

Single-Molecule Fluorescence Imaging using Polymeric Nanoholes beyond Diffraction Limit

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1. 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. To further reduce the excitation volume, in this paper, the excitation region was three-dimensionally confined in "Polymeric nanoholes (PNs)" with a diameter of less than the optical diffraction limit (Fig. 1).

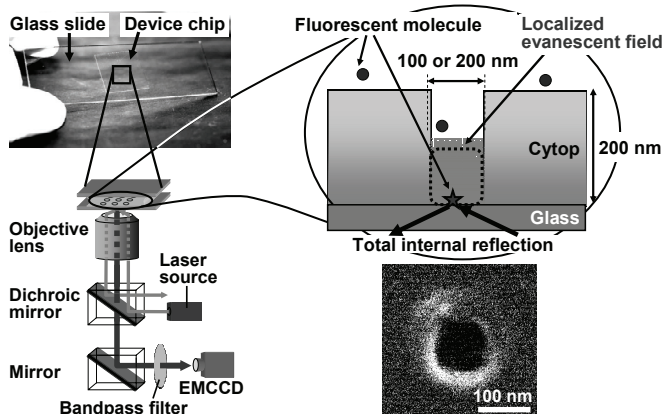


Figure 1. Schematics of the polymeric nanohole (PN). The excitation region is highly confined at the bottom of the PN. A SEM image of a PN is in the lower right.

2. Device materials and fabrication methods

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 PNs, and total reflection was observed at both the glass/liquid and glass/Cytop interfaces. One million PNs of 100 and 200 nm diameter and 200 nm depth were fabricated on a chip by the

thermal nanoimprinting using cryo-etched nanomold. After nanoimprinting, thin residual layer was etched using oxy-

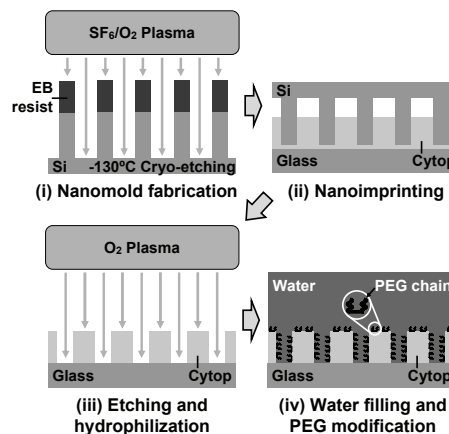


Figure 2. Schematics of the fabrication process. (i) High-aspect-ratio nanopillars of 100-200 nm diameter and 300 nm height was fabricated by electron beam lithography and cryo-etching at -130°C . (ii) The nanomold was pressed against perfluoropolymer-coated ($t=200$ nm) glass by applying a force of 1.0 kN at 125°C for 3 min. (iii) After nanoimprinting, the thin residue layer that formed at the bottom of apertures 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.

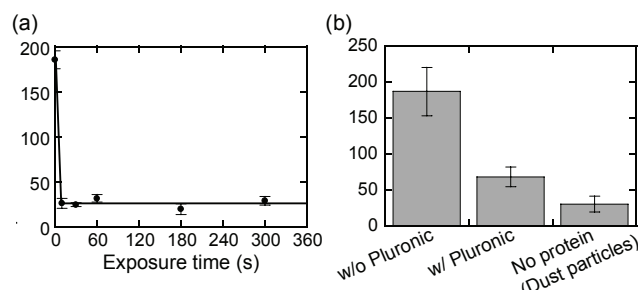


Figure 3. Effects of (a) exposure time of oxygen plasma (150 W RF, 30 mTorr) and (b) surface blocking using PEG-containing surfactant on the number of absorbed Cy5-GroEL molecules in the field of microscopic view (y axis). The concentrations of Cy5-GroEL were (a) 20 pM and (b) 30 pM. Results show averages of five points along the y axis. Error bars: one standard deviation.

gen plasma (Fig. 2) [1]. The oxygen plasma also hydrophitized the surface of the PNs and reduced the nonspecific adsorption of fluorophores on PNs to 1/5-1/6 for noise reduction (Fig. 3(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. 3(b)).

3. Principles and design of the device

PNs were designed on the basis of their theoretical performance. The Michaelis constant K_M , an index of the instability of an enzyme-substrate complex [2], is a useful parameter for evaluating the capability of PNs to visualize enzymatic reactions. To visualize an unstable complex with a high K_M , a high concentration of the fluorescent substrate and prolonged observation are required. In PNs, 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 4 shows schematics of performance of PN. Black stars, “Signal”, and circled stars indicate light-emitting fluorophores, a targeted single molecule, and fluorophores excluded by the device, respectively.

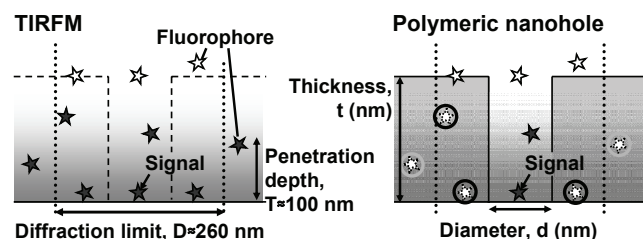


Figure 4. Schematics of the improvement of S/N ratio in PNs.

Some fluorophores inside the diffraction limit D (≈ 260 nm) are excluded by PN 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 fluorophores than that in the case of TIRFM. Some fluorophores outside D are excluded by PNs of depth t (gray circles). Evanescent light decays to $1/e$ at a distance T (≈ 100 nm) from the glass surface [1]. 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 PNs in comparison with that of TIRFM was formulated as Eq. 1.

$$K_{M,PN} / K_{M,TIRFM} = \frac{D^2}{d^2} \exp(t/2T) \quad (1)$$

Equation 1 is for evaluating the performance of PNs based on the scheme above. $K_{M,PN}$ and $K_{M,TIRFM}$ are maximum value of K_M with which reaction can be imaged at the single-molecule level using PNs and TIRFM, respectively. In PNs ($d=100$ nm, $t=200$ nm), enzymatic

reactions with K_M 20 times higher than that in the case of TIRFM can be visualized.

According to Eq. 1, in 100-nm-diameter and 200-nm-deep PNs, an enzymatic reaction with K_M 20 times higher than that observed in TIRFM is imaged at the single-molecule level, that is, an approximately 2.5-fold greater number of types of enzymatic reactions can be visualized [2].

4. Single-molecule fluorescence imaging

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

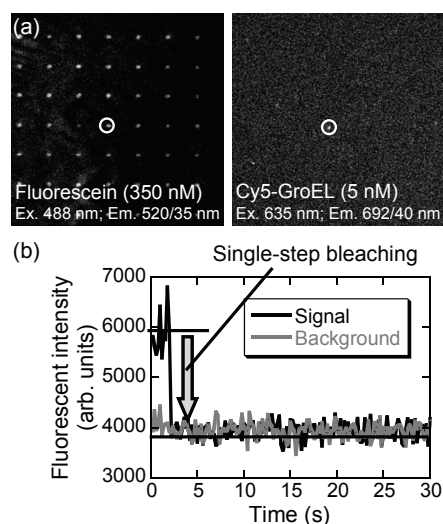


Figure 5. Single-molecule fluorescence imaging of Cy5-modified chaperonin GroEL molecule in PNs ($d=200$ nm, $t=200$ nm). (a) Fluorescence images of PNs. Position of nanoholes were identified using fluorescein. (b) Single-step bleaching in PN (white circle in (a)), which indicates a single Cy5-GroEL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

5. 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 PNs (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, PNs were designed and single protein molecule was imaged in PN of 200 nm diameter and 200 nm depth.

References

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- [2] K. T. Samiee, M. Foquet, L. Guo, E. C. Cox and H. G. Craighead, *Biophys. J.*, **88**(3), (2005) 2145.