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ScienceDirect
Journal of Luminescence 122–123 (2007) 301–303
JOURNAL OF LUMINESCENCE
www.elsevier.com/locate/jlumin

Spatial location of photosystem pigment–protein complexes in thylakoid membranes of chloroplasts of *Pisum sativum* studied by chlorophyll fluorescence

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Available online 9 March 2006

Abstract

Ultrastructure of plant chloroplasts was studied by a single-molecule spectroscopy setup at a temperature of 77 K exploring spatial location of photosystems. Two chloroplast thylakoid membrane regions were visualized by fluorescence microscopy and detected at different wavelengths. The size of these regions and the spatial resolution of the microscope allowed us to measure their chlorophyll fluorescence emission spectra of these membrane domains. While the grana regions are characterized by a predominant presence of Photosystem II pigment–protein complexes emitting at 685 nm, Photosystem I complexes are localized in stroma regions and emit at 730 nm.

Keywords: Photosynthesis; Single molecule spectroscopy; Chloroplast ultrastructure; Photosystem; Fluorescence

1. Introduction

The process of photosynthesis converts the energy of light radiation into the energy of chemical bonds. Primary processes of photosynthesis take place in pigment–protein complexes of thylakoid membranes of plants and algae chloroplasts, cyanobacteria and photosynthetic bacteria. Chloroplast thylakoid membranes are organised into two types of regions, grana and stroma. Stroma regions are free floating membranes, grana are formed by several membranes stacked together [1]. The grana and stroma are interconnected but the distribution of photosynthetic pigment–protein complexes in these two regions is different. Whereas cytochromes b_6f are distributed equally, grana regions are predominantly formed by photosystems II (PSII) with their light-harvesting complexes, and stroma regions contain mainly photosystem I (PSI) complexes [2]. The presence of chlorophyll molecules in photosynthetic pigment–protein complexes gives to these complexes distinctive absorption and fluorescence properties. While the PSII emits light in the region of 680–695 nm both at room and low temperature, the emission of PSI at room temperature is negligible and can be detected only below 200 K as a broad emission around 720–730 nm.

The spatial distribution of PSI and PSII has been observed by several destructive methods based mainly on electron microscopy or analysis of isolated thylakoid membrane fragments. The only method that was, so far, used to follow the different PSI and PSII distribution in native state of chloroplast is a spectrally resolved laser scanning microscopy [3,4]. With this method the fluorescence images of single chloroplast were obtained at different emission wavelengths, and a picture of spatial distribution of PSI and PSII was created.

In this work, we have used single molecule spectroscopy setup to detect different fluorescence emission spectra of the two major photosynthetic pigment–protein complexes

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doi:10.1016/j.jlumin.2006.01.148

of chloroplast thylakoid membrane, the PSI and PSII, in their natural environment. This approach allowed us to study non-invasively their spatial distribution.

2. Materials and methods

Chloroplasts were isolated from pea plants (*Pisum sativum*) according to Vacha et al. [4]. Chlorophyll *a* was extracted from pea leaves by 100% acetone and isolated by preparative HPLC. Isolated chlorophyll was transferred from methanol to diethylether and dried under stream of nitrogen gas.

The single molecule setup used in this work is based on Olympus IX70 inverted fluorescence microscope, long-working-distance objective (60 ×, N.A. 0.7), micro-cryostat, imaging spectrometer and liquid-nitrogen-cooled back-thinned CCD camera. The excitation was provided by 442 nm line of HeCd laser in the case of chlorophyll molecules and by a 100W mercury lamp when chloroplasts were detected. The scheme of the setup is shown in Fig. 1.

The images were recorded using mirror position of the spectrometer's turret with fully opened entrance slit (7 mm). The emission spectra of spots placed in a central region of the image (defined by the entrance slit closed to 0.5 mm) were measured by the diffraction grating. For low temperature experiments, the chloroplast suspension was placed in a cryoprotecting medium of 70% (v/v) of glycerol prior to cooling to 77 K.

3. Results and discussion

As a test sample, we have used chlorophyll *a* molecules deposited on a cover slip by spin coating in 6% (v/v) of poly(methyl methacrylate) in chloroform. Fig. 2a shows

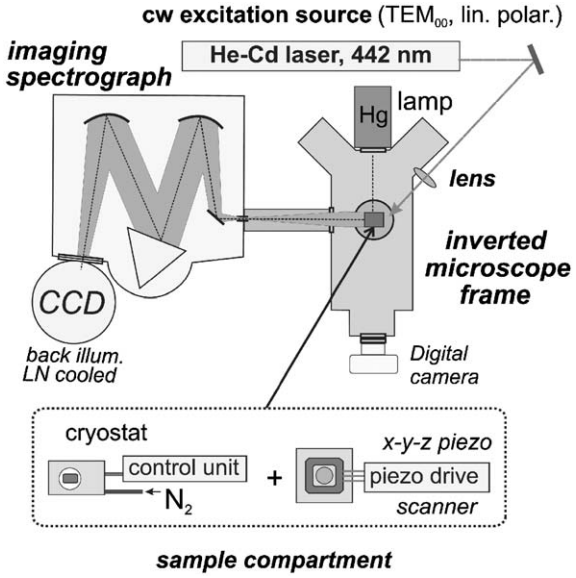

Fig. 1. Scheme of the single-molecule set up based on an Olympus IX70 inverted fluorescence microscope attached to a Jobin-Yvon Triax320 spectrograph equipped with a liquid-nitrogen-cooled CCD camera.

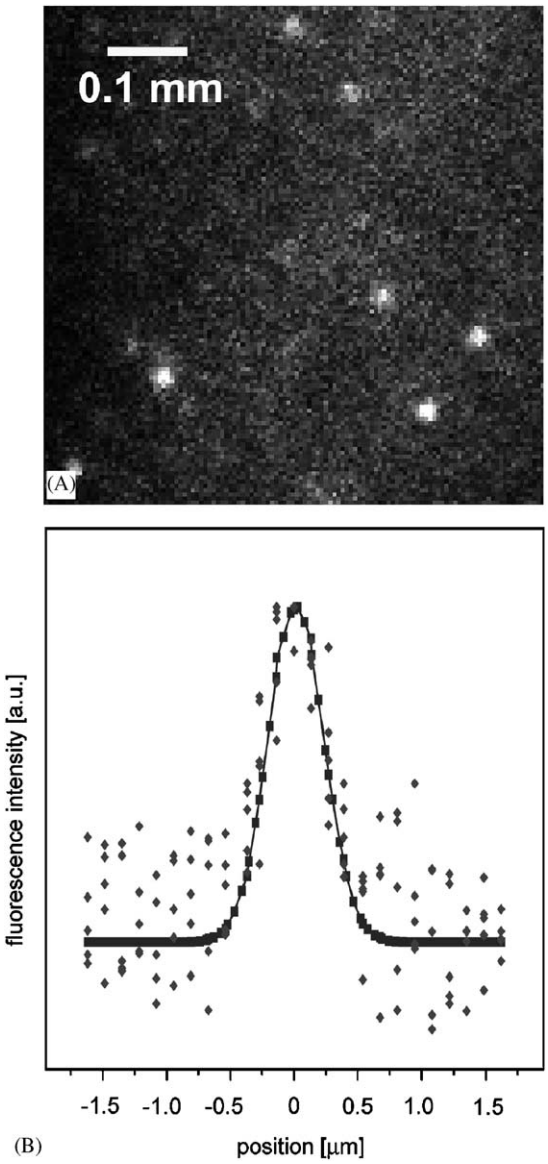

Fig. 2. (A) Microscope image of fluorescence of chlorophyll *a* molecules embedded in solid poly(methyl methacrylate) matrice by spin coating and viewed by Olympus 100 × objective, N.A. = 0.95. Number of pixels is 100 × objective, 100. The molecules were excited by a 442 nm laser line from HeCd laser in a total reflection mode. (B) Cross-section fluorescence intensity profile of the chlorophyll a fluorescence spots (diamonds). The solid line is Gausian fit of cross sections of six chlorophyll *a* molecules.

the fluorescence image of individual chlorophyll molecules. The lateral size of the dots is on the average 7.6 pixles in diameter. The Gaussian fit of cross sections of chlorophyll fluorescence dots is in Fig. 2b and gives the microscope experimental resolution (r_{exp}) of 600 ± 64 nm.This should be compared with theoretical resolution r_{th} based on the Rayleigh criterion, given by $r_{th} = 0.61\lambda/\text{N.A.}$ For the 100 × objective with N.A. = 0.95 the r_{th} is 437 nm at 680 nm, the emission wavelength of chlorophyll. Comparing r_{exp} and r_{th} we can conclude that the theoretical and experimental values of microscope resolution are in good correlation. Similar procedure can be applied to other

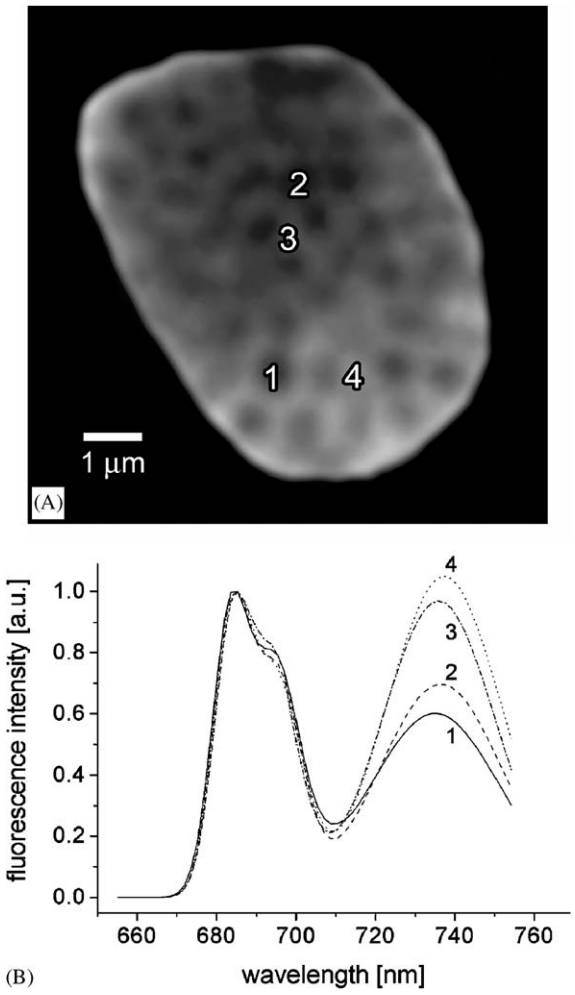

Fig. 3. (A) Fluorescence image of single chloroplast from *Pisum sativum* showing grana membrane regions in dark and stroma lamellae in light shades. Numbers show positions where the spectra were recorded from. (B) Fluorescence emission spectra recorded from four different positions in the chloroplast. Two positions, 1 and 2, represent areas of grana membranes, positions 3 and 4 represents areas of stroma membranes.

microscope objectives and it was found that the experimental resolution r_{exp} is sufficient to record the chloroplast ultrastructure and to measure the spectra of different thylakoid membrane domains.

Chloroplasts were carefully isolated to get a high yield of intactness. Fig. 3a shows the low-temperature chlorophyll fluorescence image of chloroplast processed according to Vacha et al. [4]. First, the overall fluorescence image was recorded capturing all emitted radiation above 515 nm.The image was composed of contributions of both photosystems, PSI and PSII. Keeping the low temperature during the experiment is essential since PSI almost does not emit light at a room temperature. In the next step, the fluorescence image was recorded using a long pass filter RG715 to detect only the emission of PSI complexes. Then, such image was subtracted from the overall fluorescence image to get the image of areas with fluorescence of PSII only. Finally, the "PSI" and "PSII" fluorescence images were overlaid. The resulting image shows the PSI stroma regions in light and the PSII grana regions in dark shades. The size of the chloroplast and its ultrastructure and organisation of grana and stroma membrane domains fit well with many other observation as reviewed recently [5,6].

Chlorophyll fluorescence emission spectra were measured from various sites of the imaged chloroplast. Results are shown in Fig. 3b, with the spectra normalised at 685 nm. Locations 1 and 2 were chosen to demonstrate the emission of PSII from grana membranes, location 3 and 4 to detect the presence of PSI in stroma. Spectra 1 and 2 show typical shape of PSII fluorescence emission at low temperature. They have maximum at 685 nm with a shoulder and 695 nm. The presence of emission of PSI may be explained by several reasons. (i) The spatial resolution of the optical microscope is close to the size of the grana compartments and therefore some overlap can be expected. (ii) In the inner space of the chloroplast, grana membranes and the intergranal stroma regions can occur above each other affecting the resulting spectrum by the fluorescence of PSI located out of the focal plane. (iii) PSI complexes can occur at the sides of grana membranes. Spectra 3 and 4 show the presence of both PSI and PSII complexes. The intergranal stroma membranes are known to be populated mainly by functional PSI but contain also PSII [7].

The heterogeneity in spatial distribution of photosynthetic pigment–protein complexes in thylakoid membrane of plants has been observed and reported many years ago. However, the measurements of such heterogeneity using intact systems in vivo or in situ were impossible until the development of appropriate methods. The image data we presented here are consistent with our previous measurements [4], yet this is the first spectral characterisation of the grana and stroma membrane regions in intact chloroplasts in their natural conditions.

Acknowledgements

This work was supported by grants MSM6007665808 and AV0Z50510513.

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