CHAPTER TWENTY FOUR

Development of a 3D Confocal Laser Scanning Microscope for Applying the Saturation Pulse Method to Chlorophyll *a* Fluorescence

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Abstract To analyze chlorophyll fluorescence of leaf tissue three-dimensionally using the saturation pulse method, a new real-time confocal laser scanning microscope was developed. A sensitive EM-CCD camera was installed in the rotating pinhole Nikpow disk scanning confocal laser microscope. The light intensity was controlled within 60–250 μM m^{-2} s^{-1}in actinic light and 1,400–6,000 μM m^{-2} s^{-1}in saturation light pulse by two electromagnetic shutters with neutral density (ND) filters installed in the system. The fluctuations of actinic light and of the saturation light pulse were kept at −1.46 to +1.37% and −0.45 to +0.32% of the average intensities, respectively. The shutters open within 90 ms in the dark and 60 ms under the actinic light. To acquire rapidly sufficient focal planes for constructing the 3D image, the piezo z-scan unit was installed in the system. This scan unit made it possible to capture 64 focal planes within 1.92 s. This also enabled us to capture sufficient focal planes during illumination with one saturation light pulse. The images that were captured within 30 ms were sufficiently clear for 3D reconstruction and calculation of chlorophyll fluorescence parameters such as ϕ_{PSII}.

Keywords 3D, chlorophyll fluorescence, confocal laser scanning microscope (CLMS)

Introduction

Chlorophyll fluorescence microscopy is an advanced technique of chlorophyll fluorescence imaging allowing noninvasive detection of spatio-temporal changes in photosynthetic activities of tissues, individual cells and chloroplasts (Oxborough 2004). Leaves, tissues, cells and chloroplasts have inherently complicated 3D structures. Hence, it is necessary to obtain 3D information to improve the understanding of plant functioning and responses to stresses (Rigaut et al. 1992; Schurr et al. 2006; Omasa et al. 2007). For a 3D surface reconstruction, expanded-focus 3D microscopy of chlorophyll fluorescence (Rolfe and Scholes 2002; Endo and Omasa 2007) was done by passive techniques. However, 3D anatomies of the internal parts of tissues and cells cannot be obtained by this

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technique in spite of their excellent reconstruction. Additionally, the magnifications of the specimens were not sufficient to capture high-resolution images of chloroplasts in cells.

A confocal laser scanning microscopy (CLSM) can reconstruct a 3D anatomy of the internal parts of tissues and cells by capturing fluorescence from their fluorochromes. Therefore, CLSM has been used for chlorophyll fluorescence microscopy of leaf tissue profiles (Osmond et al. 1999) and chloroplast movements in vivo (Tlałka and Fricker 1999). However, extremely strong excitation lights have been usually irradiated to specimens in order to capture clear images and it is impossible to rapidly scan and evenly illuminate the specimens with CLSM in which a galvanometer-miror is installed (Wang et al. 2005). These facts prevent the CLSM from applying the saturation pulse method. In this study, we have developed a system for applying the saturation pulse method by modifying the Nipkow disk type CLSM using high sensitivity EM-CCD camera.

Materials and methods

Boston fern (*Nephrolepis exaltata* L. cv. Bostoniensis) seedlings were acclimatized in pots in an environmentally controlled growth chamber for 4 weeks before the experiments. The pots were filled with artificial soil (mixture of vermiculite and perlite, 2:1, v/v). The plants were illuminated for 12 h each day with fluorescent lights at a PPF of 150 μM m^{-2} s^{-1}. Air temperature was 24.5°C during the day and 23°C at night. Relative humidity was 70% during the day and 90% at night. Mature intact leaves were used for the experiments. A whole leaf which was placed on a wetted cover was used as specimen for CLSM observation.

Figure 1 shows a photograph of the CLSM. The system consisted of an inverted light microscope, a confocal scanner unit, a blue laser diode system, two types of cooled CCD camera, various controlling devises and a personal computer. The Nipkow-disk-type confocal scanner unit (Yokogawa Electric, modified type of CSU-10) was installed between a highly sensitive cooled electron multiplier charge-coupled device (EM-CCD) camera (Hamamatsu Photonics, C9100-12) and an inverted light microscope (OLYMPAS, IX-71). A blue laser diode system (Yokogawa Electric, HPU50100-PFS-2, 488 nm) was used as the light source for the CLSM. A z-scan motor changed position of the objective along the z-axis and was controlled by a motor controller (Ludl, MAC5000). The position of the objective was more

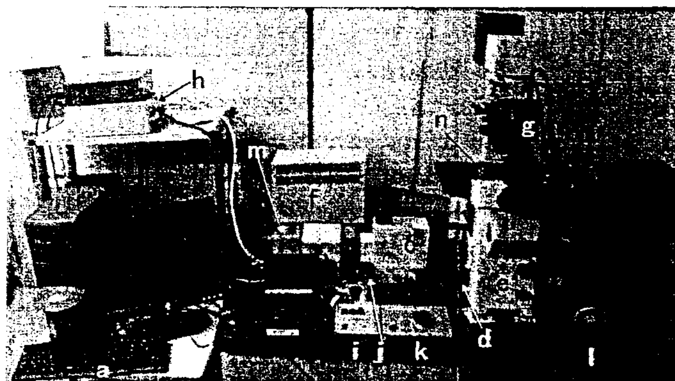

Fig. 1 Photograph of the computer-aided system for 3D chlorophyll fluorescence imaging with a CLSM. a: personal computer, b: EM-CCD camera, c: rotating pinhole disk (Nipkow disk) type confocal scanner unit, d: z scan motor, e: inverted light microscope, f: halogen lamp unit, g: color cooled CCD camera, h: blue laser diode (488nm) system, i: shutter controller, j: external ND/shutter, k: disk rotation controller, l: motor controller, m: piezo controller, n: piezo z scan unit.

precisely and rapidly controlled by a piezo z-scan unit (Physik Instrumente, PIFOC). External and built-in electromagnetic shutters attached to the confocal scanner unit were used for passing the laser beam, blocking it, or dimming it using neutral density (ND) filters. Both shutters were controlled with a shutter controller connected to the computer using software produced in our laboratory. The external shutter was selected between a hole and an ND filter and the built-in shutter selected between a hole and a non-hole. Transmitted light images of the leaves irradiated from above with a halogen lamp unit (OLYMPUS, U-LH100L-3, TH4-100) were captured with a cooled color CCD camera (Hamamatsu Photonics, C5310). The images were recorded on the computer via a digital color video recorder (Sony, DSR-V10). An immersion objective (UApo, 40, numerical aperture: 1.35) was used through experiments.

To apply the saturation pulse method, a time sequence in combination of dark, actinic light and saturation light pulse was produced by the shutters. The laser beam intensity for saturation light pulse was first determined by adjusting both the laser power and the direct measurement method using the metal pinhole disk. When both shutters opened for about 2 s in the dark, the saturation light pulse in the dark was presented. The actinic light was set by opening the built-in shutter and selecting ND filter in the external shutter. The saturation light pulse in the light was produced by about 2 s open of the external shutter under actinic light conditions.

Results and discussion

A 3D microscopy is required to simultaneously provide high lateral and axial resolution with minimal irradiation to avoid damaging the light-sensitive living cells (Wang et al. 2005). A sensitive camera and controllable light unit are needed to achieve the requirement. The installed camera has the function of electron multiplying on CCD chips, so it allows the signal to be drastically multiplied without noise. The light controlling shutters were opened within 90 and 60 ms in the dark and light, respectively. The time to maximize the light intensity of the developed system was faster than that of commercially available pulse amplitude modulation (PAM) chlorophyll fluorometers (WALZ, MINI-PAM and PAM-101).

The pinholes were gyroidally placed 250 μm apart on the disk. The pattern of the pinhole arrangement appeared at every 30° rotation of the disk, therefore a point on the leaf was illuminated from 300 to 360 times per second. This frequency is several times higher than that of the ordinary fluorescent lamp. The light intensity was controlled in ranges of 60–250 μM m^{-2} s^{-1}in actinic light and 1,400–6,000 μM m^{-2} s^{-1}in saturation light pulse. The fluctuations of actinic light and the saturation light pulse were kept at −1.46 to +1.37 % and −0.45 to +0.32 % of the average intensities, respectively.

The piezo z-scan unit made it possible to capture each image of different focal plane in 30 ms. During saturation light pulse, 64 different focal planes were captured in 1.92 s The first focal plane was located close to the abaxial leaf surface and the last one was at 80 μm depth in the direction of the adaxial surface. Each image was captured at 30 ms interval after 1.25 μm movement of the focal plane.

In the imaging with the higher resolution objective, it is essential to capture focal planes at different heights in order to focus clearly on chloroplasts, which are ubiquitous in the leaves. Figure 2 shows reconstructed 3D chlorophyll fluorescence intensity image of *Nephrolepis exaltata*. There are guard cell chloroplasts just above the center of the image and mesophyll chloroplasts scattered in the leaves. Cross-sectional chlorophyll fluorescence intensity images of leaves reveal the light distributions inside leaves (Takahashi et al. 1994; Osmond et al. 1999; Vogelmann and Evans 2002). Compared to these reports, shallower but much higher resolution profiles were captured without cutting off the leaves. This system allowed the capture of images which were sufficiently clear for 3D reconstruction and for calculation of chlorophyll fluorescence parameters such as ϕ_{PSII} even at such short exposure times.

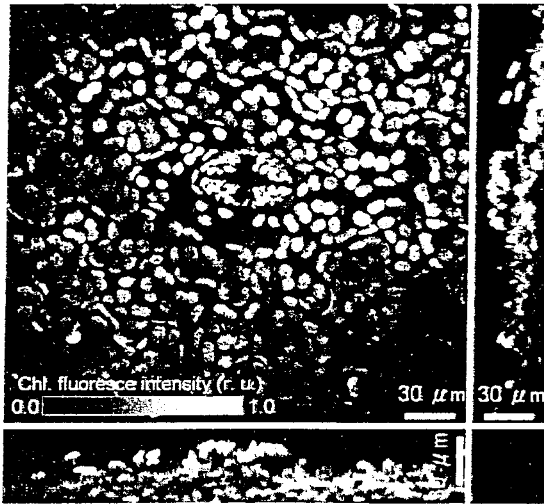

Fig. 2 3D chlorophyll fluorescence intensity image of *Nephrolepis exaltata*. The 3D image was reconstructed from 2D images captured at 64 focal planes within 1.92 s

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