Visible Fluorescence Spectroscopy of Single Proteins at Liquid-Helium Temperature

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Fluorescence spectroscopy of single proteins at liquid-helium temperatures reveals a relation between structural dynamics and biological functions of the proteins. The technical difficulties in detecting visible fluorescence are chromatic aberration and optical background. They were overcome by a new optical design using reflective optics and employing two-photon excitation. The fluorescence spectrum of single green-fluorescent proteins taken at a temperature of 1.5 K makes a distinction between different metastable conformations that last for tens of seconds.

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In their physiological condition, proteins continuously change their three-dimensional structure. The continuous change of the structure plays an important role in regulating the biological functions of the protein, such as catalysis and transportation. At liquid-helium temperature, a protein is basically fixed at one of the stable structures with residual slow change among them. Low-temperature single-protein spectroscopy based on fluorescence detection was successfully applied to study individual structures and conformational dynamics of photosynthetic pigment-protein complexes [1,2]. During the last decade after the first demonstration [3], the technique of detecting the fluorescence of a single such complex in the near infrared has been established. The extension of the detection window to cover the whole visible region would give spectroscopic access to numerous proteins carrying various biological functions [4]. In this Letter, we report the observation of the visible fluorescence of single green-fluorescent proteins at a temperature of 1.5 K. Cooling to 1.5 K enables real-time observation of the spontaneous conformational change of a single protein through a temporal behavior of the fluorescence spectrum.

Although the observation of the visible fluorescence of single proteins was established at room temperature [5–7], the observation at liquid-helium temperature was still difficult because a multicomponent objective with a high numerical aperture cannot be used. The problems that arise are chromatic aberration and optical background. To minimize the chromatic aberration, we developed a reflecting objective [8] (see below). The optical background generated by excitation in the visible range is larger than in the near infrared. The optical background is suppressed by using two-photon excitation (TPE). In the TPE process, the generation of the optical background is limited to a small volume around the focus since the TPE probability is quadratic with the excitation intensity [9]. In addition, TPE fluorescence is completely separated in wavelength from the laser light and Raman scattering since the frequency of the TPE process is twice the laser frequency (2Ω) [9]. Thus the optical background in the two-photon fluorescence spectrum was suppressed below the level of a detector noise.

Figure 1 shows our low-temperature single-protein fluorescence spectrometer. The light source was a tunable Ti:sapphire laser (pulse width, 150 fs; repetition rate, 90 MHz; wavelength range, 705–980 nm). The center wavelength was tuned at 920 nm ($\Omega = 10\,900\ \mathrm{cm}^{-1}$). Laser-beam scanning on a sample was carried out with a telecentric system consisting of a motorized flat mirror and a pair of parabolic mirrors. A home-built reflecting objective (numerical aperture, 0.6; focal length, 4 mm [8]) and a sample were immersed to superfluid helium at 1.5 K in a cryostat. The excitation light was focused on the sample by the reflecting objective with the intensity of 150 $\mathrm{kW\,cm}^{-2}$

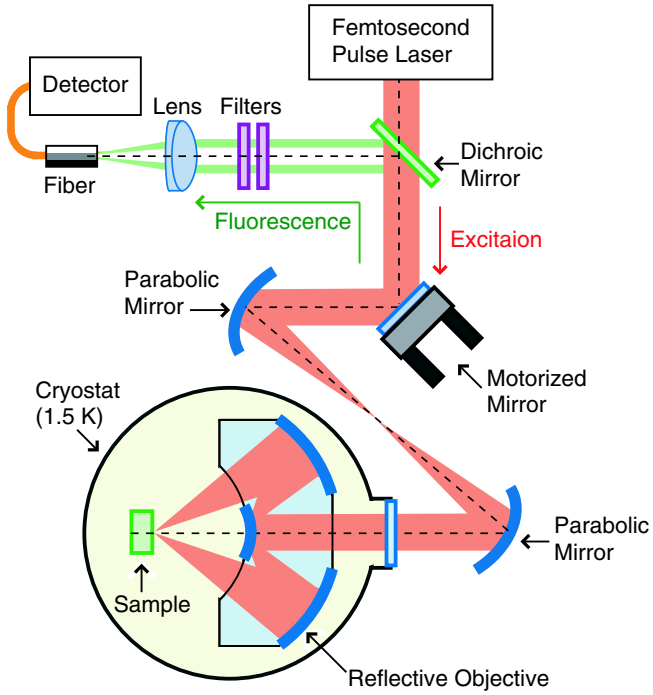

FIG. 1 (color online). Low-temperature single-protein fluorescence spectrometer using reflective optics.

at the focus. Two-photon fluorescence of individual proteins was collected by the same objective, separated from the laser light by a dichroic mirror and short-wavelength pass filters, and focused to a 100 μm core fiber by an achromatic lens (focal length, 40 mm). The fluorescence images were taken by recording fluorescence photons with an avalanche photodiode as a function of the lateral position on the sample. The fluorescence spectra were taken with a spectrograph equipped with a charge-coupled device. The resolution of the fluorescence spectrum was 1.7 nm (70 cm^{-1} at 500 nm). The exposure time for taking one spectrum was 20 sec. The key feature of our spectrometer is that reflective optics, i.e., the two parabolic mirrors and the reflecting objective, were employed in the optical path that is common to excitation and fluorescence. Consequently, the chromatic aberration of the spectrometer was negligible throughout the visible and near-infrared regions (400–1000 nm).

The sample was monomeric green-fluorescent protein, AcGFP1, derived from jellyfish *Aequorea coerulescens* (rAcGFP1, Clontech). The aqueous solution of the protein was prepared at pH = 7 in the presence of 10 mM phosphate buffer and 0.5% wt/wt polyvinyl alcohol. To have spatially isolated proteins in the fluorescence image, the 0.2 nM solution of the protein was spin coated on a sample substrate.

Figure 2(a) shows a two-photon fluorescence image of two AcGFP1s taken at 1.5 K. The fluorescence spots at $x = 0$ and -1.3 μm are of single AcGFP1s. The cross section at $y = 0$ μm of Fig. 2(a) is shown in Fig. 2(b). As seen in Fig. 2(b), the full width at a half maximum (FWHM) of the single AcGFP1 image was 0.72 μm. The value is 1.4 times of the FWHM of the theoretical diffraction-limited image of the TPE fluorescence, which is 0.5 μm at the excitation wavelength of 920 nm [8,10]. Figure 2(c) shows the detected fluorescence photons of AcGFP1s measured as a function of the excitation intensity. The quadratic behavior shows that the fluorescence is generated in the TPE process. Examples of the fluorescence time trace of single AcGFP1s are shown in Fig. 2(d). While time is negative, emission from a spot outside the fluorescing area of single AcGFP1s was measured. The count rate of 12 counts s^{-1} during this period represents the noise level of the fluorescence image. At time zero, the excitation spot was brought to single AcGFP1 to initiate the optical excitation cycle. At the liquid-helium temperature, in most of the cases the emitting rate of photons stayed constant like in the traces of Fig. 2(d). The fluorescence intensity of single AcGFP1s dropped in one step to the noise level. The one-step bleaching confirms that the fluorescence spots were generated from single AcGFP1s.

Two-photon fluorescence spectra of single AcGFP1s and an ensemble of the protein taken at 1.5 K are shown in Fig. 3. The fluorescence spectra were corrected for the total detection efficiency of the setup that is wavelength

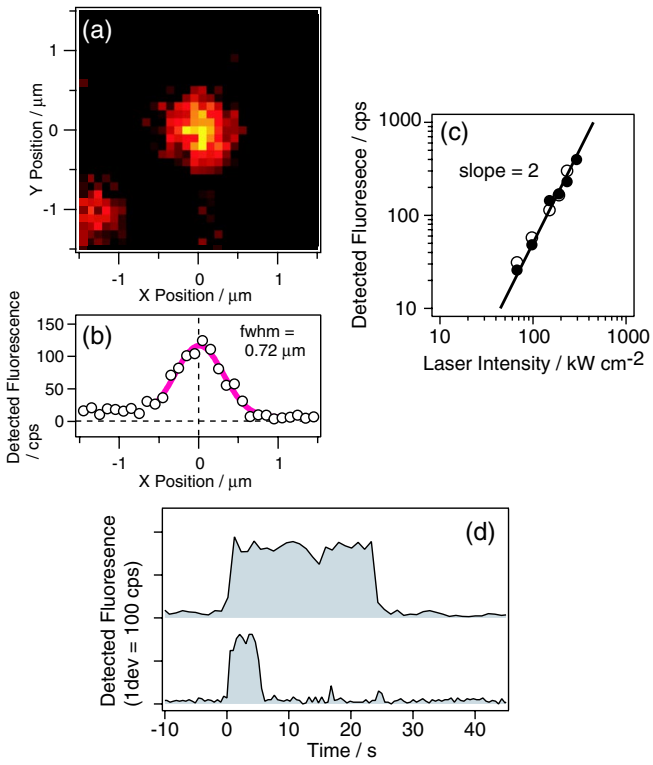

FIG. 2 (color online). (a) Two-photon fluorescence image of two single AcGFP1s at a temperature of 1.5 K. Two proteins were observed in the image (a) at $x = 0$ and -1.3 μm. (b) The cross section of image (a) at $y = 0$ μm. (c) The number of the detected fluorescence photons as a function of the excitation intensity. Open circles represent the fluorescence of the single AcGFP1 located at $x = 0$ μm in the image (a), and filled circles represent that at $x = -1.3$ μm. The fluorescence intensity of AcGFP1s increased quadratic with laser intensity. (d) Typical examples of the fluorescence time trace of single AcGFP1.

dependent. Owing to the TPE process, an optical background was negligible and the readout noise of the detector dominated the noise of the spectrum. With an exposure time of 20 sec, the readout noise corresponds to the noise level of 4 photons s^{-1}. The ensemble spectrum at the low temperature was similar to that at room temperature. The one-photon fluorescence spectrum of a mutant of green-fluorescent protein (S65T) taken at 77 K is also similar to that at room temperature [11]. In Fig. 3(a), the 0-0 transition of single AcGFP1 appears at 19 730 cm^{-1} (507 nm), which is within 250 cm^{-1} from the peak of the ensemble spectrum. The width of the 0-0 transition is 250 cm^{-1} in FWHM, which is 1/5 of the inhomogeneous width of the ensemble. The distribution of the position of the 0-0 transition of 60 AcGFP1s is shown in Fig. 4. As an advantage of the TPE excitation, the 60 AcGFP1s was randomly selected from the bulk because the spectral window of the TPE measurement completely covers the ensemble spectrum. The distribution of the 0-0 transition roughly followed the ensemble fluorescence spectrum.

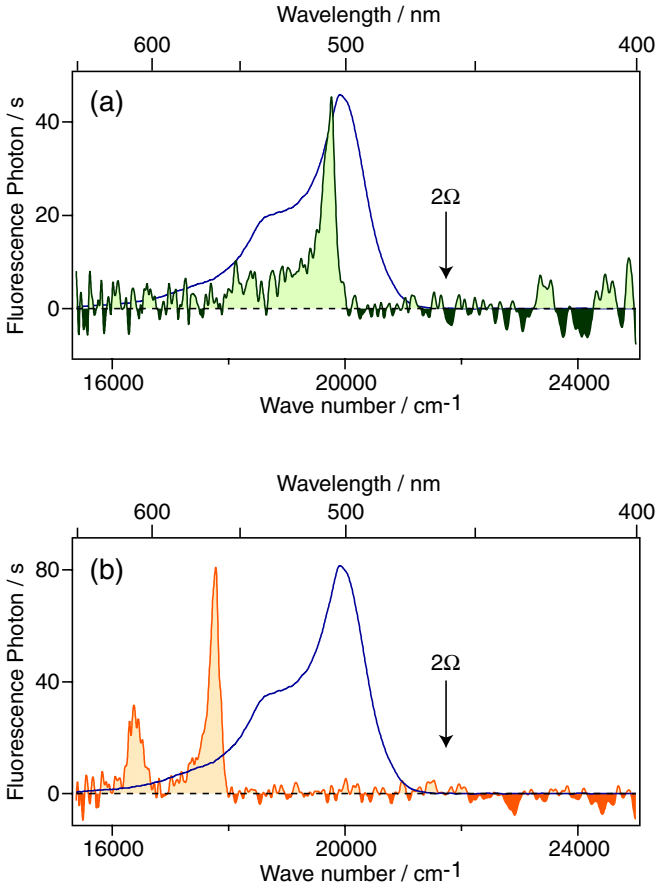

FIG. 3 (color online). Two-photon fluorescence spectra of single AcGFP1s (filled curves) with an ensemble spectrum of the protein (solid blue curves) at a temperature of 1.5 K. The vertical axis indicates the fluorescence intensity of the single protein. Twice the laser frequency (2Ω) is indicated by an arrow. The fluorescence spectra were corrected for the total detection efficiency of the setup.

Spectroscopy of single molecules enables an examination of any portion of inhomogeneous distribution on an individual molecular basis. The fluorescence spectrum of different electronic states of AcGFP1s was obtained by selecting the single proteins from different parts of the distribution of Fig. 4. The single protein of Fig. 3(a) is from the center of the ensemble distribution. A single protein from the red wing is shown in Fig. 3(b). The position of the 0-0 transition at 17 760 cm^{-1} (563 nm) is 2200 cm^{-1} redshifted from the maximum of the ensemble. The spectral feature of Fig. 3(b) makes a contrast with that in Fig. 3(a). The 0-0 transition of Fig. 3(b) is accompanied by an extra band at 16 400 cm^{-1} (610 nm) while extra bands in Fig. 3(a) are as weak as the noise. Green-fluorescent protein has several different protonation forms of the chromophore [12]. The different forms have the different positions of the 0-0 transition that spread over 2000 cm^{-1} and have different spectral features [13]. Thus the observed spectral difference suggests that the single

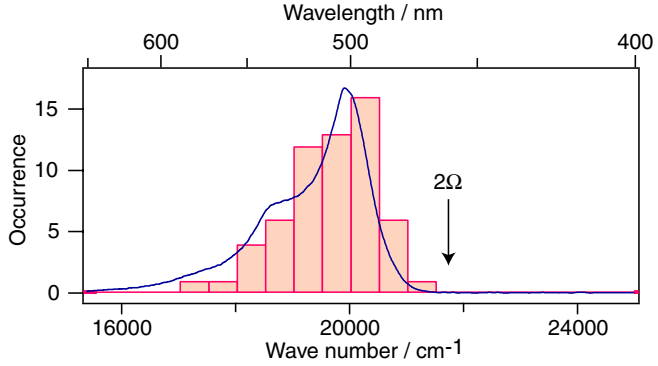

FIG. 4 (color online). Distribution of the position of the 0-0 transition of 60 AcGFP1s at 1.5 K. The ensemble fluorescence spectrum of AcGFP1 is also depicted by a solid blue curve.

AcGFP1s in Figs. 3(a) and 3(b) belong to different protonation forms.

Temporal behavior of the two-photon fluorescence of the single AcGFP1 of Fig. 3(b) is shown in Fig. 5. During 0–20 sec, the fluorescence spectrum consists of the 0-0 transition at 17 760 cm^{-1} and the second band at 16 400 cm^{-1}. During 20–40 sec, the 0-0 transition is accompanied by a new band at 17 670 cm^{-1} and the second band becomes broader towards the low energy side. During 40–60 sec, only two bands remain at the positions of ca. 100 cm^{-1} lower than the bands observed during 0–20 sec: i.e., the 0-0 transition at 17 670 cm^{-1} and the second band at 16 270 cm^{-1}. The relative position and height of these bands are the same as at the first period of 0–20 sec. After 60 sec, the signal was depleted probably due to a transition to another protonation form [13] or

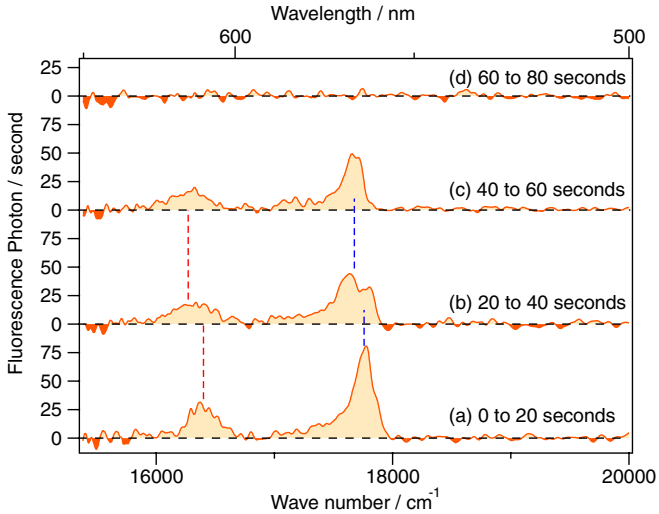

FIG. 5 (color online). Temporal behavior of the two-photon fluorescence of the single AcGFP1 of Fig. 3(b) at a temperature of 1.5 K. Four measurements were continuously repeated with the exposure time of 20 sec. Positions of the 0-0 and vibronic transitions are indicated by dashed lines. The fluorescence spectrum of Fig. 3(b) uses the data of the first exposure period.

another multiplicity of electron spin [14]. Because in the spectrum of 20–40 sec [Fig. 5(b)] both the bands observed before and after this period were present, during 20–40 sec the two bands of Fig. 5(a) have simultaneously made a discrete jump of the spectral position by the same amount of ca. 100 cm^{-1}. The lower energy band is assigned to the vibronic transition of single AcGFP1. The vibrational frequency is around 1400 cm^{-1}.

In addition to the discrete jump described above, deviation from homogeneous spectrum is also found in the line width. The width of the 0-0 transition (190 cm^{-1}) is 4 orders of magnitude broader than the homogeneous width at 1.5 K measured by hole burning spectroscopy for a mutant of green-fluorescent protein [15]. This indicates that the band is broadened during the exposure time of 20 sec. We think that the temporal behavior, i.e., the spectral jump and the broadening, reflects the dynamics of the local protein environment surrounding the chromophore of AcGFP1. Similar spectral behaviors at liquid-helium temperatures were reported for the fluorescence excitation spectrum of a single pigment-protein complex [16,17].

The visible fluorescence spectrum of single proteins was observed at a temperature of 1.5 K via the TPE process. Single-protein spectroscopy at 1.5 K is suitable for studying individual stable structures and conformational change of the proteins. The method will contribute to the understanding of the relation between structural dynamics and the biological function of visible fluorescent proteins.

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