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Article / Book Information

題目(和文)	クライオ遠視野顕微鏡による 蛍光体のナノメートル確度での相対位置決定
Title(English)	Cryogenic far-field localization microscopy of individual fluorophores with nanometer accuracy
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種別(和文)	論文要旨
Type(English)	Summary

（博士課程）
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論 文 要 旨

THESIS SUMMARY

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申請学位（専攻分野）： Academic Degree Requested	博士 Doctor of	（ 理学 ）
指導教員（主）： Academic Supervisor(main)	松下 道雄	
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要旨（英文 800 語程度）
Thesis Summary (approx.800 English Words)

One of subjects of molecular biology is to understand biochemical processes of cell in terms of chemical reactions of individual biomolecules. Among microscopic methods for non-invasive observation of cells, far-field fluorescence microscopy is unique in its high sensitivity. While the sensitivity has reached to the ultimate level of single molecules, spatial resolution of fluorescence microscopy is limited by diffraction of the light in use (around 700 nm), which is much larger than the size of biomolecules. If the three-dimensional spatial precision is improved to an angstrom level, various molecular arrangements in the cell can be visualized on an individual basis. This thesis represents development of cryogenic far-field fluorescence localization microscopy with nanometer accuracy.

By designing a mechanically stable cryogenic microscope, the imaging stability of the setup with an exposure time of 5 minutes reached 0.05 nm in standard deviation at a temperature of 1.8 K in super-fluid helium. Optically, this setup is reflecting microscope with a numerical aperture of 0.99. The essential optics to realize the performance is the reflecting objective developed by our laboratory. This objective consists of a pair of mirrors made on a single piece of fused silica. Monolithic design of the objective guarantees the optical alignment of the mirrors against temperature cycling between room temperature and liquid-helium temperatures. For fluorescence imaging, the most crucial alignment in the setup is the position of the objective with respect to the sample. The objective and the sample were fixed to a monolithic holder and were placed in super-fluid helium at 1.8 K.

In 3D localization microscopy of individual fluorophores, different methods of localization were used for the lateral (xy) and axial (z) positions. For the xy-localization, the fluorescence image on the focal plane was taken by a charge coupled device (CCD) camera, and the position of the fluorophore was determined as the centroid of the CCD image. The fluorescence intensity of individual fluorophores shows on/off behavior, which is called blinking. The observed image is free from noise due to blinking, because the CCD camera detects all of the positions on the image plane simultaneously.

The z-position of the fluorophore is determined by defocus imaging, which uses images taken at an out-of-focus position. The defocus image consists of the xy-image of the point-spread function (PSF) and diffraction rings. Since information about the z-position is contained in the width of PSF, thee image was fitted to a model function of PSF. Because the diffraction rings were found

to mislead the fitting to a false minimum, the diffraction rings were removed by on-chip binning of the CCD camera or frequency filtering in the reciprocal space. The angstrom-precision of our cryogenic localization microscopy was demonstrated by localization measurements of fluorescent molecule (ATTO647N). An individual ATTO647N at 1.8 K was localized with standard errors of 0.53 nm (x), 0.31 nm (y), and 0.90 nm (z). To build one image, 10^6 fluorescence photons from the molecule were accumulated in 5 min.

The localization measurement of individual ATTO647N was repeated on the same one fluorophore and the reproducibility of the localized position was examined. The reproducibility of the measurement is called precision. The localization precision of a single fluorophore reached to one-nanometer level. The next step towards the nanometer-level microscopy of whole cell is to localize two or more fluorophores and reconstitute the 3D arrangement of the fluorophores in the sample. The correctness of measurement of relative positions is called accuracy.

As an example of biomolecule labeled with two fluorescent dyes, we designed a dual-labeled double-stranded (ds) DNA molecule consisting of 30 base pairs (bp). The 5' and 3' ends of the DNA was labeled with an infrared and a red dyes, respectively. The NIR dye was Alexa Fluor 750 (Ax750) or sulfo cyanine 7 (sCy7) and the red dye was ATTO655 (AT655). The length of the 30-bp dsDNA is 10.2 nm. The dyes are bonded to the DNA molecule via a flexible linker of a length of a few nm. Thus, the distance between the NIR and red dyes bonded to the same DNA molecule should measure about 10 nm.

We started the 3D localization of the dual-labeled DNA molecule with position determination of the two dyes. Each of the dyes was localized with a 1 nm precision, as it was so in the case of ATTO647N. The distance between the two dyes, however, distributed from 0 to 50 nm. The discrepancy was found to be originated from the dipole character of the fluorescence emission. Since individual fluorophores are orientationally fixed at 1.8 K, the fluorescence emission from a single fluorophore should be treated as dipole emission. As a result, PSF may tilt from the z-axis and the xy-centroid may depend on the z-position at which the xy-image is taken. The amount of the z-uncertainty is the focal depth of about 0.7 μm. Because different fluorophores orient differently, the centroid of fluorophores deviates differently from the true position. Thus, relative distance between two fluorophores will suffer from z-dependent xy-deviation. This systematic error will vanish if the xy-centroid is determined on the true focal plane of $z = 0$. By taking into account of the z-dependent xy-centroid, the length of dsDNA was reproduced. As for the lateral (xy) cross-section, 19 images were taken along the z-axis with an interval of 100 nm. The distribution of D_{xy} was centered around the DNA length of about 10 nm with the standard deviation of 5 nm.

In conclusion, towards nanometer level microscopy of multiple biomolecules in cell, we demonstrate a nanometer accuracy of far-field fluorescence localization microscopy using near-infrared and red fluorophores bonded to the both ends of a 10 nm-long dsDNA.