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Near-Field Fluorescence Imaging and Fluorescence Lifetime Measurement of Light Harvesting Complexes in Intact Photosynthetic Membranes

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Fluorescence lifetime measurements with nanometer spatial resolution are made possible by incorporating time-correlated single photon counting with a near-field fluorescence microscope. Using this apparatus, single pieces of intact photosynthetic membrane from the *Chlamydomonas reinhardtii* strain doubly deficient in photosystem I (PS I) and photosystem II (PS II) are examined. Comparison of simultaneous force and emission images shows single membrane bilayers lying flat on the mica substrate with the embedded light harvesting complex (LHC II) proteins aggregated isotropicly across the membrane imaged. Fluorescence lifetimes are measured at a single spot on the membrane with picosecond time resolution.

Introduction

Photosynthesis in green plants takes place on the thylakoid membranes which contain a number of transmembrane proteins to which pigments such as chlorophyll and other important prosthetic groups are bound. Each of these protein complexes is embedded and distributed in the thylakoid membrane in a specific fashion to facilitate light trapping, electron transfer, proton pumping, and other enzymatic reactions. In the primary events of photosynthesis, light-induced electronic excitation of chlorophyll in the light harvesting antenna complexes is rapidly transferred among the pigments and trapped in reaction centers where fast electron transfer occurs with high efficiency.

In recent years, crystal structures of several isolated protein complexes of chlorophyll systems such as light harvesting complexes (LHC),[1] photosystem I reaction centers,[2] and photosystem II reaction centers[3] have become known. However, the relative orientation and distribution of LHC, PS I, and PS II within the thylakoid membrane are less well understood. This information is needed for a complete understanding of the energy disposal mechanisms operating between the various complexes. Previous knowledge of the spatial distribution was based on freeze fracture techniques in which the topology of a metal replica of a fractured membrane is examined by electron microscopy.[4] Although electron microscopy provides the high spatial resolution required, it has two disadvantages. The first is the destructive nature of its preparatory procedure. Second, such a technique can only provide information regarding the size and the shape of the various features attributable to the protein complexes but completely loses their spectroscopic identities. LHC, PS I, and PS II all have distinct spectral properties such as fluorescence lifetimes, which can be used to identify the various complexes.

Time-resolved fluorescence spectroscopy is a powerful tool to study the energy and electron-transfer processes in photosynthetic proteins. Use of this technique has resulted in a great deal of information on the dynamical processes occurring in these systems.[5] To date, most of the informative experiments were done with big ensembles of isolated protein complexes in buffer solutions. The heterogeneity of the sample is often problematic, contributing to the complexity of the fluorescence decay kinetics. Isolation of the protein from the membrane also tends to reduce the integrity of the membrane protein. Conducting time-resolved fluorescence measurements with nanometer spatial resolution on intact thylakoid membranes makes it possible to study the energy and electron-transfer dynamics on a single protein basis, free from the above complications.

Near-field optical microscopy allows one to conduct optical spectroscopy at the nanometer scale.[6] This technique can now provide optical measurements with tens of nanometer resolution, comparable to that of a low-resolution electron microscope. Furthermore, the near-field technique is particularly sensitive in emission measurements. Recently, several groups have reported fluorescence detection of single molecules.[7-9] These experiments were similar to previous single molecule detection experiments in that the signal averaging is done by exciting the single molecule many times.[10,11] In addition to the advantages of high spatial resolution, high sensitivity, and easy rejection of the background signal, the near-field technique allows the examination of native biological samples in an ambient environment. We recently reported the near-field fluorescence imaging of allophycocyanin, a photosynthetic antenna protein which contains only six chromophores.[9] The results indicated that the fluorescence count rate was sufficient to spread the counts in time.

In this Letter, we report results obtained using a near-field fluorescence microscope combined with a time-correlated photon counting apparatus. The microscope has an instrumental response (fwhm) of 120 ps with high sensitivity and low background signal. Using this apparatus, we obtained simultaneous shear force images and total fluorescence images of intact thylakoid membranes. The thylakoid membranes used were isolated from the C2 mutant strain of the green alga *Chlamydomonas reinhardtii*, which is deficient in both PS I and PS II reaction centers and core antenna complexes but produces the peripheral light harvesting chlorophyll–protein complexes, LHC II.[12] The shear force mechanism is noninvasive to the membrane structure, providing high spatial resolution force images. The emission images were collected simultaneously with the force images. In addition, the fluorescence lifetime of the light harvesting antenna proteins embedded in the membrane was measured by time-correlated single photon counting at specific locations. We believe that this technique offers a unique approach for studying photosynthetic membranes.

Experimental Section

Excitation light is coupled into a single mode optical fiber (Corning Flexcore 633) whose end has been drawn down to a fine point (usually 100 nm) using a Sutter Instruments P-2000 fiber

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puller. The sides of the tapered tip are evaporatively coated with aluminum to prevent light leakage, resulting in the confinement and delivery of an extremely small point of light.[6] Typically, tips with 100-nm apertures attenuate the light by 6 orders of magnitude, resulting in the delivery of approximately 10^{10} photons/s with 5 mW coupled in. The high sensitivity of fluorescence detection results from the high net photon flux that the coated fiber delivers through the near-field zone to the far field, as well as the strong evanescent field which can excite fluorophores in the near-field region. This source of illumination is positioned in close proximity (approximately 5–10 nm) to the sample in order to obtain a spatial resolution beyond the diffraction limit. To maintain this small gap while the sample is scanned in a raster fashion, a shear force technique is implemented. In this method, the fiber tip is dithered laterally approximately 5 nm at its resonant frequency. As the tip approaches the sample surface, the oscillation becomes damped which is optically detected and utilized in a feedback loop similar to those previously reported.[13,14] This results in a simultaneous force mapping of the sample surface. The scanning of the sample stage and tip feedback loop was incorporated into a Nanoscope III controller (Digital Instruments). Great care was taken in designing the optical train of the microscope that results in a high emission collection efficiency coupled to a high quantum yield detector. The near-field assembly is supported on an inverted fluorescence microscope (Nikon Diaphot), and the emission from a transmissive sample is collected with a high numerical aperture (1.3 NA) oil immersion 100× objective. A combination of a holographic beam splitter and notch filter (Kaiser Optical Systems) is used to separate the residual excitation from the emission. The total emission is imaged onto an avalanche photodiode (APD) (EG&G SPCM-200) modified for fast time response,[15] while maintaining a high quantum efficiency (70% at 650 nm) and low dark noise (<30 counts/s). The nearly background-free emission measurements are possible because of the small illumination area of the near-field probe and the effective stray light rejection of the optics train.

For the time-resolved experiments described here, pulses from a synchronously pumped dye laser system were coupled into the optical fiber. The output of a Coherent Antares 76-YAG was frequency doubled to 532 nm (2 W, 80 ps fwhm, 76 MHz) and used to synchronously pump a Coherent 702 dye laser operating with sulfarhodamine 640 lasing at 633 nm (100 mW, 5 ps fwhm, 76 MHz). The output of the dye laser was directed through a pulse selector (Coherent 7220) to reduce the repetition rate to 9.5 MHz and a holographic band-pass filter (Kaiser Optical Systems) to eliminate the spontaneous emission. The light was attenuated down to 2 mW average power before being coupled into the single mode optical fiber. We have shown that at this power level no broadening of the picosecond pulses occurs while passing through the aluminum-coated fiber tips.[16] The fluorescence decay times were measured using the time-correlated single photon counting technique in the reverse counting arrangement. The output of the APD was amplified 100× (Phillips Scientific Model 6954), sent to a leading edge discriminator (Phillips Scientific Model 710), and used as the start pulse for a time-to-amplitude converter (TAC) (Tennelec TC 864). The stop pulse for the TAC was generated from a fast photodiode monitoring the dye laser output of the pulse selector. The analog signal from the TAC was digitized using an Ortec 800 A-to-D converter coupled to a home-built histogram memory where it was read with a 486 computer. The combination of the particular APD and the detection electronics results in an instrumental response time of 120 ps full width at half-maximum (fwhm) with a full-width at one-hundredth maximum of 710 ps. This is shorter than that previously reported using an APD detector.[15] The time resolution can be improved further using a microchannel plate photomultiplier tube at the expense of detection quantum yield.

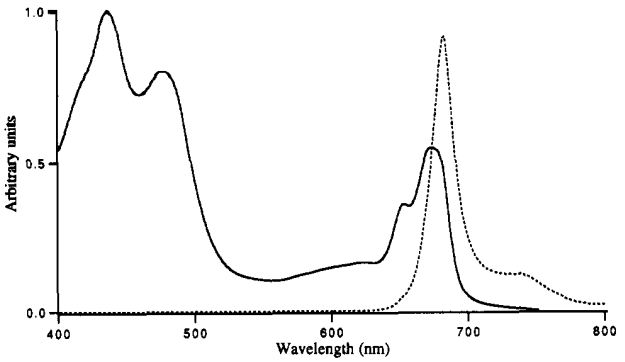

Figure 1. Steady-state absorption (solid line) and emission spectra (broken line) of a suspension of thylakoid membranes from the *Chlamydomonas reinhardtii* PSI-PSII doubly deficient C2 strain in buffer solution at room temperature.

Thylakoid membranes from the *Chlamydomonas reinhardtii* PS I-PS II doubly deficient C2 strain were prepared as previously described.[17] This strain lacks the chloroplast tscA and Psba genes necessary for the synthesis of the proteins and core antenna complexes associated with PS I and PS II, respectively. The complete complement of peripheral antenna LHC proteins, however, is retained. The C2 strain cells were broken in a French press. After separation on a sucrose gradient, the stacked thylakoid membrane pieces were treated with EDTA solution at 0 °C. The unstacked bilayer membrane is kept cool to prevent vesicle formation. The absorption and emission spectra of the membrane sample suspended in solution are shown in Figure 1. Based on the absorption spectra, the Chl a/Chl b ratio was estimated to be around 1.4. A drop of ice cold membrane suspension was dispersed on a freshly cleaved mica substrate, rinsed with deionized water, and air-dried at room temperature. Single layers of bilayer membrane pieces were laid down on the mica substrate, as seen by electron microscopy and the shear-force microscope discussed below. All the near-field measurements were done on the dried sample at room temperature.

Results and Discussion

The top image in Figure 2 is a 1.3 μm × 1.3 μm shear force image of membrane bilayers from the C2 strain lying flat on a mica surface. The unfiltered image shown has a high signal-to-noise ratio which results in a vertical resolution of <1 nm. The lateral resolution appears to be greater than that expected from a coated tip diameter of 250 nm which may reflect the presence of a sharp feature on the end of the coated tip. Clearly there are four membrane fragments (two big and two small) in this image. The thickness of these membrane fragments is measured to be approximately 6.5–9.5 nm, which is in close agreement with the known bilayer membrane thickness of 6–7 nm. From this we can conclude that the membranes are deposited on the mica surface as single intact bilayers. Stacking of the membranes was also observed in some regions (data not shown), but the high vertical sensitivity of the shear force microscope makes these areas easily distinguishable from the desired single bilayer regions.

In recent years, atomic force microscope has been elegantly used to image many biological samples including membranes.[18,19] We note that one advantageous feature of the shear force microscope may be its noninvasive nature, particularly suitable for biological membranes. The frictional/attractive forces in the shear force technique are significantly smaller than the repulsive forces inherent in contact force microscopes. As a result, the shear-force technique is gentler on the surface being imaged. The force curve for a dry sample allows the tip to be positioned and held ~10 nm above the sample, appropriate for near-field fluorescence measurements. Shorter gaps can possibly lead to a significant fluorescence quenching by the aluminum coating or perturb the optical spectra of the sample.

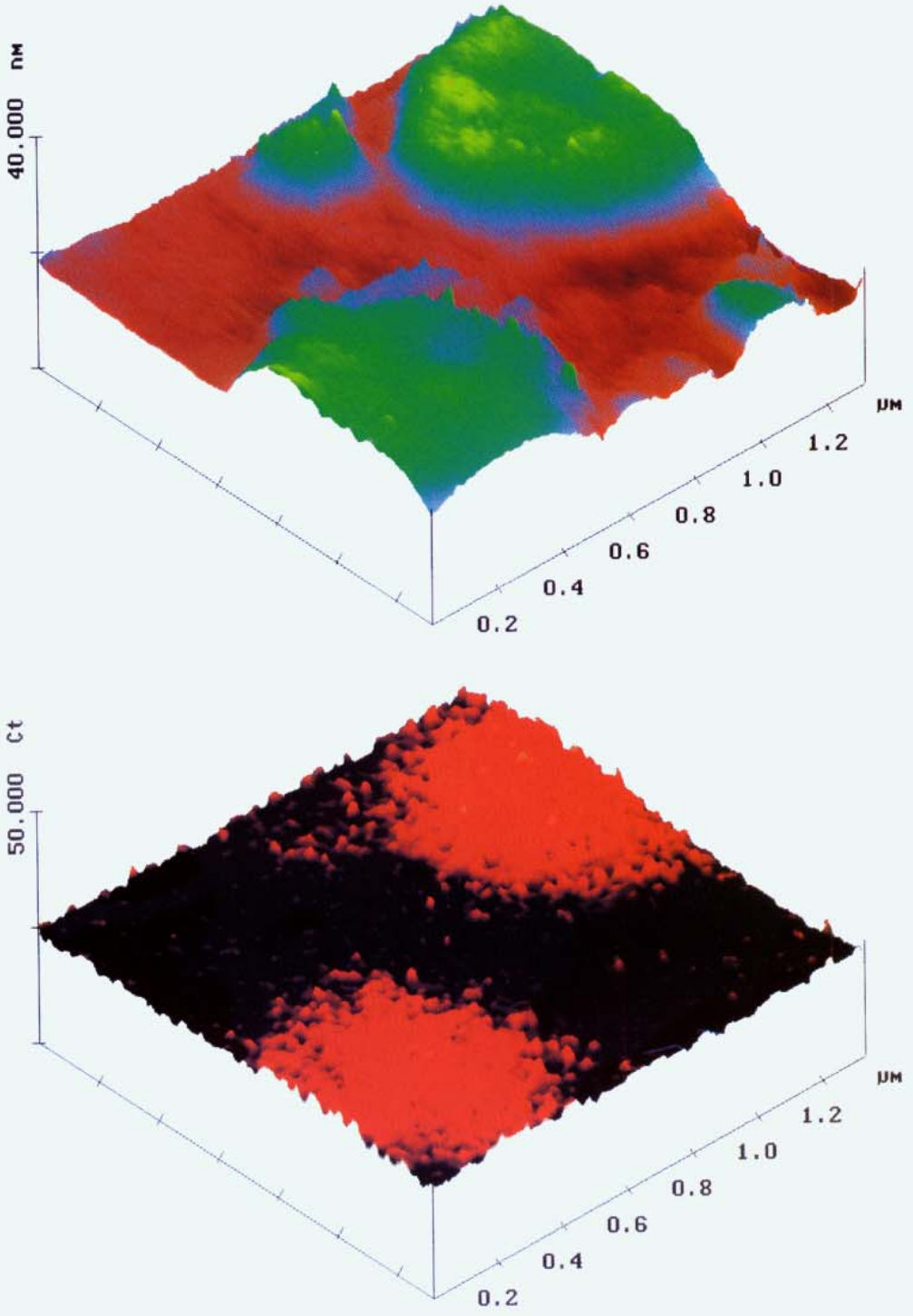

Figure 2. Top image: 1.3 μm × 1.3 μm shear force image taken with the near-field microscope showing intact photosynthetic membrane fragments. The high signal-to-noise ratio results in a vertical resolution of <1 nm. The height of the membrane pieces lying flat on a mica surface is measured to be 6.5–9.5 nm, consistent with the thickness expected for single bilayer membranes. Bottom image: 1.3 μm × 1.3 μm fluorescence image taken with the near-field microscope simultaneously with the above shear-force image. Homogeneous emission is seen in both of the large membrane pieces due to the close-packed network of LHC II proteins embedded in the membrane.

The bottom image in Figure 2 is the 1.3 μm × 1.3 μm near-field fluorescence image obtained simultaneously with the force image shown above. The 165 × 165 pixel image was accumulated with a collection time of 4 ms per pixel. The emission is from the chlorophyll *a* molecules bound to LHC II membrane proteins. Subsequent images of the same area shows the same features but with reduced intensity due to the photobleaching. For this reason, the image shown in Figure 2 was collected with the light output of the near-field aperture reduced to 10^8 photon/s to allow time-resolved measurements afterward. The emission image in Figure 2 clearly shows two features corresponding to the two large membrane pieces seen in the force image. Other regions of the same sample scanned with the same tip showed emission features as small as 90 nm (data not shown), breaking the diffraction limit. The spatial resolution is only limited by the size of the particular near-field aperture with resolution <25 nm possible at

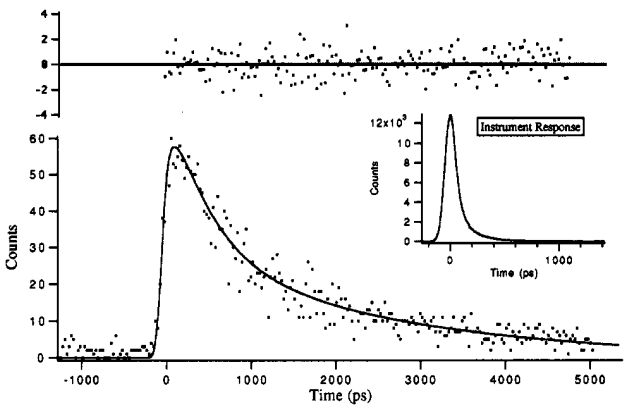

Figure 3. Fluorescence decay of LHC II proteins in an intact membrane taken with the near-field microscope. The decay is fit with a sum of exponentials with lifetimes of 450 ps (65%) and 2.7 ns (35%), with the weighted residuals shown at the top. The inset shows the instrumental response function which has a fwhm of 120 ps.

the expense of signal. While we are currently improving our resolution for the spectroscopic mapping of the thylakoid membrane, here we demonstrate the sensitivity of the near-field technique for fluorescence measurements on photosynthetic membranes.

The simultaneous shear-force and near-field fluorescence imaging capabilities allow a unique comparison of the topographic and spectral information. For example, the two small membrane pieces seen in the force image in Figure 2 are dark in the emission image. This demonstrates the power of combining the optical detection with shear force to distinguish membranes with and without fluorophores. The light harvesting complexes (LHC II) not connected to reaction centers, as in strain C2, have a high fluorescence quantum yield and are responsible for the bright fluorescence from the two big membrane pieces. It appears from comparing the force image and emission image that the emission is homogeneous throughout the membranes. Kuhlbrandt and co-workers[1] have resolved the structure of the LHC II protein and have found that the LHC II complex forms trimers with three symmetrically arranged monomers. Each monomer contains three trans-membrane alpha helices plus 15 chlorophyll molecules (8 Chl a + 7 Chl b and carotenoids). The cylindrical trimer has a diameter of 7.3 nm and a thickness of 6.0 nm. It has been shown that the trimers tend to aggregate in the membrane to form a two-dimensional lattice.[1] Electron micrographs from freeze fracture studies clearly show this closed packed arrangement.[4,20] We therefore attribute the lack of detailed emission features across the membranes imaged to this closed-packed LHC II lattice structure.

A particular advantage of the near-field technique over other scanning probe techniques is the high time resolution afforded by its optical nature. Coupling ultrafast pulses through the near-field optics will allow dynamical processes such as electron or energy transfer to be examined at the molecular level. As a first step, our group[16] and others[21] have shown that these pulses can be passed through the near-field aperture without damage to the tip or broadening of the pulses. We report here the demonstration of fluorescence lifetime measurements using the near-field technique.

Figure 3 shows the fluorescence decay data taken by moving the fiber tip to the center of the membrane piece in the upper portion of Figure 2 and keeping the feed back loop on. The decay is significantly longer than the instrumental response function (fwhm 120 ps) shown in the inset of Figure 3. The decay can be fit with a sum of two exponentials; the short component has a lifetime of 450 ps (65%), and the long component has a lifetime of 2.7 ns (35%). The solid line is the fitted decay convoluted with the instrumental response function, with the weighted residuals shown on top. The data collection time was limited to approximately 10 s due to bleaching of the sample mentioned earlier. Although the carotenoid in LHC II presumably serves to quench the triplet state of Chl a, the fast excitation rate in the near-field experiments apparently still leads to photodestruction. We are currently trying to use a degassed liquid cell to avoid the quenching of the sample by singlet oxygen.

It is important to stress that the time-resolved fluorescence measurements were conducted on a single intact membrane with nanometric spatial resolution, free from the complications of sample heterogeneity. Time-resolved fluorescence measurements on single fluorophores have been demonstrated previously at liquid helium temperature[22] and with flow cytometry techniques.[23] For biophysical applications, however, combining the high time resolution and high spatial resolution of the near-field technique should be particularly informative. Fluorescence lifetime imaging of biological samples with confocal microscopy, for example, has undergone rapid developments in recent years.[24,25] The technique presented in this paper allows a higher spatial resolution and higher detection sensitivity for two-dimensional samples.

Time-resolved fluorescence measurements on the same membrane samples in solution have been carried out on the picosecond[12,26] and femtosecond time scales.[27,28] A double-exponential decay with a short component of 839 ps (16%) and a dominant long component of 2.7 ns (84%) were reported using time-correlated photon counting.[12] The short component was attributed to a small amount of concentration quenching. Our result has an identical decay rate but a smaller amplitude for the long component. One possibility for the faster decay (450 ps) and larger amplitude (65%) for the short component could be singlet–singlet annihilation.[29] We are working on the intensity dependence study to examine this possibility. The faster decay could also be due to fluorescence quenching by the aluminum coating on the fiber probe. This is a technical issue yet to be resolved in the development of near-field fluorescence imaging. Time-resolved emission measurement should be particularly useful in the characterization of this effect. While we are currently examining this phenomenon in detail over a wider tip–sample separation range, these initial results are encouraging with respect to applying the near-field time-resolved technique to probe the spectroscopic properties of photosynthetic membranes. Fluorescence measurements on the nanometer scale offers exciting possibilities for the spectroscopic mapping and detailed study of the electron and energy-transfer mechanisms in these systems.

Conclusions

We have demonstrated that intact photosynthetic membranes can be examined with both high spatial and temporal resolution using a near-field fluorescence microscope. For the photosynthetic membranes containing only LHC II, shear-force images show features 6.5–9.5 nm thick, attributable to single membrane bilayers lying flat on the mica surface. The simultaneous fluorescence images show that the emission from the membranes is isotropic, consistent with the known closely packing arrangement of LHC II within the membrane bilayer. We report near-field fluorescence lifetime measurements conducted by time-correlated single photon counting with an instrumental response time of 120 ps (fwhm) and a spatial resolution of 100 nm. The lifetime for the LHC II aggregates measured at a single spot on the membrane is best fit by a double exponential with lifetimes of 450 ps (65%) and 2.7 ns (35%). We believe the high time resolution, spatial resolving power, single molecule sensitivity, and the noninvasive nature make this technique a powerful approach for the study of photosynthesis and other biological systems.

Note Added in Proof. Since the submission of this paper, W. Patrick Ambrose and co-workers at Los Alamos Laboratory reported at the SPIE'94 meeting that they have measured the fluorescence lifetime of single rhodamine 6G molecules using the near-field technique. Recently, we have done similar picosecond

experiments on single Texas Red molecules. In addition, we have carried out near-field fluorescence lifetime imaging on single chromophores using the same apparatus reported here.

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