Development and Evaluation of Local Illumination Device beyond Diffraction Limit using Polymeric Nanohole Array

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1. Introduction

A localized illumination is an effective approach for single-molecule fluorescence imaging to obtain a high signal/noise ratio due to a reduction in background noise. Single-molecule imaging is currently recognized as a promising approach for elucidating biomolecular functions and biochemical reaction mechanisms that cannot be revealed in the statistically averaged behaviors of massive molecules. Total internal reflection fluorescence microscopy (TIRFM) is a representative method for this purpose. TIRFM confines its illumination region using an evanescent field at the glass/liquid interface. However, at a high fluorescent molecule concentration, the background noise becomes a serious issue because the illumination region is limited only in the vertical direction in TIRFM. Moreover, a metal nanohole array, called the zero-mode waveguide (ZMW), further limits the illumination volume at the bottom of the nanoholes, but it has also some issues, such as the alteration of the fluorescence lifetime or nonradiative damping of fluorescent molecules due to the electron leakage through a conductive device material [1], or the temperature increase caused by light absorption. To overcome these issues, a polymeric nanohole array was fabricated using an amorphous perfluoropolymer, Cytop™ (Asahi Glass Co., Ltd.) (Fig. 1).

In this device, illumination is three-dimensionally confined by the evanescent field and polymeric nanoholes. Cytop hardly causes the electrical or exothermal issues because of its electrical insulation and high optical transparency. Furthermore, the leakage of the laser beam is avoidable since Cytop has a refractive index similar to that of water (1.34), and the total reflection occurs at both glass/liquid and glass/Cytop interfaces.

2. Experimental and Results

The depth of nanoholes was determined from the penetration depth of the evanescent field. From the effects of Cytop thickness on fluorescence intensity, a 200 nm depth was estimated to be adequate for reducing the background noise (Fig. 2).

Fig. 2 Effects of Cytop thickness on background fluorescence intensity. Cytop layers with various thicknesses were coated on glass substrates, and 100-nM fluorescent molecules (Alexa647-ATP) were introduced into them. Fluorescent molecules were then illuminated in an evanescent field (λ=635 nm). In the Cytop layers, evanescent light intensity exponentially decayed from the glass surface. The background fluorescence emitted by fluorescent molecules attenuated to the noise level with a Cytop layer of more than 200 nm thickness. Averages of 3 or 4 points along x-axis and 5 points along y-axis. Error bars: one standard deviation.
To ensure a smaller illumination volume than that of TIRFM, the aperture diameter was required to be less than the diffraction limit. In this study, the diffraction limit was calculated to be approximately 270 nm from the 635 nm emission wavelength and 1.45 numerical aperture of the objective lens used. Therefore, a 100-nm-diameter aperture was designed.

The device was fabricated by thermal nanoimprint followed by the oxygen plasma etching of a thin residual layer (Fig. 3). The perfluoropolymer was hydrophilized during the oxygen plasma etching process, and therefore, a water solution was easily introduced to the fabricated nanoholes.

Fig. 3 Schematics of fabrication process. (i) A 200-nm-thick Cytop film was spin-coated to glass substrate. (ii) A cryo-etched micromold was pressed against the perfluoropolymer-coated glass using a nanoimprinter by applying a force of 1.0 kN at 125ºC for 3 min. (iii) After nanoimprinting, a thin residue layer remained at the bottom of apertures. (iv) These layers were removed by oxygen plasma treatment, which simultaneously hydrophilized the device surface.

To avoid silicon mold breakage during demolding, the mold was etched by a cryogenic process at -130ºC using a SF6/O2 mixture plasma for a smooth surface that cannot be obtained by a standard micromachining process, such as the Bosch process (Fig. 4a). The above-mentioned nanofabrication processes resulted in high-aspect-ratio nanoholes of 100 nm diameter and 200 nm depth (Fig 4b).

Figure 5 shows the fluorescent image of the Cy5 fluorophore in the device. The background noise was effectively reduced outside the apertures.

![Fluorescent Image of Cy5](image)

**3. Summary**

A novel imaging device with a localized illumination volume beyond the diffraction limit was fabricated by the high-aspect-ratio nanofabrication of a perfluoropolymer. The device had nanoimprinted perfluoropolymer apertures whose diameter and depth were 100 nm and 200 nm, respectively, and was used with a TIRFM. This device is applicable to single-molecule imaging at a high concentration of fluorescent molecules and free from electron leakage and thermal disturbance.

**Reference**