

Multiphoton Confocal Microscopy Using a Femtosecond Cr:Forsterite Laser

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Summary: With its output wavelength covering the infrared penetrating window of most biological tissues at 1200–1250 nm, the femtosecond Cr:forsterite laser shows high potential to serve as an excellent excitation source for the multiphoton fluorescence microscope. Its high output power, short optical pulse width, high stability, and low dispersion in fibers make it a perfect replacement for the currently widely used Ti:sapphire laser. In this paper, we study the capability of using a femtosecond Cr:forsterite laser in multiphoton scanning microscopy. We have performed the multiphoton excited photoluminescence spectrum measurement on several commonly used bioprobes using the 1230 nm femtosecond pulses from a Cr:forsterite laser. Efficient fluorescence can be easily observed in these bioprobes through two-photon or three-photon excitation processes. These results will assist in the selection of dichroic beam splitter and band pass filters in a multiphoton microscopic system. We have also performed the autofluorescence spectrum measurement from chlorophylls in live leaves of the plant *Arabidopsis thaliana* excited by 1230 nm femtosecond pulses from the Cr:forsterite laser. Bright luminescence from chlorophyll, centered at 673 and 728 nm, respectively, can be easily observed. Taking advantage of the bright two-photon photoluminescence from chlorophyll, we demonstrated the two-photon scanning paradermal and cross-sectional images of palisade mesophyll cells in live leaves of *Arabidopsis thaliana*.

Key words: multiphoton microscopy, scanning microscopy, Cr:forsterite laser, multiphoton luminescence

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Introduction

Confocal laser scanning microscopy provides a significant improvement in axial resolution over conventional epifluorescence microscopy by eliminating out-of-focus fluorescence with a spatial filter in the form of a confocal aperture. Combining two-photon-induced fluorescence with laser scanning microscopy, Denk *et al.* (1990) achieved high axial discrimination even without a confocal aperture in front of the photodetector. Strong fluorescence is only induced in the vicinity of the focal point due to the quadratic dependence of the two-photon absorption on the laser intensity. This optical sectioning with a soft aperture effect also provides high depth discrimination capability. Taking advantage of the nonlinear effect, multiphoton microscopy can also avoid unnecessary out-of-focus bleaching and photo damages. Among the excitation wavelengths applied in multiphoton confocal microscopy, near infrared (NIR) wavelength shows a low linear absorption coefficient in most biological specimens and leads to greater penetration depth in most materials, providing the opportunity for imaging thicker samples. Taking advantage of the recent rapid development and commercial availability of femtosecond Ti:sapphire lasers operating at NIR, two-photon microscopy has been applied extensively in biology. However, recent studies on human skin and other biological specimens have suggested that the lowest light attenuation (absorption and scattering) in most biological materials exists around 1200 to 1250 nm (Anderson and Parrish 1981, Cheng *et al.* 1998), which is not available with the Ti:sapphire lasers. The high linear absorbance of plant material in the 700 to 800 nm range made the use of Ti:sapphire lasers in multiphoton fluorescence not much different from single-photon fluorescence microscopy. The photo damage induced by the Ti:sapphire wavelength also indicates the importance of finding an alternative light source for multiphoton scanning microscopy. It is thus important to develop multiphoton confocal scanning microscopy techniques based on 1200 to 1250 nm infrared laser light.

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For 1200 to 1250 nm infrared laser light, the Cr:forsterite laser with an output wavelength operating from 1170 to 1340 nm is the most suitable candidate for this application. Not only does its output wavelength match the transparent window of most biological tissues, but also the entire visible spectrum and NIR regions are open for fluorescence detection both in two- and three-photon excitation. Besides, many mode-locking techniques, such as Kerr Lens Mode-locking (KLM), have been applied to Cr:forsterite laser (Seas *et al.* 1992, 1993; Sennaroglu *et al.* 1994; Slobodchikov *et al.* 1996; Zhang *et al.* 1997) and a pulse as short as 14 femtoseconds has been reported (Chudoba *et al.* 2000). The wavelength of approximately 1300 nm provided by the Cr:forsterite laser corresponds to the zero dispersion wavelength in optical fibers, which makes it compatible with the femtosecond laser-based microscopic system fiber. Combining its high output power of about 300 mW and its short pulse width, the Cr:forsterite lasers show an advantage in multiphoton microscopy, which requires high peak intensity to achieve nonlinear process.

In this paper we employed a semiconductor saturable absorber mirror (SESAM) mode-locked Cr:forsterite laser with its output wavelength centered at 1220 to 1240 nm to excite the fluorescence of several widely used bioprobes, including Alexa Fluor 594 (Model: U-21654), SYTO 17 (Model: S-7579), BODIPY TR (Model: D-6116), LysoTracker Red (Model: L-7528), and MitoTracker (Model: M-7512), all from Molecular Probe Inc., Eugene, Ore., USA). Multiphoton spectroscopic studies and multiphoton confocal microscopy were then performed on the leaf of *Arabidopsis thaliana* to demonstrate the capability of section imaging with Cr:forsterite laser.

Materials and Methods

Bioprobes and Live Plant Materials

Five widely used fluorescence probes were prepared for multiphoton excited spectroscopic measurement with a femtosecond Cr:forsterite laser. Alexa Fluor 594 was prepared as an unbuffered H₂O solution; SYTO 17 was dissolved in dimethylsulfoxide (DMSO) at a concentration of 5 mM; methanol solution of BODIPY TR, LysoTracker Red, and MitoTracker Red were also used. A depression slide with a coverslip was used to contain the solution during measurement. In addition, live leaves of *Arabidopsis thaliana* were used for spectroscopic studies and multiphoton confocal microscopy.

Femtosecond Cr:Forsterite Laser

Structure of the Cr:forsterite laser resonator is shown schematically in Figure 1. The home-built Cr:forsterite laser constructed for this study uses a 19 mm long Brewster-cut Cr:forsterite crystal with its b axis on the horizontal plane, resulting in horizontal polarization for the

lasing fundamental beam. The crystal temperature was kept at approximately 2°C. The laser was pumped with an 8 W of 1064 nm light from a diode-pumped Nd:YVO₄ laser (Model: Millennia IR, Spectra-Physics, Mountain View, Calif., USA). At the output of the pump laser, a pump lens was placed to adjust the size of the pumping laser beam. After turning polarization of the pumping laser beam by a periscope, it was focused with a 10 cm lens through a high reflecting cavity mirror onto the Cr:forsterite crystal. The laser cavity was a Z cavity consisting of M1, M2, M3 mirrors with a 4.5% output coupler (M4) and a semiconductor saturable absorber mirror (SESAM). For confocal microscopy, the employment of the SESAM initiates and stabilizes the femtosecond pulse generation even with back reflection from the external cavity. In our study, the SESAM consisted of 25 periods of GaAs/AlAs quarter wave layers, followed by an Al_{0.48}In_{0.52}As quarter wave layer with two embedded Ga_{0.47}In_{0.53}As quantum wells. To provide the saturable absorber nonlinearity for initiating and stabilizing the Cr⁺:forsterite laser, the quantum well structure was designed to have the heavy-hole excitonic resonance at 1232 nm at room temperature. The insertion loss of the SESAM is 2.5%, with a saturation energy ~50 μJ/cm². The use of SESAM initializes and stabilizes the Kerr lens mode-locking mechanism in the Cr:forsterite laser and makes this laser less sensitive to weak feedback light from the microscopy system. M1, M2, and M3 were mirrors with 10 cm radius of curvature. All mirrors and the output coupler were high reflection-coated (>99%) for the spectral range of the fundamental output (1200–1270 nm). An SF₆ prism pair was inserted to provide intracavity group velocity dispersion compensation. Before the output coupler, a slit was used to tune the fundamental wavelength. Outside the cavity, we used another SF₆ prism pair to achieve both beam shaping and dispersion compensation of the fundamental output. Figure 2 shows a typical output spectrum. The output wavelength was centered at 1230 nm with a full-width half-maximum (FWHM) spectral width of 20 nm. Its corresponding autocorrelation trace is depicted in Figure 3, showing an FWHM of 215 fs. Assuming a square hyperbolic second pulse shape, an output pulsewidth of ~140 fs can be obtained. Pulsewidth

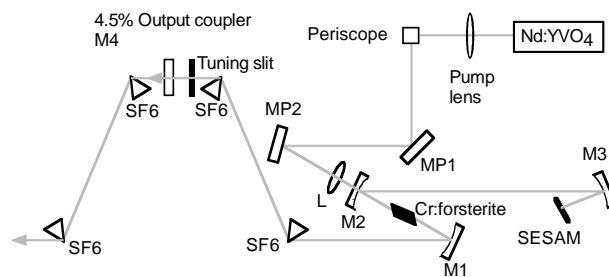


FIG. 1 Schematic diagram for the cavity of SESAM mode-locked Cr⁴⁺:forsterite laser.

between 80 and 150 femtoseconds can be obtained daily. The maximum average output power was approximately 350 mW, with 125 MHz repetition rate. Without the SESAM, the output wavelength can be extended to 1270 nm, which was limited by our mirror and output coupler coating.

Experimental Setup

Figure 4 shows a schematic representation of the experimental setup used for analyzing fluorescence spectrum and for scanning microscopy. The experiments were performed by using the SESAM mode-locked Cr:forsterite laser at 1230 nm wavelength with a ~ 100 femtosecond pulse width. The average output power of the mode-locked Cr:forsterite laser is on the order of 300 mW. A dichroic beam splitter (Model: 700DCSPXR, Chroma Technology, Brattleboro, Vt., USA), which is highly reflected at 1230 nm but highly transmissive for visible spectrum, was employed to reflect the Cr:forsterite laser

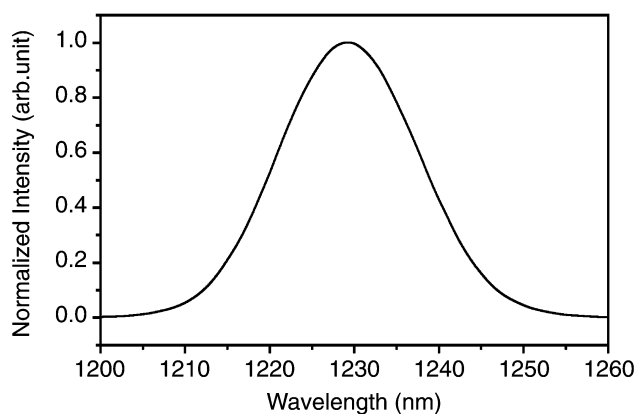


FIG. 2 Spectrum of the Cr⁴⁺:forsterite laser output, which centers at 1230 nm, with 20 nm bandwidth.

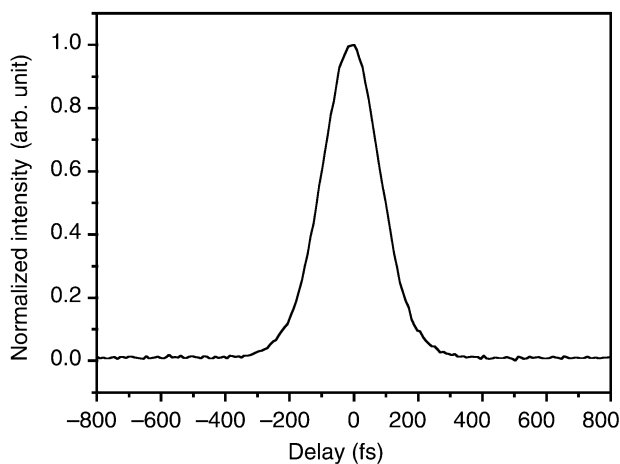


FIG. 3 The autocorrelation trace of the Cr⁴⁺:forsterite laser output, which shows 215 femtosecond (fs) full width-half maximum of autocorrelation trace.

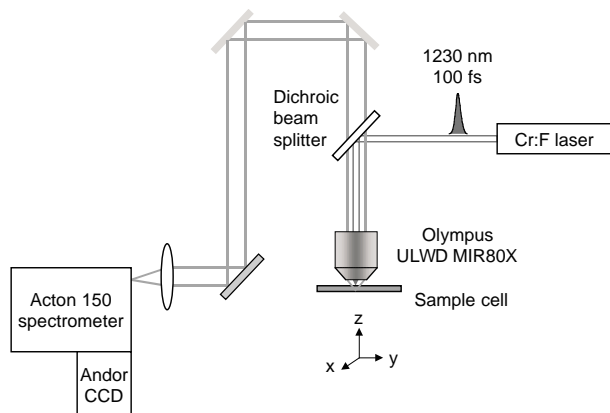


FIG. 4 Schematic diagram for the spectroscopic studies and multiphoton confocal microscopy with femtosecond (fs) Cr:forsterite laser. CCD=charge-coupled device.

beam to an 80 \times objective (NA= 0.75) (Model: ULWD MIR 80 \times , Olympus, Melville, N.Y., USA) to focus onto the sample with 15 mW average power. The sample was held in the chamber of a depression slide when performing spectroscopic study. As for multiphoton confocal microscopy, two pieces of cover glass were used to clamp the leaf of *Arabidopsis thaliana* for sample flatness consideration. They were fixed on a three-axes translation stage of submicrometer step resolution to accomplish scanning. The multiphoton-induced fluorescence was collected by the same objective, transmitting through the dichroic beam splitter, and directed into a cooled charge-coupled device (CCD)-based spectrometer (Acton Research, Acton, Mass., USA, or Andor Technology, Belfast, Northern Ireland) for spectral measurement by three mirrors, M1, M2, M3, and lens L1. The inlet of the spectrometer is a tuning slit with 10 μ m minimum width, serving as a confocal pinhole. The CCD was operating at -40°C for spectroscopic study and at -60°C for multiphoton confocal microscopy to achieve a high signal-to-noise ratio. The image of a specific fluorescence wavelength was taken by recording the light intensity on the specific CCD pixel corresponding to the desired wavelength while scanning the sample position in XYZ directions.

Results and Discussion

Multiphoton Fluorescence of Common Bioprobes

We first performed the multiphoton-excited photoluminescence spectrum measurement using the 1230 nm femtosecond pulses from the Cr:forsterite laser. From the results on several widely used bioprobes, fluorescence excited by high power femtosecond Cr:forsterite laser at 1230 nm can cover from the visible to the near in-

frared part of the spectrum by multiphoton nonlinear processes. Efficient fluorescence can be observed in these bioprobes by two- or three-photon excitation processes. These results show the ability of efficient fluorescence emission by multiphoton processes with this new excitation wavelength of 1230 nm, which assists in the selection of dichroic beam splitter and band pass filters in a multiphoton microscopy study.

Alexa fluor 594: Alexa Fluor 594 is a protein labeling dye. Proteins labeled with the dye have single-photon absorption and fluorescence emission maxima at 590 and 615 nm, respectively (Haugland 1999). Figure 5 shows the fluorescence spectrum excited with the 1230 nm femtosecond light from the Cr:forsterite laser. The excitation power is 15 mW with 4.8 s CCD integration time. A broad emission centered at 617 nm can be observed, which is similar to its single-photon luminescence spectrum. It is interesting to note that the emission spectrum extends into the wavelength region shorter than the two-photon wavelength (615 nm) of the 1230 nm excitation, indicating a mixture of two- and three-photon-induced photoluminescence.

SYTO 17: SYTO dyes are cell-permeant nucleic acid stains that differ from each other in one or more characteristics, including cell permeability, fluorescence enhancement upon binding nucleic acids, excitation and emission spectra, DNA/RNA selectivity, and binding affinity. SYTO 17 is one of the SYTO series dyes that has absorption and fluorescence emission maxima at 621 and 634 nm, respectively (Haugland 1999), and has been used to assess the effects of cytotoxic agents on *Escherichia coli* by flow cytometry (Comas and Vives-Rego 1997). The spectrum of two-photon fluorescence excited by 1230 nm femtosecond Cr:forsterite laser with 4.8 s CCD integration time is shown in Figure 6; the excitation power is 15 mW. Its multiphoton excited fluorescence peaks at a wavelength of 643 nm, similar to its single-photon photoluminescence. However, the multiphoton-excited spectrum is accompanied by a second emission shoulder at a longer wavelength of 686 nm, which has not been ob-

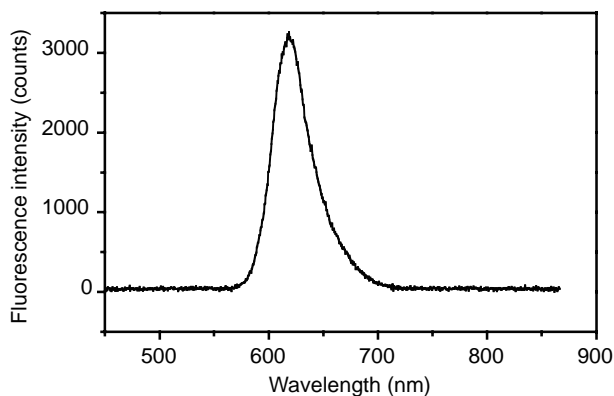


FIG. 5 Multiphoton fluorescence spectrum of Alexa 594, excited by femtosecond Cr:forsterite laser operating at 1230 nm.

served in previous single-photon spectra (Haugland 1999).

BODIPY TR: For DNA sequencing application, the amine-reactive BODIPY dyes are useful because they are isomerically pure and cause little perturbation to the mobility of DNA fragments (Metzker *et al.* 1996). BODIPY TR is one of the BODIPY dyes that has absorption and fluorescence emission maxima at 588 and 617 nm, respectively (Haugland 1999). When excited by a 1230 nm light from the Cr:forsterite laser, strong photoluminescence can be easily observed. Figure 7 shows the measured fluorescence spectrum excited by 15 mW 1230 nm femtosecond light from the Cr:forsterite laser with a 1 s CCD integration time. Due to the fact that the two-photon excitation energy is close to its single-photon emission peak, the observed two-photon emission peak is slightly red shifted to 630 nm. A shoulder of approximately 675 nm, which also showed in a previous single-photon spectrum, can be observed (Haugland 1999).

LysoTracker Red: The LysoTracker probes are fluorescent acidotropic probes for labeling and tracking acidic organelles in live cells. They have high selectivity for acidic organelles, effective labeling of live cells at nanomolar concentrations, and are available in several

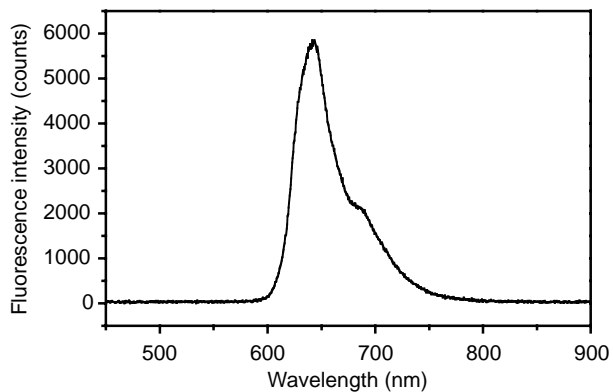


FIG. 6 Multiphoton fluorescence spectrum of SYTO 17, excited by femtosecond Cr:forsterite laser operating at 1230 nm.

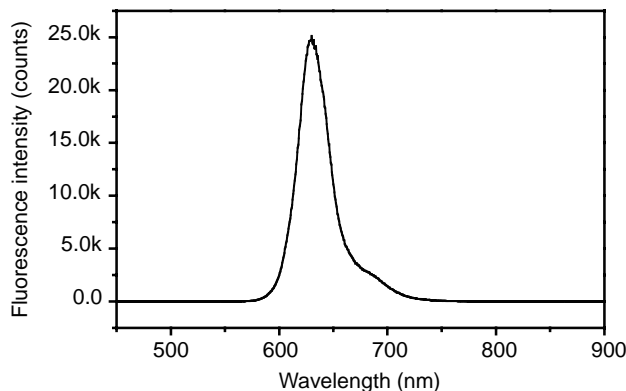


FIG. 7 Multiphoton fluorescence spectrum of BODIPY TR, excited by femtosecond Cr:forsterite laser operating at 1230 nm.

fluorescent colors, making them especially suitable for multicolor applications. The LysoTracker used in this experiment is LysoTracker Red DND-99 and has absorption and fluorescence emission maxima at 577 and 590 nm, respectively (Haugland 1999), which are both shorter than the two-photon excitation wavelength of 615 nm. Figure 8 shows the fluorescence spectrum excited by 15 mW femtosecond Cr:forsterite laser light with 24 s integration time. Due to the combination of two- and three-photon excitations, four emission peaks at 594, 627, 646, and 668 nm can be observed, which is very different from the single-photon excited spectrum. The strongest emission peak centered at 594 nm is shorter than the two-photon wavelength of 615 nm, indicating its three-photon excitation nature; this emission peak is similar to its single-photon spectral peak. Other emissions at 627, 646, and 668 nm could be contributed from two-photon excitation processes.

MitoTracker Red: MitoTracker probes are mitochondrion-selective dyes that are well retained during cell fixation. The checked MitoTracker is MitoTracker Red CMXRos and has absorption and fluorescence emission maxima of approximately 579 and 599 nm, respectively (Haugland 1999), both of which are also shorter than the two-photon wavelength of 615 nm of our excitation. The 1230 nm light excited spectrum presented in Figure 9 shows a three-photon fluorescence peak at 600 nm, which is close to its single-photon excited spectral peak. The spectrum was excited by 15 mW infrared light with 1 s CCD integration time. A weak shoulder of approximately 650 nm was also observed, which could be contributed from the two-photon excitation process.

Multiphoton Fluorescence of *Arabidopsis thaliana* Leaves

We then focus on the study of autofluorescence from chlorophylls in live *Arabidopsis thaliana* leaves excited by the 1230 nm femtosecond pulses from the Cr:forsterite

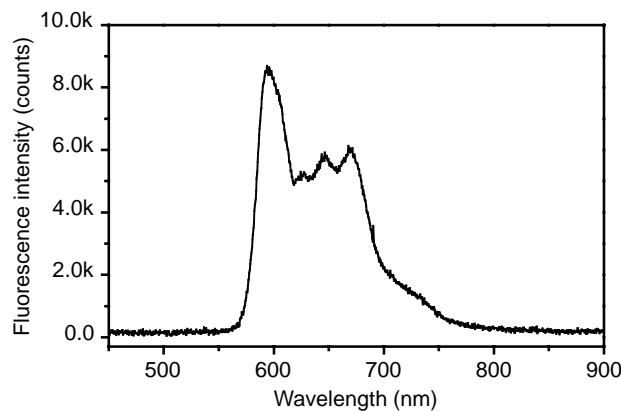


FIG. 8 Multiphoton fluorescence spectrum of LysoTracker, excited by femtosecond Cr:forsterite laser operating at 1230 nm.

laser. *Arabidopsis thaliana* is unique among plant model organisms in having a small genome (130–140 Mb), excellent physical and genetic maps, and little repetitive DNA. A coordinated international effort to sequence the *Arabidopsis* genome was initiated in late 1996 and was completed in the year 2000 (The Arabidopsis Genome Initiative 2000). The nucleotide sequences and annotations of two of the five chromosomes, number 2 and 4, have been reported. In our experiments, live leaves of *Arabidopsis thaliana* were excited with femtosecond Cr:forsterite laser and the fluorescence emission spectrum was measured. With 4.8 s integration time, the obtained multiphoton spectrum (see Fig. 10) shows maximum intensity at 673 nm with a shoulder of approximately 728 nm, indicating the characteristic response of the chlorophylls. Due to the fact that the obtained spectrum is on the low-energy side of the two-photon energy, the excitation should be originated from the two-photon absorption process. Bright luminescence can also be easily obtained because of its two-photon excitation nature.

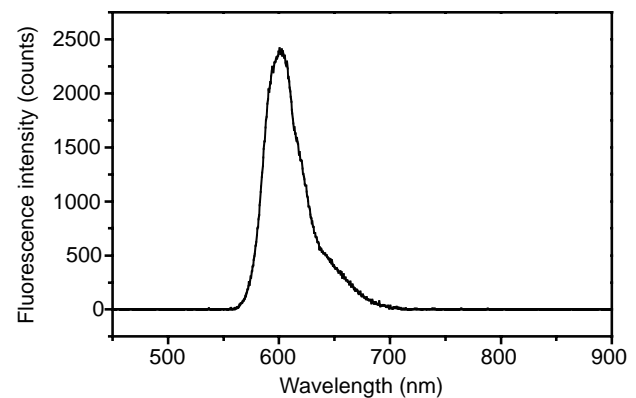


FIG. 9 Multiphoton fluorescence spectrum of MitoTracker, excited by femtosecond Cr:forsterite laser operating at 1230 nm.

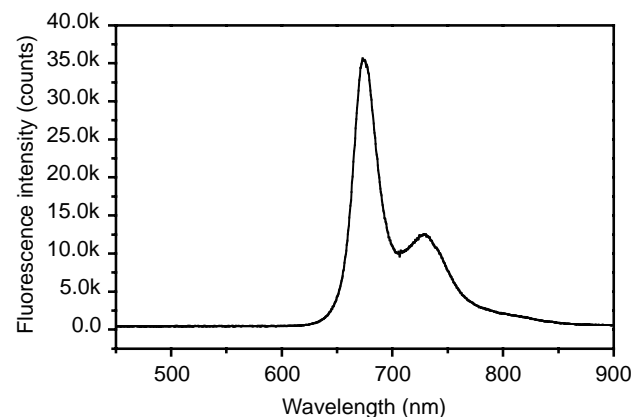


FIG. 10 Multiphoton fluorescence spectrum of a leaf of *Arabidopsis thaliana*, excited by femtosecond Cr:forsterite laser operating at 1230 nm.

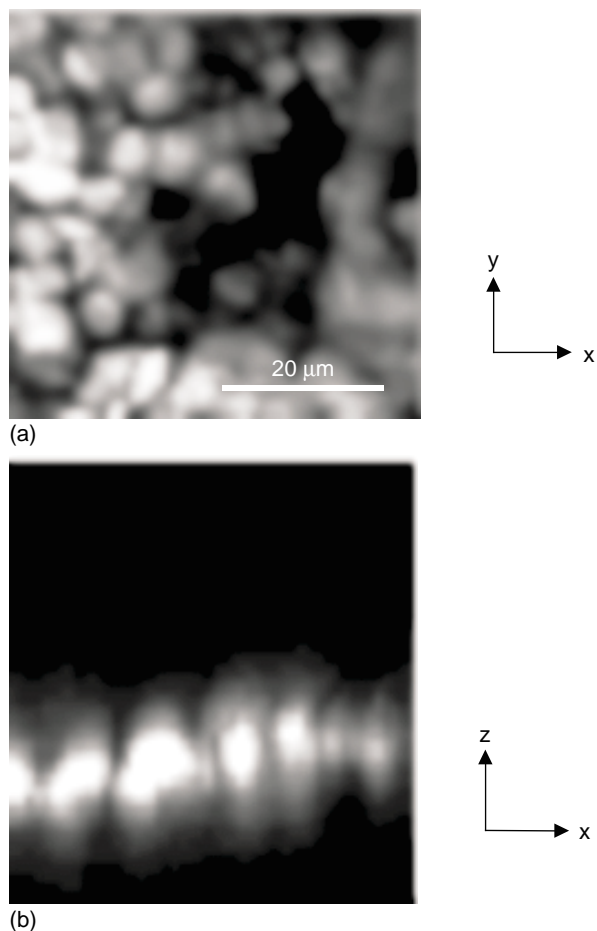


FIG. 11 (a) Transverse section image (xy plane) of the 673 nm photoluminescence in the leaf of *Arabidopsis thaliana*, and (b) its corresponding cross-sectional image (xz plane). The horizontal field width is 50 μm .

Two-Photon Fluorescence Image of *Arabidopsis thaliana* Leaf Cells

Taking advantage of the bright two-photon photoluminescence from the chlorophylls of approximately 673 nm with the 1230 nm femtosecond light excitation, we demonstrate for the first time two-photon scanning microscopy with the Cr:forsterite laser in a plant material, the leaf of *Arabidopsis thaliana*. We selected the maximum intensity at 673 nm as image wavelength, with multiphoton confocal microscopy performed by scanning the XYZ stages. The sample was placed on the xy -plane as defined in Figure 4, and we searched the signal along the z axis to find the optimal point as our scanning depth. Figure 11(a) shows a transverse image of the 673 nm photoluminescence, corresponding to a paradermal view of the palisade mesophyll cells below the upper epidermis of the leaf, where chloroplasts are highly abundant. Because of our specific wavelength selection, strong response occurred only when chlorophylls exist, which indicates the localization of mesophyll cells with micrometer-order

resolution. Figure 11(b) shows a cross-sectional (xz -plane) image of the palisade mesophyll cells in the leaf, corresponding to the bottom part of Figure 11 (a). *Arabidopsis thaliana* is known for having a single layer of palisade mesophyll cells interior to the upper epidermis. The success of obtaining a mesophyll section image beneath the epidermis of the leaf demonstrates the penetrating capability through biotissue with femtosecond Cr:forsterite laser in the multiphoton confocal microscopy system.

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