Supporting Information for
Single-Molecule Surface Enhanced Resonance Raman Spectroscopy of the Enhanced Green Fluorescent Protein (EGFP)

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Purification of EGFP

Standard methods were used for in vitro DNA manipulations. The gene coding for EGFP was first removed from pEGFP (Clontech) as an 800 bp *Eco*RI-*Pst*1 fragment and subsequently inserted into pBAD/HisA (Invitrogen). The expression of the protein was induced for 12 h in *Escherichia coli* Top10 cells grown at an optical density of 0.5 at 600 nm using 0.2 % arabinose. The His-tagged protein was subsequently isolated and purified under native conditions by Ni-chelation chromatography using the Xpress Protein Purification System. Purity of the samples was confirmed by polyacrylamide gel electrophoresis. Finally, the proteins were concentrated in a Vivaspin 6 concentrator, and were stored in a phosphate buffer saline solution (PBS, pH 7.4, Sigma).

Sample preparation for SM-SERRS measurements

Ag sols were prepared according to the description of Lee and Meisel.[1] A 45-mg sample of silver nitrate was added to 250 ml of ultra pure water, purged with Ar, and heated to 100 °C under vigorous magnetic stirring. 5-ml of a 35 mM sodium citrate solution (1 % sodium citrate dihydrate by weight) was injected. The resulting solution was refluxed for 1 h. A 250 µl aliquot of silver colloid was incubated with 1250 µl of 1 mM sodium chloride solution containing EGFP (2×10^{-10}) for 1 h. 40 µl of the sol was deposited on a polylysine coated cover slip, and the water was allowed to evaporate at room temperature. Under these conditions, each particle carries an average of one EGFP molecule. The colloidal particles were immobilized on a polylysine coated glass surface due to the electrostatic interactions between the negative charges on the particles and the positive charges of the surface. After evaporating the solvent, the cover slips were rinsed with ultra pure water to remove loosely bound colloidal particles, and dried with nitrogen flow.

Experimental set up

SM-SERRS measurements were performed by using a confocal microscope[2] (Diaphot 200, Nikon) with an oil immersion lens (100×, NA 1.4). The excitation source was the 488 nm line of an Argon ion laser (Stabilite 2017, Spectra Physics). All spectra were measured with the collection time of 5 seconds. The incident laser power was 250 nW. The SERRS signal was collected by the same objective, passed through a dichroic mirror (Chroma Technology) and through a notch filter (Kaiser Optical System), divided into two beams by using a beam-splitter cube (05BC17MB.1, Newport), and focused on an avalanche photodiode (APD)

(SPCM AQ15, EG&G) and into a 250-mm polychromator (250IS/SM, Chromex) coupled to a cooled CCD camera (LN/CCD-512SB, Princeton Instruments). The wavelength resolution of the system is approximately 10 cm^{-1}. The SERRS signal detected by the APD was registered by a time-correlated single-photon counting PC card (SPC 630, Picoquant) by using the FIFO-mode.[3] The data set allows the reconstruction of the SERRS time trace with a minimum dwell time of 50 ns.

Autocorrelation analysis

The autocorrelation function $G(\tau)$ is defined as

$$G(\tau) = \frac{\langle F(t)F(t+\tau)\rangle}{\langle F(t)\rangle^2}$$

where $F(t)$ is the SERRS wavelength integrated intensity obtained at time t (angled brackets denote average), and τ is the correlation time. The autocorrelation function was computed from the data set with a homemade program. The obtained autocorrelation function was analyzed using an exponential decay model function.

Adsorption state of EGFP on the Ag particles

The ratio of EGFP to Ag nanoparticles (1:1) is a standard condition for single-molecule SERRS measurements. EGFP molecules distribute statistically on the Ag particles. Some particle will carry a few molecules, and some of them will not carry any molecules. Such distribution, however, has no effect on the measurements because there is a small number of the active sites on the Ag surface and because the size of the active sites is very small (such as add atom or atomic cluster).

At the measurement condition (pH = 7.4), the ensemble spectrum has a major contribution of the deprotonated form and a minor contribution of the protonated form.[4] In order to determine the acid-base equilibrium between the protonated and the deprotonated form on the Ag particles, we have tried to measure the absorption spectrum of EGFP adsorbed on the Ag articles. However, this was not possible because of the huge scattering and plasmon absorption of the Ag particles. Since the relative amplitude of the Raman band of the protonated form is higher than that measured at the similar pH in the solution,[5] the acid-base equilibrium might be slightly modified due to the adsorption of EGFP on the Ag particles.

Assignment of the Raman spectra obtained from SM-SERRS measurements

The excitation wavelength (488 nm) corresponds to the absorption maximum of the deprotonated form of the chromophore, resulting in a resonance enhanced Raman spectrum. For the protonated form, there is almost no absorption at the excitation wavelength. However, excitation at 488 nm results in a pre-resonance Raman enhancement which allows one to detect the Raman signal from the chromophoric site without spectral interference from the surrounding environment.[5,6]

The Raman bands obtained from our SM-SERRS were assigned based on the ensemble Raman spectrum measured in solution.[5] The literature spectrum that is cited is not the spectrum of EGFP (F64L/S65T), but the spectrum of the mutant S65T. Compared to the latter, in EGFP the amino acid residue at the position 64 is mutated from phenylalanine to leucine. However, this amino acid residue is not located in the vicinity of the chromophore of the protein and is not involved in the hydrogen-bonding network surrounding the chromophore.[7,8] Moreover, the spectroscopic properties as well as pKa of the mutant S65T and EGFP are almost the same.[4,9,10] Consequently, it is reasonable to cite the spectrum of the mutant S65T as the reference spectrum.

The Raman bands of the protonated and the deprotoanted form obtained from our SM-SERRS measurements do not perfectly agree with the values reported by Bell (1560 cm^{-1} and 1537 cm^{-1} for the protonated and the deprotonated form, respectively) determined based on ensemble measurements.[5] However, it is well known that Raman spectra obtained from a single molecule show spectral fluctuations, especially in the case of protein molecules.[11,12] Taking into account the wavelength resolution of our experimental set up (10 cm^{-1}) together with the large spectral fluctuations, the deviation of the Raman peaks obtained by the single molecule measurements from the ensemble spectrum can be expected to be a common phenomena in single protein Raman spectroscopy.

Origin of the spectral jumps observed for SM-SERRS measurements

The spectral jumps observed in SM-SERRS spectra were assigned to switching between the protonated and the deprotonated form. These jumps in the Raman spectrum were observed for about 15 % of the molecules studied. Another possible origin of the spectral jumps is denaturation of the protein. Denaturation of heme proteins on Ag particles was reported previously.[13] However, the possibility of the denaturation is unlikely in our system for the following reasons. First, the Ag colloidal particles prepared by the Lee and Meisel citrate reduction method have almost no influence on the protein structure because the citrate ions act as a coating which prevent denaturation of the protein.[14] Second, the reversible conversion between the protonated and the deprotonated form is expected only when the protein has its intact tertiary structures. If the protein denatures on the Ag surface, the chromophore would have the protonated form at the measurement condition (pH = 7.4) because the pKa of the chromophore not stabilized by the protein barrel is 8.1.[15,16] Consequently, the spectral jump from the protonated to the deprotonated form should correspond to the renaturation of the protein in that case. However, a denaturation/renaturation cycle within the measurement time scale (seconds) is quite unlikely. For these reasons, the possibility of the denaturation of the protein is unlikely. In our case, the origin of the spectral jump is the spontaneous conversion between the protonated and the deprotonated form in the ground state although a photo-induced protonation of the deprotonated form can not be excluded.

The protonation/deprotonation of green fluorescent proteins (GFP's) has been extensively studied. Peterman et al. suggest that the protonation/deprotonation of the chromophore is probably not the origin of on/off blinking in the fluorescence intensity traces of single EGFP molecules.[17] This discussion is based on the fact that the on-time of the fluorescence from a single EGFP molecule is independent of pH (in the pH range from 6-10). However, it has been demonstrated that the photoconverted GFP relaxes back toward equilibrium in the dark.[18] Photo-induced conversion between the deprotonated and the protonated form has been demonstrated by single-molecule fluorescence spectroscopy[19] as well as hole-burning spectroscopy.[20] Hole-burning spectroscopy also revealed that the energy level of the protonated form is close to that of the deprotonated form for EGFP, suggesting a potential ground state conversion between the two forms. Furthermore, the presence of the protonated and the deprotonated form has been suggested at a single molecule level. Simultaneous two-color excitation in the absorption bands of the protonated and the deprotonated form leads to an enhancement of the fluorescence intensity, strongly suggesting that the chromophore can convert between the protonated and the deprotonated form.[21] Taking into account these findings, conversion between the protonated and the deprotonated form is principally possible, either via a photo-induced process or acid-base-equilibrium in the ground state. The observed Raman spectra of a protoned and a deprotonated chromophore further substantiate these suggestions. In the case of Peterman et al., the on-time was strongly dependent on the excitation power,[17] indicating that on-time of the fluorescence is determined by a photo-induced process which results in an unknown dark state. Photo-induced on-off behavior has

been reported by several groups.[22,23] The protonation/deprotonation of the chromophore is, therefore, probably hidden by that photo-induced process. It should be mentioned that the long off-times in the fluorescence were observed even when exciting both the protonated and the deprotonated form simultaneously.[21] In our case, the excited state is quenched rapidly by resonance energy transfer from the molecule to the Ag particles. Consequently, the conversion between the protonated and the deprotonated form observed in our SM-SERRS measurements will be the result of the acid-base equilibrium in the ground state rather than the photo-induced protonation/deprotonation.

Relation between the fluorescence and SERRS intensity time trace

Figure S1 displays a typical intensity time trace of the fluorescence from a single EGFP molecule, which shows fast on-off blinking (order of milliseconds) as well as slow on-off blinking (order of seconds). The intensity time trace of the fluorescence from a single EGFP molecule measured with the same excitation power and wavelength as used in the SERRS experiment is depicted in Figure S2. Although the intensity of the fluorescence in Figure S2 is lower than that in Figure S1 due to the lower excitation power, on-off blinking of the fluorescence can be seen clearly. As mentioned above, the origin of the slow on-off blinking is not clear although this is a common phenomenon of GFP's. The autocorrelation analysis of a single burst (indicated by arrow in Figure S2) leads to the correlation curve shown in Figure S3. The correlation curve can be fitted by a single-exponential decay function, giving an auto-correlation time of 4.7 ms. The intensity time trace of the SERRS signal also displayed intensity fluctuation (See Figure S4). However, the time trace shows much more dynamics as compared with the fluorescence time trace. In contrast to the fluorescence time trace which reflects directly the nature of the chromophore, the fluctuation of the SERRS time trace is the result of a complicated combination of factors including both the nature of the chromophore and the interaction between the protein and the Ag particles. The complicated dynamics can be seen clearly in the autocorrelation curve (see Figure 2 in the communication). Because all the different conformations (the protonated form, the deprotonated form, and a possible unknown conformation which corresponds to the dark state in the fluorescence) of the chromophore are principally active for SERRS enhancement and because the interaction between the protein and the Ag particles is crucial for SERRS enhancement, the fluorescence and the SERRS intensity time trace can not be directly compared. The reported dynamics in the SERRS signal might be correlated with the change in the environments around the chromophore as well as with the dynamic character of the Ag-protein interaction and of the electromagnetic field enhancement. More investigations are being carried out in order to elucidate to what extend the different factors contribute.

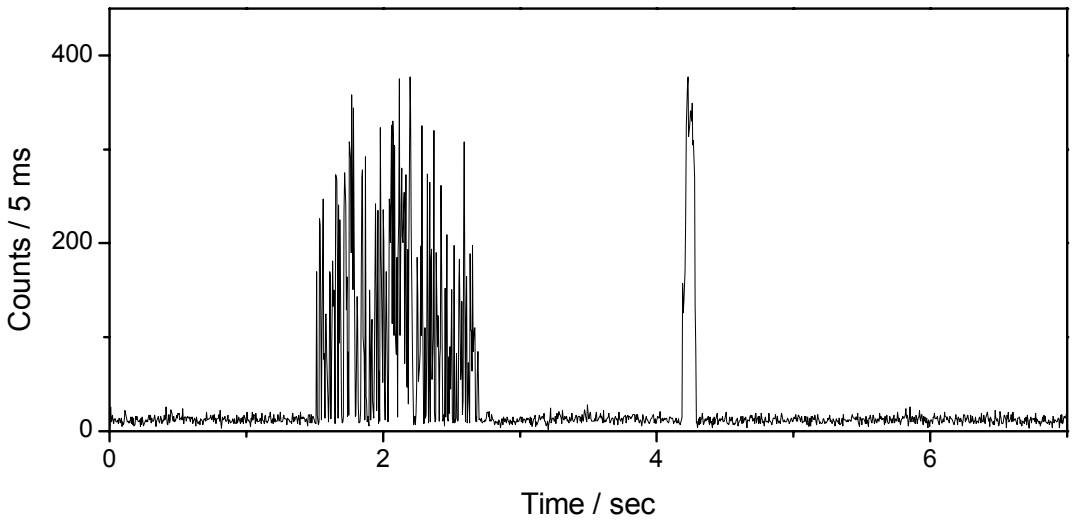

Figure S1 Typical intensity time trace of the fluorescence from a single EGFP molecule fixed in a polymer matrix of poly-vinyl alcohol.

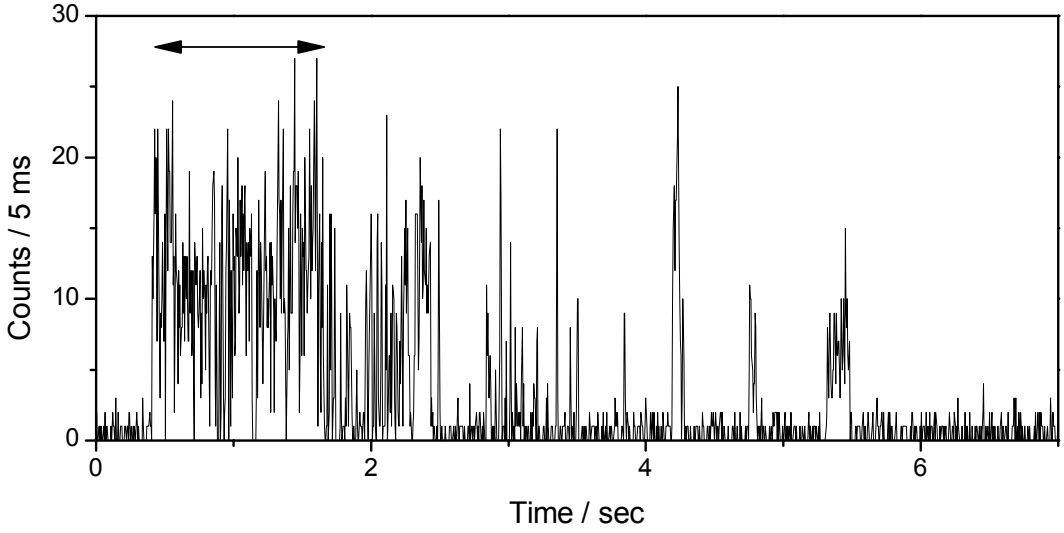

Figure S2 Intensity time trace of the fluorescence from a single EGFP molecule fixed in the polymer matrix of poly-vinyl alcohol measured with the same excitation power and excitation wavelength as used in the SERRS experiment.

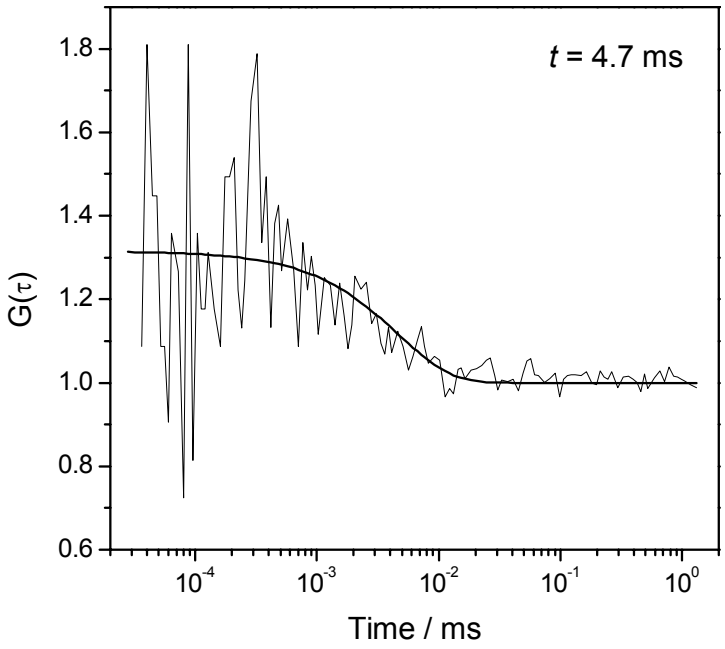

Figure S3 Autocorrelation curve obtained from a single burst (indicated by arrow in figure 2) in the fluorescence time trace of EGFP displayed in Figure 2 and the single-exponential fit yielding a correlation time of 4.7 ms.

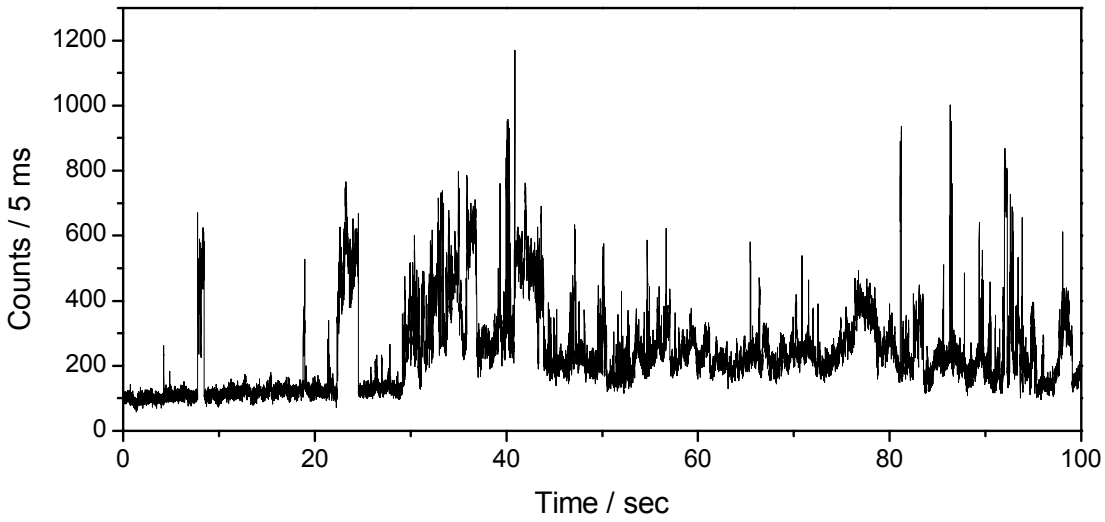

Figure S4 Intensity time trace of the wavelength integrated SERRS signal detected from a single EGFP molecule.

References for Supporting Information

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