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Development of a novel cryogenic microscope with numerical aperture of 0.9 and its application to photosynthesis research

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ABSTRACT

A novel cryogenic optical-microscope system was developed in which the objective lens is set inside of the cryostat adiabatic vacuum space. Being isolated from the sample when it was cooled, the objective lens was maintained at room temperature during the cryogenic measurement. Therefore, the authors were able to use a color-aberration corrected objective lens with a numerical aperture of 0.9. The lens is equipped with an air vent for compatibility to the vacuum. The theoretically expected spatial resolutions of 0.39 μm along the lateral direction and 1.3 μm along the axial direction were achieved by the developed system. The system was applied to the observations of non-uniform distributions of the photosystems in the cells of a green alga, *Chlamydomonas reinhardtii*, at 94 K. Gaussian decomposition analysis of the fluorescence spectra at all the pixels clearly demonstrated a non-uniform distribution of the two photosystems, as reflected in the variable ratios of the fluorescence intensities assigned to photosystem II and to those assigned to photosystem I. The system was also applied to the fluorescence spectroscopy of single isolated photosystem I complexes at 90 K. The fluorescence, assigned to be emitted from a single photosystem I trimer, showed an intermittent fluctuation called blinking, which is typical for a fluorescence signal from a single molecule. The vibronic fluorescence bands at around 790 nm were observed for single photosystem I trimers, suggesting that the color aberration is not serious up to the 800 nm spectral region.

1. Introduction

Combinations of the optical microscope technique with various fluorescence-spectroscopic techniques have become promising approaches to various biological investigations. This is especially the case for the investigation of photosynthesis, since in-situ fluorescence spectroscopic analyses offer much information directly related to the functions of photosynthetic pigment–protein complexes. Actually, microscope measurements of in-situ fluorescence spectra [1,2] and their temporal variations with a picosecond time resolution [3,4] have exerted a powerful impact on photosynthesis research. On the other hand, fluorescence microspectroscopy studies of living oxygenic photosynthetic organisms have had the problem of overlaps of a lot of spectral components, often resulting in ambiguous interpretations. Since oxygenic photosynthesis is managed through the cooperation of a wide variety of pigment–protein complexes [5], the coexistence of many fluorescence spectral components is inherent to such organisms.

It has long been known that the problem of spectral overlap can be reduced by conducting experiments at cryogenic temperatures. In general, decreasing the temperature results in a drastic sharpening of the fluorescence spectrum of each component, leading to a much improved spectral resolution. From the fluorescence spectrum of a living leaf taken at room temperature, it is almost impossible to distinguish between the fluorescence spectral components of photosystem I (PS I) and photosystem II (PS II). In contrast, a leaf at 77 K typically shows the fluorescence spectrum of the PS II component, with a double-peaked band at 686 nm and 695 nm, which is clearly distinct from that of the PS I component, which shows a specific red-shifted band at around 710 to 735 nm depending on the organisms [6,7]. We know that the two peaks at 686 nm and 695 nm of the PS II component mainly come from the chlorophylls (Chls) bound to its core–antenna complexes, CP43 and CP47, respectively [8–11]. Another benefit of microspectroscopy at a low temperature is much milder photodamage to samples by high-intensity laser irradiation. Additionally, unwanted physiological responses of living samples can be suppressed at

Abbreviations: PS I, photosystem I; PS II, photosystem II; Chl, chlorophyll; RC, reaction center; NA, numerical aperture; APD, avalanche photodiode; CCD, charge-coupled device; LED, light-emitting diode; DM, n-dodecyl-β-D-maltoside; FWHM, full width at half maximum; PSF, point-spread function

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low temperatures. This is extremely useful for observations of a biological sample because its fluorescence images in specific physiological states can be recorded without further perturbing the sample.

Another typical application of fluorescence microspectroscopy is single-molecule fluorescence spectroscopy, which has become a powerful tool to study conformational dynamics of photosynthetic pigment–protein complexes [12–18]. It is necessary to conduct experiments at cryogenic temperatures for fluorescence detection of a single photosynthetic reaction-center (RC) complex because the complex generally has a very low fluorescence quantum yield at room temperature due to the competing, extremely efficient charge-separation. PS I has a very low fluorescence quantum yield at room temperature, whereas it has a rather strong fluorescence emission below 100 K [14,19].

A drawback of cryogenic microspectroscopy is its limited spatial resolution. Basically, there are two methods of cryogenic microscopy. In one method, the objective lens is set inside the cryostat and immersed in the cooling medium [12,13,20]. On the other method, the lens is set outside the cryostat [21–23]. Since, in the former case, the microscope objective is cooled to a cryogenic temperature, one has to use a single lens made of fused quartz that has an extremely low heat-expansion coefficient. In this case, it is difficult to achieve an accurate color-aberration correction and a high numerical aperture (NA). In the latter case, one has to use an objective lens with a long working distance, again resulting in a limited NA. The NA of an objective lens used for cryogenic microspectroscopy hardly exceeds ca. 0.7. The limited NA of an objective lens results in not only a limited spatial resolution but also a limited collection efficiency of the fluorescence. Improvement of the collection efficiency of fluorescence is critical to the single-molecule fluorescence spectroscopy.

Here, we propose a novel cryogenic microscope set-up in which the objective lens is set inside the adiabatic vacuum space of the cryostat. This arrangement allows a drastically shortened working distance. Since the objective lens in this arrangement is adiabatically isolated from the cooled sample holder, we can use a semi-conventional objective lens composed of a set of glass lenses fixed inside a metal tube. Here, we use the word *semi-conventional* because the lens has to be equipped with an air vent for usage in the vacuum. This type of microscope has already been developed and applied in observations of solid surfaces [24]. However, no one has tried to develop it for observations of biological samples. Here, we demonstrate for the first time a high NA of 0.9 for measurements of biological samples at 90 K by the developed microspectroscopy system. The temperature can be further decreased to 10 K if liquid He is used as a cooling medium. Using the developed system, we reveal the non-uniform distributions of PS I and PS II inside a cell of a green alga, *Chlamydomonas* (*C.*) *reinhardtii*. We also demonstrate that the developed system is applicable to observations of the single-molecule fluorescence spectra of isolated PS I at 90 K. The improved spatial resolution of the developed system will be further applicable to the correlative microscopy, which is a recently emerging technique combining the cryo-electron tomography and the fluorescence microscopy ([25,26] and the references therein).

2. Materials and methods

2.1. Optical set-up

Fig. 1 is a schematic description of the developed cryogenic microscope. We adopted the inverted laser-scanning confocal microscope configuration. A home-built vacuum chamber contains an objective lens (Plan Apo HR100× NA0.9 custom-tailored for compatibility to the vacuum, Mitutoyo, Kawasaki), a piezo actuator (NS7120-C custom-tailored for compatibility to the vacuum, Nanocontrol, Tokyo) to control the fine focus, a hand-made copper sample holder, and a quartz stage (QS) to support the holder. The objective lens is designed for observation through a 0.3-mm-thick quartz cover slip. A sample solution is sealed in a cavity between two circular quartz plates (10-mm diameter) with a thickness of 0.3 mm. A Teflon spacer with a typical thickness of 0.05–0.2 mm is clipped between the quartz plates. The copper sample holder is fixed to the quartz sample stage through four rods made of thermally insulating polyphenylene sulfide (PPS) resin. The quartz stage is mounted on vacuum-compatible X–Y stages, and its position can be coarsely adjusted from outside of the chamber through the two right-angle arranged linear manipulators. The sample holder is connected to the cold head of the cryostat (Microstat, Oxford Instruments, Eynsham) through a copper braid to maintain the flexibility of the relative position of the holder with respect to the cold head. The bellows component allows the vertical movement of the bottom plate of the chamber fixing the objective lens. The focus can be coarsely adjusted by the vertical movement of the bottom plate.

Either a He–Ne laser (1137P, JDS Uniphase, Milpitas) or the second-harmonic generation light from a Ti:sapphire laser (MaiTai, Spectra-Physics, Mountain View) is used for the excitation light source. The excitation beam is reflected by a dichroic mirror and a pair of galvanic mirrors (VM500 plus, GSI, Bedford) and enters the objective lens through a quartz window at the bottom of the chamber. The lateral scanning of the focal point is carried out with the galvanic-mirrors. The fluorescence collected by the same objective lens passes in the opposite direction to the excitation beam, goes through the dichroic mirror, and is focused into the entrance slit of the polychromator (MS2004i, SOL instruments, Minsk). The fluorescence signal is detected with either an avalanche photodiode (APD) (id100-MMF50, ID Quantique, Geneva) or a liquid-nitrogen cooled charge-coupled device (CCD) camera (PyLoN:100BR eXcelon, Princeton Instruments, Trenton). The wavelength-dependent sensitivities of the system are estimated by measurements of the emission spectrum of a standard halogen lamp located at the sample position. As shown in Fig. S1, the sensitivity curve does not show any sharp wavelength dependence over the spectral range of interest. In the present study, therefore, we omitted the sensitivity correction.

The upper surface of the vacuum chamber is closed with a transparent acrylic window in order to maintain optical access from the upper side. A white light-emitting diode (LED) (MCWHL2-C3, Thorlabs, Newton) and a set of lenses and irises realizing the Kohler illumination are set over the acrylic window for measurements of a transmission image through the sample. When a transmission image is measured, the reflecting mirror is replaced with a half mirror, and the light through the sample is focused with an imaging lens onto a CMOS camera (Wraycam-G500, Wraymer Inc., Osaka).

2.2. Fluorescence beads spin-coated on a quartz surface

A suspension of fluorescence beads with a diameter of 200 nm (F-8807, Life Technologies Japan, Tokyo) was appropriately diluted with a 1% (w/v) polyvinyl alcohol aqueous solution. The solution was then spin-coated on a circular quartz plate with a thickness of 0.3 mm to obtain a thin film. The spinning rates were at 500 rpm for the initial 15 s and 2000 rpm for the following 60 s [27].

2.3. Sample preparation of C. reinhardtii living cells

Cells of wild-type *C. reinhardtii* strain 137c were grown in a Tris-acetate-phosphate (TAP) medium [28] under low light (~20 μmol photons m^{-2} s^{-1}). The concentration of the cells was adjusted by centrifugation and re-suspension to the culture medium. The culture medium was mixed with the same volume of glycerin to maintain the transparency of the solution and was sealed in the sample holder. The cells were incubated under dark for ca. 10 min and irradiated with PS I light (the white-LED light through a R72 long-pass filter, ~8 μmol photons m^{-2} s^{-1}) to induce state I. After the 10-min irradiation, the cooling process was started with a continuous liquid-nitrogen flow. The irradiation was continued until the sample temperature was below ca. 0 °C.