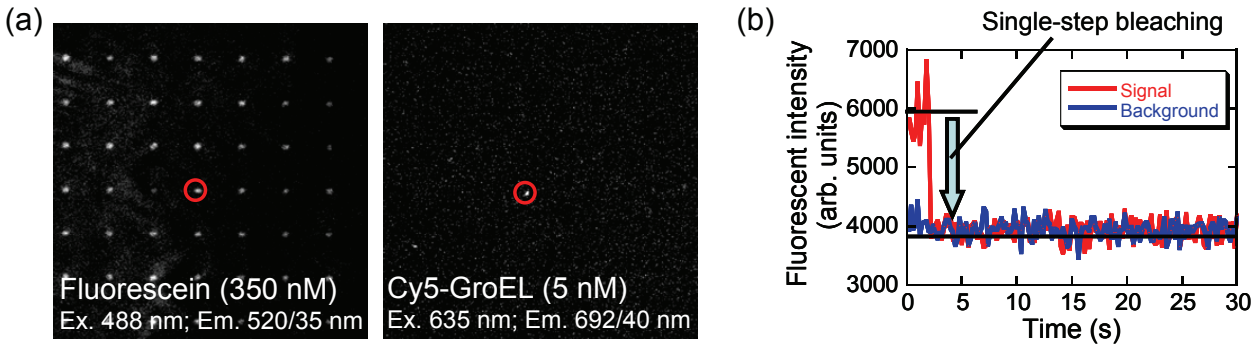

Figure 5. Single-molecule fluorescence imaging of Cy5-modified chaperonin GroEL molecule in PNH (d=200 nm, t=200 nm).
(a) Fluorescence images of PNH. Position of nanoholes were identified using fluorescein.
(b) Single-step bleaching in PN (red circle in (a)), which indicates a single Cy5-GroEL molecule.

CONCLUSIONS

A single-molecule imaging device using polymeric nanoholes was developed. It localizes an excitation volume at the bottom of its nanoholes for the reduction in background noise. Owing to this reduction, the number of types of enzymatic reactions visualized at single-molecule level in PNH (200 nm depth and 100 nm diameter) was estimated to be approximately 2.5 times greater than that observed by TIRFM. On the basis of this estimate, PNH were designed and single protein molecule was imaged in PNH of 200 nm diameter and 200 nm depth.

REFERENCES

[1] K. T. Samiee, M. Foquet, L. Guo, E. C. Cox and H. G. Craighead, Biophys. J., **88**(3), pp. 2145-2153, (2005).
[2] T. Ono, R. Iizuka, T. Akagi, T. Funatsu and T. Ichiki, μTAS 2011, W2F, pp. 1801-1803, (2011).

CONTACT

Takao Ono ono@bionano.t.u-tokyo.ac.jp http://bionano.t.u-tokyo.ac.jp/ono.html