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## Near-field fluorescent imaging of single proteins

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### Abstract

We report the results of our initial efforts to image photosynthetic proteins using the near-field technique. Allophycocyanin trimers, which contains six chromophores each, were dispersed onto a glass cover slip, and the fluorescence was imaged using a recently constructed near-field microscope. The results show that the emission from single proteins can be detected and localized on the nanometer scale.

### 1. Introduction

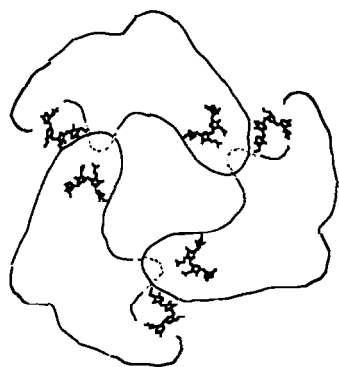
We report our initial results using a recently constructed near-field scanning optical microscope (NSOM) which demonstrate its single molecule sensitivity and sub-diffraction limit spatial resolution. In the NSOM technique [1], a spot of light smaller than the optical wavelength is scanned in close proximity to the sample such that far-field diffraction effects cannot occur. By doing this, the spatial resolution of the optical technique is not diffraction limited but is only limited by the size of the probe, which can be produced with apertures less than 100 nm. This provides a means of studying systems with a spatial resolution comparable to scanning electron microscopes but with the wealth of information

available from optical techniques. Furthermore, because the light is confined to pass through a small aperture (100 nm), a high photon flux can be delivered to the end of the probe with relatively small powers (nanowatts). This enables nearly background free detection of emission signals with single molecule sensitivity [2]. We report here the near-field fluorescence imaging of single trimers of allophycocyanin (APC) dispersed on a glass substrate.

APC is one of the phycobiliproteins contained in phycobilisomes, a light harvesting protein complex in cyanobacteria [3]. In phycobilisomes, APC and other specialized phycobiliproteins facilitate the collection and funneling of light energy towards reaction centers where electron transfer processes occur with high efficiency. The crystal structure of APC has not been solved, but comparison with the crystal structure of a closely related phycobiliprotein, C-phycocyanin [4], suggests that it has the trimeric structure shown in Fig. 1. Each monomer unit of the trimer structure contains two open chain, tetra-pyrrole chromo-

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|----- 11 nm -----|

Fig. 1. Proposed structure of APC trimer based on the crystal structure of the similar phycobiliprotein, C-phyocyanin. There are two tetra-pyrrole chromophores per protein monomer, and the entire structure has a  $C_3$  symmetry axis. The trimer is disk-shaped with a diameter of approximately 11 nm and a thickness of 3 nm.

phores covalently bound to the protein structure. These chromophores give rise to strong absorption and emission bands in the visible spectrum peaked at approximately 650 and 670 nm, respectively. APC has long been known for the intensity of its fluorescence and its inherent photostability occasioning its use as a test of single molecule sensitivity. In addition to this, we chose to initiate the present study on APC because of our interest in applying the near-field technique to address issues involving photosynthesis [5]. The results presented here demonstrate the ability of the near-field technique to detect and localize single proteins on the nanometer scale. Furthermore, the emission signal sizes that we measured for single APC trimers are such that measurements which spread the emission in both the frequency and time domains are possible allowing one to make spectroscopic and dynamic measurements on single proteins.

## 2. Experimental

The near-field fluorescent microscope is similar to the scanning aperture based designs re-

ported previously [1] and is shown schematically in Fig. 2. The output of a laser is passed through a bandpass filter and subsequently through both half wave and quarter wave plates to control the polarization of the light before being coupled into a single mode fiber. For the experiments reported here, a 5 mW HeNe laser operating at 633 nm was used. The light travels through the fiber to a tip that has a diameter of approximately 100 nm and is coated with 50–100 nm of aluminum to prevent leakage from the sides [6]. The tip is mounted in a piezo-tube that controls the tip to sample distance, and the sample is mounted on a  $x$ - $y$  piezo-stage which is used to scan the sample under the tip. Both the tip and the sample are in turn supported on an inverted Nikon Diaphot microscope.

The height regulation of the tip above the surface is critical in the near-field technique and is controlled through the shear force feedback technique recently introduced [7,8]. In this method, the fiber tip is dithered approximately 5 nm laterally at its resonant frequency. This oscillation can be monitored optically and processed using phase sensitive detection. When the tip nears the surface, the oscillation is damped and a feedback loop can be implemented to maintain a constant damping. As an example, Fig. 3 shows the shear force image obtained by scanning the fiber tip over a sample consisting of latex spheres

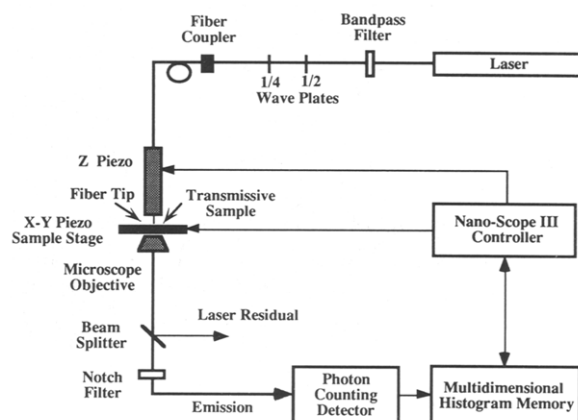


Fig. 2. Schematic diagram of the near-field microscope (see text for details).

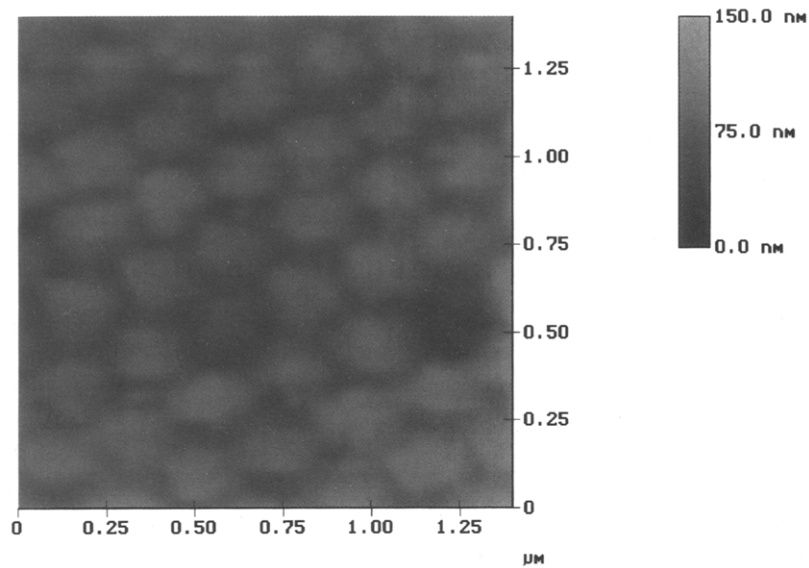


Fig. 3. Shear force image of 210 nm latex spheres dispersed on mica collected using the near-field microscope. Visible on the right of the image is a site which is unoccupied.

dispersed on a cleaved mica surface. The spheres were 210 nm in diameter and were spin coated onto the mica surface to produce a smooth monolayer. The image clearly shows the close-packed

arrangement of the spheres on the mica and is able to resolve the missing sphere in the right of the image.

In designing the microscope, particular atten-

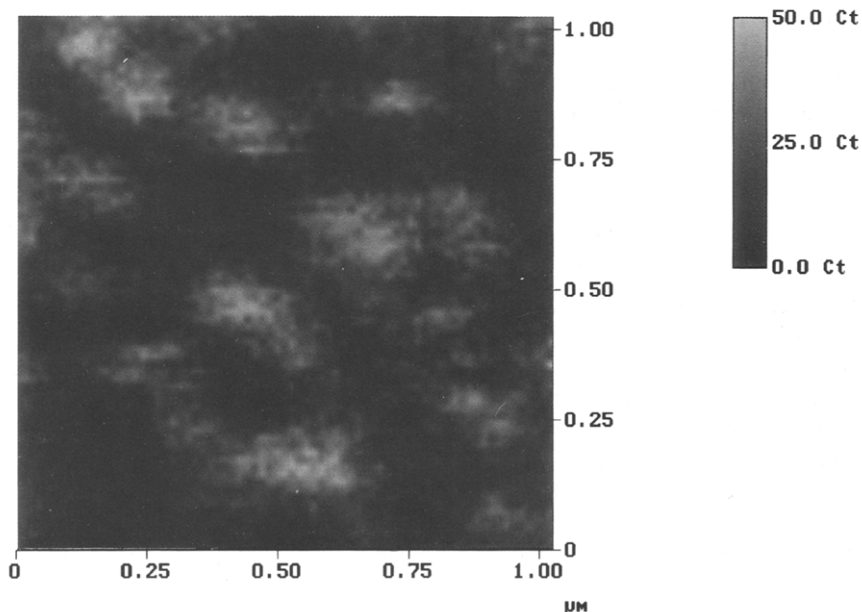


Fig. 4. Near-field fluorescence image of single APC trimers dispersed on a glass cover slip. The FWHM of the individual features is 100 nm, which is limited by the diameter of the tip used. The extrapolated signal for positioning the tip above a peak is 5000–10 000 counts/s, which is enough to carry out spectroscopic measurements on a single protein basis.

tion was paid to the optics train in order to maximize stray light rejection and optimize the collection efficiency of the emission. This results in low background counts and provides the sensitivity needed to detect single protein molecules. A high numerical aperture oil emersion objective is used to collect the emission and the residual excitation light. The light is then sent through a combination of a beam splitter and notch filter to separate the two. The laser residual, separated by the beam splitter, is used to generate the feedback signal and the emission, which passes through the beam splitter and notch filter, is detected with a high quantum efficiency, low noise avalanche photodiode (APD). The signal from the APD is sent to multidimensional histogramming memory which keeps track of the position of the sample and deposits the observed counts into the correct  $x$ - $y$  coordinates. The tip and sample piezos are controlled with a Nanoscope III from Digital Instruments.

Cross-linked allophycocyanin trimer was purchased from Molecular Probes. The allophycocyanin was dialyzed in 0.1M phosphate buffer at pH 7.0 resulting in a concentration of  $1 \times 10^{-6}$  M. The sample was further diluted by a factor of 20 in deionized water and dispersed on a cleaned glass cover slip by spin coating. Unlike normal APC, cross-linked APC can exist in very dilute buffer solutions without disrupting the trimer structure [9].

### 3. Results

Fig. 4 shows a  $1 \mu\text{m} \times 1 \mu\text{m}$  ( $75 \times 75$  pixel) near-field fluorescent image of single APC trimers dispersed on a glass cover slip. Distinct features with full-width-half-maximums (FWHM) of approximately 100 nm are clearly discernible. The 100 nm spatial resolution, which is well beyond the diffraction limit, corresponds to the diameter of the tip aperture used to image the proteins. The image in Fig. 4 was collected while maintaining a tip to sample height of approximately 6 nm. Maintaining tip to sample distances closer than this (i.e. more force) resulted in the sweeping away of the protein by the tip. We found that

there was little loss in the intensity of the individual features following several scans of the same region reflecting the photostability of the APC trimer. No triplet states have been observed for APC [10] and the protein matrix reduces the probability of photobleaching.

We observe a small variation in the emission intensities of the features in Fig. 4 and there are several possibilities that can account for this. One possibility is that the proteins may tend to aggregate or decompose into the monomers. However, the cross-linked APC samples have been shown not to aggregate or dissociate to the monomer. Another possibility is that the APCs orient on the surface with different tilting geometry. From SEM images [11,12], the individual APC trimers are 11 nm in diameter and 3 nm thick, resembling a disk-like structure. This favors a flat orientation on the surface due to the more favorable interactions with the surface, suggesting that tilt angle is not responsible for the intensity variation. Linearly polarized light was used in these experiments, and the small intensity variation may result from the different orientation of the proteins on the surface. We are currently investigating the effects of incident polarization on the observed emission images, which may also provide structural information. Finally, complications may arise from working with dry samples. We are presently extending this study to image APC samples in a liquid cell.

As mentioned above, the diameter of APC is only 11 nm so its internal features are unresolvable in the emission image shown Fig. 4. It is important to point out, however, that this is not the goal of the present study. The image in Fig. 4 demonstrates that single proteins can be detected and localized optically on the nanometer scale, which is well beyond the optical diffraction limit. Furthermore, by taking the scan speed of 4 ms/pixel used to collect the image in Fig. 4 and the number of counts observed, one can estimate the count rate expected for positioning the tip above a single APC trimer at 5000–10 000 counts/s. This count rate is more than enough to spread the counts in frequency or time and make spectroscopic measurements on the individual protein trimers. This will provide unique informa-

tion on the protein's particular environment and its interactions with its surroundings.

#### 4. Conclusions

We have demonstrated the fluorescence imaging of single trimers of APC dispersed on a glass substrate using the near-field technique. We observe that the FWHM of the features are 100 nm, limited only by the aperture diameter of the fiber probe. The count rate of a single APC is such that spectroscopic measurements on individual proteins is possible. Currently we are studying the effects of excitation polarization on the observed APC emission images. We are also interested in what affect, if any, quenching of the fluorescence by the metal coating of the tip may have on the observed signal size. We are incorporating a picosecond time-correlated single photon counting capability into our microscope to investigate the magnitude of this effect as well as energy transfer dynamics of this system.

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