Non-de-scanned parallel recording two-photon hyperspectral microscopy with high spectral and spatial resolution

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Abstract: A two-photon hyperspectral microscope with non-de-scanned geometry and parallel recording scheme was constructed which had a low dwell time, high spectral resolution, and high spatial resolution. The dwell time was effectively decreased to reduce photodamage through parallel recording, while the non-de-scanned geometry led to a considerable reduction in the signal loss and spectrum distortion. Two experiments were performed to show that this system can solve crosstalk issues and spectrally resolve the intrinsic fluorophores in optically-thick tissues without staining and sectioning.

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optically-thick tissues. In contrast to single-photon excitation, two-photon fluorescence were developed to have the optical sectioning power necessary for the investigation of filter device, such as a liquid crystal tunable filter (LCTF) or acoustic-optical tunable filter wavelength-dependent information for spectral analysis [13]. The other method uses a tunable information. Excitation wavelength scans have to be applied to obtain the excitation-filters to divide the signals have been demonstrated. One offers limited channels for spectral division of the signals may produce serious crosstalk issues. The hyperspectral technique is a good candidate to overcome this problem [12,13].

Due to the high optical intensity required for two-photon excitation, the point-scanning geometry is commonly used. In order to acquire the spatial and spectral information simultaneously, different configurations based on the point-scanning system have been proposed and demonstrated. Two different configurations operating on the concept of using filters to divide the signals have been demonstrated. One offers limited channels for spectral information. Excitation wavelength scans have to be applied to obtain the excitation-wavelength-dependent information for spectral analysis [13]. The other method uses a tunable filter device, such as a liquid crystal tunable filter (LCTF) or acoustic-optical tunable filter (AOTF). Such systems can achieve higher spectral resolutions; therefore, only one excitation wavelength is required [14,15]. The monochromator configuration also enables the signal to be divided and the spectral resolution can be adjusted by tuning the slit width [16]. Multiple photomultiplier tubes (PMTs) can be used together to increase the acquisition rate. However, when the required number of bands is more than the number of the PMTs, sequential image acquisition is needed and this results in inefficient data acquisition and requires longer recording times. To record all wavelength bands simultaneously, detector arrays such as the PMT array and charge-coupled devices (CCD) have been used to record the fluorescence signals dispersed by a grating or prism [17–20]. To apply the dispersive elements for spectrum detection, it is common to employ the de-scanned geometry [17,18,20] or sample scanning [19] in the system. PMT array-based systems can provide a very high recording rate. However, the number of recording bands is limited by the PMT element size and the dead zone between two adjacent elements, which can lead to spectral information loss [21]. On the other hand, CCD-based systems can provide more recording bands, that is, higher spectral resolution, thanks to the smaller pixel size. Nevertheless, the recording time for a complete spectrum is limited by the read-out speed of the CCD [17,19,22]. Utilizing de-scanned geometry, a 2-dimensional (2D) electron-multiplying CCD (EM-CCD) design was proposed...
capable of increasing the acquisition speed and sensitivity [17]. However, the dwell time was limited by the integration time for one spectrum recording. Moreover, the de-scanned geometry can lead to additional signal loss and spectrum distortion.

A two-photon hyperspectral microscope with non-de-scanned geometry and parallel recording scheme is constructed in this work. The system provides a short dwell time, high spatial resolution, and high spectral resolution. The application of the system to two types of samples containing mixed fluorescent beads or fresh leaves without staining and sectioning will be demonstrated in order to illustrate the system's capability for biological applications in optically-thick tissues.

2. System configuration

Figure 1 illustrates the concept of the non-de-scanned two-photon hyperspectral microscope. A 1D galvo mirror and a 1D translation sample stage are responsible for the point scanning in the $x$- and $y$-directions. The fluorescence signals are scanned at the image plane due to the non-de-scanned geometry. When combined with a 2D CCD, parallel recording of the spatial-spectral ($x$-$\lambda$) information is enabled (Fig. 1). The frame is read out after a series of spectral information in the $x$-direction has been recorded. By integrating the $x$-$\lambda$ information several times for a frame, the dwell time can be effectively decreased which reduced photodamage. Fewer optical elements on the non-de-scanned detection light path can considerably improve the signal loss and spectrum distortion.

Figure 2(a) shows the configuration of the home-built non-de-scanned two-photon hyperspectral microscopic system. For two-photon excitation, a 1064-nm femtosecond fiber laser (FPL-03UFF0 with pulse compressor, Calmar) with ~200-fs pulse width, 40-MHz repetition rate, and ~200-mW average power output is used. The laser beam is guided into a 1D galvo mirror (GVSM002/M, Thorlabs) that accounts for the scanning in the $x$-direction. A telescope, $T_1$, is used to collimate the laser beam and expand the beam diameter to meet the required spot size of the galvo mirror. Passing through the galvo mirror, the laser beam is deflected at the desired scanning angles. $T_2$, comprised of a pair of tube lenses, serves two purposes in this system. One is to refocus the deflected laser beam into the back aperture of the water-immersion objective (UPLSAPO 60XW, NA 1.2, Olympus), Obj. The other is to expand the laser beam to fill the back aperture of the objective. The laser beam is tightly focused by the objective onto the samples which are mounted on a motorized translation stage, the $y$-stage, which is responsible for scanning the sample in the $y$-direction. The emitted two-photon fluorescence signals are collected backward by the same objective. The dichroic beamsplitter (DBS) transmits the excitation laser wavelength and reflects the fluorescence.
signals into the home-built spectrometer. L1 and L2 form a telescope with individual focal lengths of 175 mm and 60 mm, respectively. An adjustable slit (AS) is set at the confocal plane of L1 and L2. The x-direction spatial information carried by the fluorescence signals is imaged onto the x-axis of the CCD (CoolSNAP HQ2, Photometrics) through the imaging lens, L3, with a focal length of 60 mm. The fluorescence signal is dispersed by a blazed reflection grating with a line density of 300 grooves/mm. The spectral information is then expanded and projected along the y-axis of the CCD through L3. By tuning the width of AS, the spectral resolution can be adjusted. The CCD has 1040 and 1392 pixels on the x- and y-axis, respectively. The field of view on the sample plane in the x-direction is set to 100 μm; the spectral range is set to 480-780 nm.

To acquire a complete 3D (x-y-λ) hyperspectral fluorescence image, the galvo mirror, y-stage, and CCD have to be perfectly synchronized, as shown in Fig. 2(b). The galvo mirror, accounting for fast scanning in the x-direction, is set to have a period of T. To reduce photodamage in the samples, the period is set to be around 2 ms to obtain a short dwell time of 1.92 μs. As the galvo mirror starts to scan, the CCD begins to record data simultaneously. To enhance the signal-to-noise ratio (SNR), the exposure time of the CCD is set to be nT, where n is an integer number, in order to record the complete x-λ information at a single y-position. Once the recording process of the CCD is completed, the CCD transfers the recorded frame to the memory, and the y-stage moves one step during this readout period. The pixel spacing of the CCD is about 100 nm mapped to the sample plane in the x-direction; therefore, the step size of the y-stage is also set to be 100 nm. The next recording process of the CCD begins right after the frame readout and the stage movement are both accomplished. A full-size image containing a 100 μm (x) × 100 μm (y) × 300 nm (λ) data stack or 1040 × 1000 × 1392 pixels requires 1000 recording-readout cycles. In the general acquisition mode, the frame rate of the CCD is 10 frames/s without pixel binning. Limited by the CCD frame rate, the minimum acquisition time of this system is about 100 s for a full-size data stack. The minimum number of laser pulses required for each pixel is ~3840. Due to the higher sampling rates in both the spatial (~100 nm/pixel) and spectral (~0.3 nm/pixel) dimensions, this system can achieve high spatial and spectral resolution hyperspectral image stacks.
3. Sample preparation

3.1 Mixed fluorescent beads

Two types of 100-nm diameter fluorescent beads, F8800 and F8801, that are suitable for 1064-nm two-photon excitation, were obtained from Molecular Probes. The two types of fluorescent beads were mixed and distributed sparsely by mixing 1-μl F8800 and 1-μl F8801 together well and then diluting this with DI (de-ionized) water. Drops of the diluted mixture were placed between the slide and cover glass for imaging.

3.2 Epipremnum aureum leaf

Both green and yellow leaves were obtained from a potted *Epipremnum aureum* (*E. aureum*). The leaves were removed from the plant and cut into 1 cm × 1 cm pieces for imaging without the application of sectioning, fixing, or staining processes. The hyperspectral images were all acquired within 3 hrs after the leaves were taken off the plant.

4. Experimental results

4.1 Calibration and spectral resolution

The designed spectral range of the hyperspectral microscope was from 480 nm to 780 nm. To calibrate the detected spectrum, three light sources, Hg-Ne lamp, He-Ne laser, and a 671-nm laser were used. The five known wavelengths, 546 nm, 577 nm, 579 nm, 633 nm, and 671...
nm, were used to calibrate the hyperspectral microscope. Figure 3(a) shows the relation between the wavelengths and the CCD pixel numbers. The fitting curve (red line in Fig. 3(a)) matched the theoretical curve (black line in Fig. 3(a)) very well. Since the difference in the diffraction angles of 480 nm and 780 nm was only 7°, the pixel number was approximately linear to the wavelength. The slope of the fitting curve was ~0.3 nm/pixel throughout the entire detection range. Figure 3(b) shows the calibrated spectra of the three light sources measured by the hyperspectral microscope. Figures 3(c) and 3(d) show an enlargement from Fig. 3(b), and the measured spectra of the three lines of the Hg-Ne lamp. The full-width at half maximums (FWHMs) of the three lines at 546, 577, and 579 nm were 2.41, 1.96, and 1.52 nm, corresponding to 8, 7, and 6 pixels. It should be noted that in Fig. 3(d), the two close peaks can be clearly resolved based on the high sampling rate and resolution in the spectral dimension. Figures 3(e) and 3(f), enlarged from Fig. 3(b), show the spectra of the laser lines at 633 and 671 nm, respectively. The bandwidths of these two laser lines are both well-below the 20 pm needed for spectral resolution measurement. The FWHMs of the laser lines at 633 and 671 nm were 1.05 and 0.91 nm, corresponding to 4 and 3 pixels. According to the measured FWHMs of the laser lines, the spectral resolution was estimated to be ~1 nm. This high spectral resolution can help to increase the accuracy of spectral analysis.

4.2 Spatial resolution

The spatial resolution of the hyperspectral microscope was measured using a sample of 100-nm F8801 fluorescent beads. Figure 4(a) shows the 3D image stack (100 μm (x) × 100 μm (y) × 300 nm (z)) of the fluorescent beads, reconstructed from 1000 x-z images recorded by the CCD. Figure 4(b) shows the spectrum at the position indicated in Fig. 4(a), while Fig. 4(c) shows the spatial distribution of the beads obtained at the peak wavelength, 610 nm, of the spectrum illustrated in Fig. 4(b). Figures 4(d) and 4(e) show the average intensity profiles of the fluorescent beads along the x- and y-directions, respectively. The measured resolutions were 482 nm and 557 nm in the x- and y-directions, respectively. The reason why the resolution in the x-direction is better than that in the y-direction is due to different image
formation mechanisms along these two directions. Since the image in the $x$-direction was effectively formed by wide-field image projection, the point spread function (PSF) of two-photon excitation was involved in the image formation. The diffraction limit is $0.43\lambda_{ex}/NA$ [23], where $\lambda_{ex}$ is the excitation wavelength. Under a 1064-nm excitation, the diffraction limit was around 381 nm. In the $y$-direction, since the image was formed by point-by-point recording, the PSF of the imaging system was involved in the image formation. The diffraction limit is $0.61\lambda_{em}/NA$, where $\lambda_{em}$ is the peak wavelength of the fluorescence emission. With a peak emission wavelength of 610 nm, the theoretical spatial resolution was around 310 nm. The discrepancy between the theoretical and experimental resolution may be due to aberration in the optical system.

![Image](image_url)

**Fig. 4.** (a) The 3D image stack of F8801 fluorescent beads with a 100-s acquisition time. The dimensions along the $x$, $y$, and $\lambda$ axis are 100 $\mu$m, 100 $\mu$m, and 300 nm, respectively. (b) The emission spectrum of the beads obtained at the position indicated by the arrow in (a). (c) The spatial distribution of the beads obtained at the peak emission wavelength, 610 nm, indicated by the arrow in (b). (d) and (e) show the averaged intensity profiles of the beads along the $x$- and $y$-directions, respectively. The corresponding spatial resolutions are 482 nm and 557 nm.

### 4.3 Hyperspectral imaging of mixed fluorescent beads

Traditional fluorescence microscopy utilizes beamsplitters or filter sets to separate the fluorescence signals. Therefore, samples containing multiple fluorophores which have overlapping emission spectra can cause crosstalk issues. In contrast, the hyperspectral system records the complete spectral information and separates the fluorescence signals of different fluorophores distinctly through spectrum analysis. A sample containing mixed fluorescent beads, F8800 and F8801, which has overlapping spectra, was measured by the hyperspectral microscope in order to demonstrate its capability for solving the problem of crosstalk.

The normalized two-photon fluorescence spectra of the F8800 and F8801 fluorescent beads measured by the hyperspectral microscope are shown in Fig. 5(a). Figure 5(b) shows the 3D image stack of the mixed fluorescent beads with dimensions of 100 $\mu$m ($x$) $\times$ 100 $\mu$m ($y$) $\times$ 300 nm ($\lambda$). Take the spectrum at one position (black line in Fig. 5(c)) for instance. This spectrum is the combination of the emission spectra of F8800 and F8801 which have different signal strengths. The images obtained by traditional fluorescence microscopy using filters are simulated by separating the spectral data at 590 nm (dashed line in Fig. 5(c)) to imitate long pass and short pass filters at this wavelength. The selection of this wavelength is based on the cross point of the normalized spectra of F8800 and F8801 (Fig. 5(a)). Figures 5(d) and 5(e) show the simulated traditional fluorescence microscopic images of the F8800 and F8801 fluorescent beads using filters. The images were obtained by integrating the fluorescence signals with wavelengths shorter and longer than 590 nm, respectively.
In the hyperspectral microscope, the fluorescence spectrum can be well-decomposed into two spectra (blue and red lines in Fig. 5(c)) by applying the linear unmixing algorithm [24, 25]:

\[
I(\lambda) = \sum_{i=1}^{n} C_i R_i(\lambda),
\]

where \(I(\lambda)\) is the measured spectrum, \(R_i(\lambda)\) is the reference spectrum of each individual fluorophore, \(C_i\) is the concentration of each individual fluorophore, and \(i\) represents the index of the fluorophore. The reference spectra \(R(\lambda)\) are taken from the measured spectra shown in Fig. 5(a). The summation of these two decomposed spectra (green line in Fig. 5(c)) is shown to fit the measured spectrum very well. Figures 5(f) and 5(g) show images of the F8800 and F8801 fluorescent beads which were separated using the linear unmixing algorithm. Comparison of Fig. 5(d) and 5(f) shows that the crosstalk signals from F8801 to F8800 can be found in the results of traditional fluorescence microscopy (arrow in Fig. 5(d)) but not in the hyperspectral microscope (arrow in Fig. 5(f)). These results clearly prove that the hyperspectral microscope can solve the crosstalk issue and that it is able to obtain molecular images of two different fluorescent beads with overlapped emission spectra.

4.4 Hyperspectral imaging of photosystems in the epipremnum aureum leaf

Photosynthesis is a process that is performed by plants to convert light into chemical energy. To accomplish the process of photosynthesis, both photosystems I (PS I) and II (PS II) are required. Photosystems are the protein complexes that are responsible for absorbing light, converting light into electrons, and transferring the electrons during photosynthesis. PS I and PS II have different reactive center chlorophylls. Named after the wavelengths to which the photosystems are most reactive, PS I and PS II are also called P700 and P680. Since both photosystems emit fluorescence after absorbing light, a two-photon hyperspectral microscope can be applied in order to investigate the activity of these two photosystems. To reveal the
relationship between the fluorescence intensity and the photosystem activity, both a healthy green leaf (GL) and a withered yellow leaf (YL) were observed.

Figures 6(a) and 6(e) show examples of the two-photon fluorescence spectra corresponding to the GL and YL (black lines), respectively. Without the spectral data of PS II and PS I, curve fitting was applied to the spectrum in Fig. 6(a) based on Gaussian functions [26]. Two Gaussian functions with peak wavelengths at 685 nm (blue line) and 727 nm (red line) were obtained for PS II and PS I, respectively. The summation of these two spectra (green line) fit the measured spectrum well. By applying the linear unmixing algorithm based on the obtained Gaussian functions, the spectrum of the YL (Fig. 6(e)) can also be separated into two spectra (blue line for PS II; red line for PS I). The summation of these two spectra also fits the measured spectrum well.

Figures 6(b) and 6(c) show the PS II and PS I images of the GL, separated by using the linear unmixing algorithm, while Figs. 6(f) and 6(g) show the PS II and PS I images of the YL. Both the hyperspectral images of GL and YL were obtained at a depth of 70 μm beneath the leaf surface. Both PS II and PS I signals were found in the mesophyll cells. In the observed layer of the GL, the mesophyll cells formed a palisade structure (Fig. 6(b) and 6(c)), while the structure of the mesophyll cells became blurred and unorganized in the YL (Fig. 6(f) and 6(g)). Figures 6(d) and 6(h) respectively show superpositioned PS II and PS I images of the GL and the YL. The colors in Figs. 6(d) and 6(h) show the signal ratio distribution of PS II and PS I within the sample and indicate the variation in the PS II and PS I activity from cell to cell. The greenish color in Fig. 6(h) indicates that the intensity of the PS I signal was generally much lower than the PS II intensity everywhere in the YL. This might be because PS I plays an essential role in photosynthesis and the degradation of the PS I activity can directly cause the withering of the leaf.

Fig. 6. (a) Two-photon fluorescence spectrum of the GL (black line). The spectrum was decomposed into PS II (blue line) and PS I (red line) spectra by applying Gaussian curve fitting (green line); (b), (c), and (d) show the PS II, PS I, and the superpositioned images of the GL with a 300-s acquisition time. (e) The two-photon fluorescence spectrum of the withered yellow leaf (black line). Based on the spectra shown in (a), the spectrum was decomposed into PS II (blue line) and PS I (red line) spectra by applying linear unmixing; (f), (g), and (h) show the PS II, PS I, and superpositioned image of the YL with a 300-s acquisition time. Both the hyperspectral images of GL and YL were obtained at a depth of 70 μm beneath the leaf surface. Scale bar: 25 μm.

5. Conclusions

In this study, a method for two-photon hyperspectral microscopy with non-de-scanned geometry and parallel recording has been demonstrated. Based on the high sampling rate in both the spatial and spectral dimensions, this system can provide two-photon hyperspectral imaging with a high spatial resolution of ~500 nm and high spectral resolution of ~1 nm. Parallel recording can help to increase the recording speed for recording such a large amount
of data, while simultaneously reducing the dwell time. Combined with the linear unmixing algorithms, this system shows the capability of identifying different fluorophores and solving the crosstalk issue. The spatial distribution of the PS I and PS II activity in fresh unstained leaves is revealed using this system. Two-photon microscopy provides a powerful less-invasive tool for in vivo investigation of photosynthesis-related plant physiology. The high spectral resolution of this system can further increase the accuracy of the spectral analysis and enable the decomposition of fluorophores in more complex bio-tissues.

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