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Mechanical stability of a microscope setup working at a few kelvins for single-molecule localization

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ABSTRACT

A great advantage of single-molecule fluorescence imaging is the localization precision of molecule beyond the diffraction limit. Although longer signal-acquisition yields higher precision, acquisition time at room temperature is normally limited by photobleaching, thermal diffusion, and so on. At low temperature of a few kelvins, much longer acquisition is possible and will improve precision if the sample and the objective are held stably enough. The present work examined holding stability of the sample and objective at 1.5 K in superfluid helium in the helium bath. The stability was evaluated by localization precision of a point scattering source of a polymer bead. Scattered light was collected by the objective, and imaged by a home-built rigid imaging unit. The standard deviation of the centroid position determined for 800 images taken continuously in 17 min was 0.5 nm in the horizontal and 0.9 nm in the vertical directions.

1. Introduction

Fluorescence microscopy in the visible wavelength region is one of the non-invasive methods to investigate three-dimensional structure of the sample. Owing to highly sensitive photon-counting devices, the sensitivity of fluorescence detection reaches single-molecule level. When performed on single molecules, fluorescence microscopy provides a potential to characterize structure of the sample on individual molecular level. As a far-field optical method, conventional optical microscopy suffers from poor spatial resolution. The diffraction of the optical wave limits the resolution to about a size of the wavelength in use. For conventional visible light sources of $\lambda > 500$ nm, where λ denotes wavelength of light, diffraction limit is roughly 300 nm, which is 1–2 orders larger than the size of molecules.

If the source of fluorescence is a single emitter, its image carries spatial information finer than the diffraction limit. The position of the emitter can be determined as the centroid of the fluorescence image. Localization precision is no longer limited by diffraction but is limited only by the finite number of the detected photons [1]. One of the earliest applications of single-molecule localization was observation of diffusion of single molecules in phospholipid membrane. The position of single dye-labeled phospholipid molecules were followed with an accuracy of 30 nm by taking images with 5 ms illumination [2]. More precise localization of immobile molecules was carried out at lower temperature. At 1.5 K three-dimensional localization was demonstrated [3] and influence of mechanical stability on the localization precision was addressed [4]. At a few kelvins, homogeneous linewidth of the zero phonon line of the electronic 0-0 transition of a single molecule often gets close to the lifetime-limited width of around 0.0005 cm^{-1}. In contrast the inhomogeneous linewidth of the spectrum of an ensemble is typically several to tens of cm^{-1}. The ratio of the homogeneous to the inhomogeneous linewidth can reach 10^4 or more. When spatial density of the sample molecule is too high, additional spectral selection is helpful to realize single-molecule observation. Making use of the large ratio of the homogeneous to inhomogeneous linewidth, about 300 molecules residing within a diffraction-limited focal volume were individually localized in 8 h with average precision of about 25 nm [5]. When the inhomogeneous broadening is very large to yield hundreds cm^{-1} of the inhomogeneous width, spectral selection is so effective that spatial selection is no longer necessary for single-molecule observation. In a wide-field fluorescence imaging of a sample film of dimensions $75 \times 75 \times 0.5$ μm, about 300,000 single molecules were observed during the excitation frequency scan over 700 cm^{-1} [6].

An essential prerequisite for localization of single molecules at a few kelvins is mechanical stability of the microscope setup, because the stability limits the acquisition time of signal photons. In view of influence of stability on the image, the most vital elements of microscope are the sample and the objective. The mechanical drift of the position of the objective relative to the sample is directly translated into the same amount of drift of the object. In low-temperature measurement, in addition, the sample and the objective are both immersed in liquid helium. Since the

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cooled elements are held in the liquid helium bath, their position relative to the rest of the microscope set up cannot be fixed as rigidly as the elements fixed on an optical table.

In the present work we examined the mechanical stability of the cooled elements of the sample and objective relative to the rest of the microscope setup. All the elements fixed on the optical table were replaced by a home-built simple rigid imaging unit. Mechanical stability of the cooled sample and objective was evaluated by localization precision of a point source at 1.5 K imaged by the imaging unit. The position was determined as the centroid of the image. The standard deviation (σ_{sdev}) of the centroid position determined for 800 images continuously taken in 17 min with acquisition time for one image of 1.3 s at 1.5 K was 0.5 nm in the horizontal and 0.9 nm in the vertical directions.

2. Experimental

2.1. Imaging unit and light-source unit

Fig. 1 shows the optical imaging unit to examine mechanical stability of the sample and objective of a microscope setup and a light-source unit to test stability of the imaging unit. The imaging unit consists of an imaging lens of the focal length (f) of 200 mm and a complementary metal oxide semiconductor (CMOS) camera (ARTCAM-130MI-BW, frame rate $> 15\ s^{-1}$, pixel size 5.2 μm $\times$ 5.2 μm). The lens and the CMOS camera are fixed to a single aluminum rod of 50 mm square with a 25 mm-diameter partly tapped hole through the center axis. Because the CMOS camera was connected on a printed circuit board, space between the camera and the board was filled with glue of epoxy resin to fix the camera firmly to the aluminum body. The z-position of the lens can be adjusted with a standard screwed mount in the tapped hole. The light-source unit was made in the same way as the imaging unit except that the CMOS camera is replaced by a pinhole of 10 μm diameter. The pinhole was illuminated by a laser light at a wavelength (λ) of 633 nm. Influence of the pointing instability of the laser was minimized by making the diameter of the laser beam to be 600 μm at the pinhole. The light from the pinhole was collimated by the collimator lens of f = 200 mm and the diameter of the parallel beam was reduced by a mask to 2.4 mm, the same diameter as the pupil of the objective. With the same diameter of the collimated beam, the image of the point light-source has the same size of diffraction pattern as the cooled objective would produce.

2.2. Objective and sample of the microscope setup tested with the imaging unit

Fig. 2 shows the optical arrangement for evaluating mechanical stability of the low-temperature part of the microscope setup. In

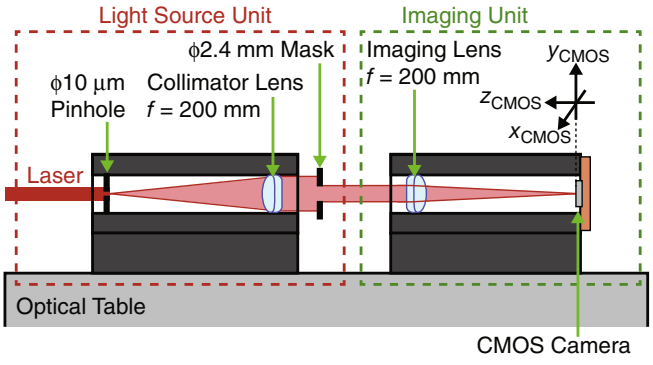

Fig. 1. The optical imaging unit developed to evaluate mechanical stability of a microscope setup and the light source unit to test the stability of the imaging unit.

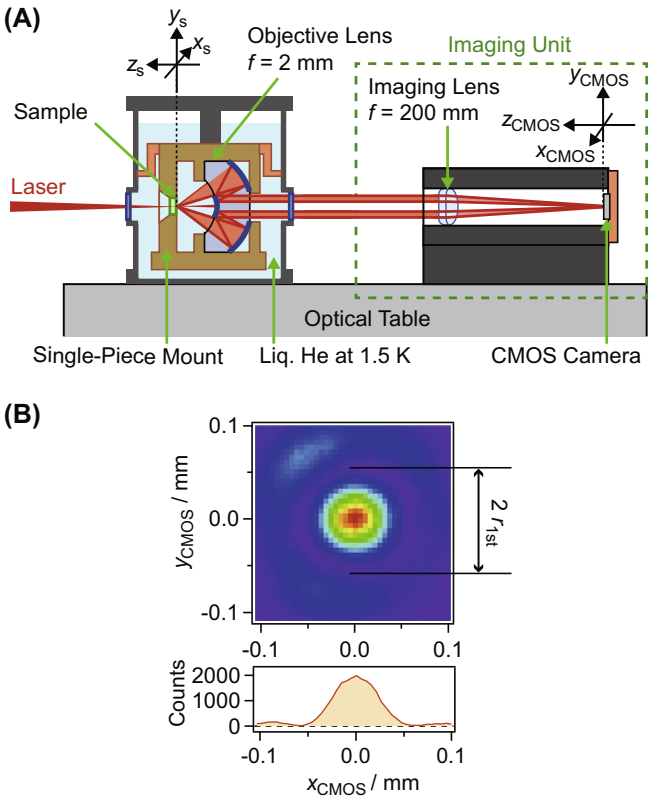

Fig. 2. (A) Experimental setup for evaluating mechanical stability of the sample and microscope objective in superfluid helium at 1.5 K. When the objective is combined with the imaging unit shown in Fig. 1, the optical system makes a 100-times-magnified image of the sample on the CMOS camera. Note that coordinates of the sample space are denoted by x_s, y_s, z_s, and those in image space on the CMOS camera are denoted by x_{CMOS}, z_{CMOS}, and z_{CMOS}. (B) An example of a diffraction pattern of the scattering image of a polymer bead.

view of the optical design of microscope, the most crucial alignment is that of the objective with respect to the sample. In our fluorescence microscope setup developed for single-protein microspectroscopy at 1.5 K [7–9], the objective and the sample were immersed in superfluid helium in a cryostat bath. In order to suppress shift of the objective to the sample, we follow the hinged single-piece design of the mount employed in the single-molecule localization experiments at a few kelvins [5,10]. In these experiments the sample and the objective are fixed on the same mount. The mount has a hinge mechanism to allow fine focus adjustment [5].

The objective and the sample were cooled at 1.5 K by immersing them in superfluid helium which was pumped in a cryostat below λ point. The stainless-steal cryostat for liquid helium is about 1 m high, directly fixed by M6 screws onto the optical table. The cryostat consists of a 2 mm thick outer shell of a diameter of 18 cm, an intermediate shield of liquid nitrogen, and a 0.5 mm thick liquid helium bath of a diameter of 9 cm. Three parts are mechanically combined at their o-ring sealed top flanges.

The mechanical stability was measured as a drift of the centroid of the scattering image of a polystyrene bead of a diameter of 100 nm. The beads were spin-coated on a glass substrate at a surface density of 10^{-3} μm^{-2}. At this density, scattering images of single beads do not overlap each other. The position of beads can be individually determined as the centroid of the image. The sample was irradiated by a laser of λ = 633 nm. The scattering light from a bead was collected by the single-component reflecting objective of f = 2 mm and numerical aperture (NA) of 0.53 [11]. The collimated scattering light was introduced to the imaging unit, and focused on the CMOS camera to form a 100 times magnified image.