Fabrication of Small Fluorescence Scale Patterns by Electron Beam Drawing Using Polymer Film Containing Fluorescence Dye

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We report the fabrication of a resolution-evaluation chart and a scale for a fluorescence microscope. Small fluorescence patterns were fabricated by irradiating an electron beam (EB) in a thin film prepared by dispersing fluorescence dye (rhodamine 590) in poly(methyl methacrylate) (PMMA) and by development to remove EB-irradiated parts. As a result, the fluorescence emission pattern of nanometer order (a line width of 110 nm and a line interval of 370 nm) with sufficient fluorescence intensity was obtained, and a 1 μm bright fluorescence scale for the laser scanning fluorescence microscope was fabricated. These fluorescence patterns are handled easily and are effective for measurement using a fluorescence microscope. © 2006 The Optical Society of Japan

Key words: fluorescence scale, fluorescence microscope, resolution evaluation, high-resolution microscope

1. Introduction

With the rapid progress of biotechnology, the technological means of observing a small domain without damaging to biomolecules is strongly desired. An optical microscope is believed to be the most suitable tool for this because of its capability to observe in solution with little damage to biomolecules.1) The demand for observation of a smaller domain using an optical microscope has energized research on development of a scanning fluorescence microscope or nonlinear microscope that can achieve highly resolved imaging and the analysis of smaller biomolecules.2–8) A high-resolution fluorescence imaging technique requires development of a small fluorescence pattern to evaluate the resolution (hereafter referred to as a “resolution-evaluation chart”). Resolution is defined as the minimum distance between two features that can be resolved with a certain contrast.9) The two-line chart shown in Fig. 1(a) is useful to roughly evaluate the resolution of a microscope. When a large diameter beam scans fluorescence lines, two line images overlap and the lines cannot individually be discriminated; however, when scanned with a small diameter beam the lines can be discriminated.

In the fluorescence microscope no resolution-evaluation chart exists except for a microfabrication chart that is very expensive and difficult to handle because the observer has to drip fluorescence material on the chart surface. Fluorescence beads can be used to evaluate the resolution, but it is difficult to control the diameter or alignment of the particles. Moreover, if the fluorescence scale can be fabricated on the substrate of the sample, we can directly measure sample size at the same time [Fig. 1(b)]. Recently, a nonlinear microscope two-photon absorption microscope has been used for the observation of biomolecules and the demand for fluorescence scales has increased. Particularly, the fabrication of such fluorescence scales are strongly desired in the research field, where the size of microorganisms is measured, because the evaluation time is expected to be shortened by using such scales.

Fukushima et al. reported the fabrication of fluorescence patterns for memory applications by means of the electron-beam (EB) lithography in dye-doped sol–gel films.10) Size of the pattern is the order of several micrometers, but contrast of the pattern is inadequate. Nanoprinting or a self-organizing process for fabricating micro-nano structures are suitable for the mass production,11,12) but it is difficult to fabricate many kinds of patterns and the processing accuracy is still inferior compared with EB lithography. Advantages of EB drawing are the easy fabrication of complicated patterns controlled by a personal computer.13,14)

In this paper, we describe a resolution-evaluation chart and a fluorescence scale, both of which are fabricated simply and handled easily. First of all, thin films are formed, which are prepared by dispersing fluorescence-dye (rhodamine derivative) in the polymer matrix [poly(methyl methacrylate); PMMA]. After the EB irradiation, the line and scale patterns are formed by removing EB-irradiated parts. As a result of optimization of the experimental conditions, we succeeded in forming of small fluorescence patterns of sub-micrometer size using dye-doped polymer films. We also fabricated a fluorescence scale to aid in determining the size and number of samples to be measured. Detailed methods are described in the following section.

2. Experimental

2.1 Preparation of sample film

Fabrication processes of the fluorescence patterns used in this study are shown in Fig. 2. The PMMA [molecular weight: (7.0–7.5) × 10⁴] and rhodamine 590 (molecular weight: 543.1) were mixed with chloroform solution. This solution was spin-coated onto glass substrates, 11 × 13 mm² in size which were cut out from a slide glass (size: 76 × 26 mm², thickness: 1 mm). Substrates were scrubbed with neutral detergent and washed by with ultrasonic wave in a methanol and acetone solution [Fig. 2(a)]. Conditions of the rotational speed, rotation time, and concentration of PMMA
Fig. 1. Illustration of (a) a resolution-evaluation chart and (b) a small scale pattern for the fluorescence microscope.

to chloroform were: constancy (5000 rpm, 30 s, 0.6 mol/l for a line pattern and 0.3 mol/l for scale patterns, respectively) [Fig. 2(b)]. The film thickness was 1.2 μm for a line pattern. For scale patterns, the film thickness was adjusted to 0.3 μm to avoid a blurred image based on the focus depth. The concentration of dye to PMMA was adjusted to 0.5 wt % for a sample of 1.2 μm thickness. To form high luminance fluorescence substrates for samples 0.3 μm thick, the concentration was adjusted to 2.0 wt %. After the spin coat, annealing was done for 1 h at 160°C, which is higher than the glass transition temperature of PMMA in order to form a uniform film. Before EB irradiation, a coating of the electroconductive agent (Showa Denko: s-300) was applied.

2.2 EB irradiation and evaluation method

Figure 3 shows a schematic illustration of EB drawing equipment used in this experiment; it consists of the source of EB, an EB control system, a pattern generator, and a computer. Using the EB irradiation technique, 100 nm three-dimensional desired patterns are created on the sample surface for desired magnification [Fig. 2(c)]. In forming the line pattern, EB drawing equipment that partially improved the scanning electron microscope (SEM; JEOL JSM-5300) was used. A special machine for EB lithography [ELIONX ELS-3300LB(II)] was used for the nanometer line pattern, and for a scale pattern, equipment combining a field emission scanning electron microscope (FE-SEM; JEOL JSM-6335F) with a pattern generator was used. As a parameter, the energy of EB (acceleration voltage) and the amount of electrons (exposure time and beam current) were changed. After EB irradiation, the electroconductive coating agent was removed by pure water. The patterns were formed by the removal of the EB-irradiated parts using the developer (Tokyo Ohka Kogyo OEBR-1000 developer, 25°C, 4 min) [Fig. 2(d)]. Two-line patterns were observed by fluorescence microscope (FM), NIKON ECLIPS E600
with an excitation wavelength of 510–560 nm, a fluorescence wavelength of >590 nm and a detector which was a charge-coupled device (CCD) of a digital camera on the market. Scale patterns were observed by a laser-scanning-fluorescence microscope (LSFM), ZEISS LSM510. The excitation wavelength was 488 nm, fluorescence wavelength was >505 nm and the detector was a photomultiplier. Using fluorescence micrographs, the contrast and brightness of the pattern were evaluated. The contrast ratio (CR) was defined as the ratio of fluorescence intensity of an emission area \( I_{\text{max}} \) and a center area between emission area \( I_{\text{min}} \): \( \text{CR} = I_{\text{max}} / I_{\text{min}} \).

3. Results

The pattern of a two-line structure was formed as a fundamental pattern, and the EB drawing conditions were examined. The acceleration voltage, exposure time, and beam current was 20 kV, 2 µs, and 200 pA, respectively. Figure 4 shows the results of a fluorescence two-lines pattern [(a) SEM image and (b) fluorescence micrograph]. Figure 4(a) shows that a two-line pattern fabricated with a line width of 2.0 µm and a line interval of 5.2 µm consisting of fluorescence film. A clear pattern can be easily observed in Fig. 4(b). The value of CR was 6.0 [Fig. 4(c)]. It was found that the two lines were clearly separated and the contrast was sufficient.

Figure 5 shows the LSFM images of narrower line and space patterns [fringe spacing: (a) 2.0, (b) 1.6, (c) 1.2, and (d) 0.8 µm]. For the pattern of fringe spacing at 0.8 µm, a clear fluorescence pattern was observed [Fig. 5(d)]. Moreover, we succeeded in fabricating an even smaller and uniformity two-line pattern (line width: 110 nm, line interval: 370 nm, pattern height after development: ∼1 µm). Figure 6 shows the SEM image and the fluorescence intensity profile of the smaller two-line pattern; two lines can be seen to have been physically formed [Fig. 6(a)]. This time, the resolution of the FM we used was set to 1.2 µm because wavelength of fluorescence was 600 nm and the numerical aperture of the objective lens was 0.3. Therefore, two clear peaks were not observed in the fluorescence intensity curve, however, it was found that sufficient fluorescence intensity was obtained even for a 100 nm small pattern [Fig. 6(b)]. This result indicates that this pattern supports a rough evaluation of the resolution of the microscope.

Based on these results, we attempted the fabrication of various fluorescence patterns such as lattice type, wedge type, and scale type. The fluorescence micrographs of these patterns are shown in Fig. 7. Each pattern was designed in a 100 x 100 µm² area and fabricated on the same sample [Fig. 7(a)]. Five minutes or less was taken to draw the pattern in the area of 100 x 100 µm². For a lattice type pattern, size of the main square lattice and sub square lattice was 10 and 5 µm, respectively. The line width of the main lattice was 1 µm and the sub lattice was 0.5 µm [Figs. 7(a)-(c) and 7(b)]. The wedge type pattern consisted of two-line pairs that expand radially from the center in 8 directions and the centerline interval has been extended to 2 µm [Figs. 7(a)-(γ), 7(c), and 7(d)]. For instance, a lattice type pattern is useful to count the number of microorganisms on the filtration filter in a specific area. A wedge type pattern is useful for correction of an optical axis of a microscope. The FE-SEM image and LSFM image of the fabricated scale pattern are shown in Figs. 8(a) and 8(b), respectively. For a scale pattern, we assume observation by LSFM with high resolution. Using an objective lens with high numerical aperture (1.3) and light source of λ = 600 nm, the theoretical value of resolution becomes about 0.3 µm. Therefore, to observe it clearly, a minimum unit of the scale was adjusted to 1 µm. From Fig. 8(a), the design pattern is physically fabricated. The fluorescence scale with a minimum unit of 1 µm was observed clearly with high contrast (CR = 8.3).
Fig. 5. Fluorescence microscope images of line and space patterns. Fringe spacing (Λ) is (a) 2.0, (b) 1.6, (c) 1.2, and (d) 0.8 μm, respectively.

Fig. 6. Observation results of fluorescence small two lines pattern: (a) SEM image and (b) fluorescence intensity.

This value provides enough contrast for observation by fluorescence microscope. We also fabricated a smaller fluorescence scale of 0.5 μm, which is close to the resolution (0.3 μm). Fluorescence intensity and the value of CR were decreased compared with the scale of 1 μm, however, the fluorescence pattern was observed clearly. Such a fluorescence scale is thought to be very important in the observation of biomolecules.

Fig. 7. Fluorescence microscope images of various patterns: (a) whole image, (b) a lattice type pattern [magnified image of (α)], (c) a wedge type pattern [magnified image of (γ)], and (d) an expanded image of (c).
4. Conclusions

In this paper, we formed a small fluorescence pattern by irradiating EB in a thin film that was prepared by dispersing fluorescence dye in PMMA with the aim of fabricating a resolution-evaluation chart and fluorescence scales. A fluorescence two-line pattern (a line width of 110 nm and a line interval of 370 nm) with sufficient fluorescence intensity was fabricated. This result indicates that this two-line pattern aids in roughly evaluating the resolution of the microscope. Regarding fluorescence scales, we fabricated lattice, wedge and scale patterns that were able to measure sub-μm size, advantages of these scales are easy fabrication and handling. They are also effective in observing biomolecules by the fluorescence microscope and in correcting the optical axis of the microscope. In future work, photobleaching of the fluorescence dye is a problem that remains to be solved. We are investigating fabrication of fluorescence patterns using materials with excellent photostability.

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