MULTIFOCAL MULTISPECTRAL DESCANNED DETECTION IN TPLSM

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Abstract

We present a new detection method for multifocal two-photon laser scanning microscopy (TPLSM) that allows a fast and easy access to spectrally resolved, three-dimensional images. In our setup eight fluorescent foci are directed through a descanned tube lens combination and a straight vision prism. This prism spectrally splits up the fluorescence beamlets, resulting in eight parallel spectral fluorescence lines. These lines are imaged onto a slit block array in front of a 8x8 multi anode PMT. Each PMT row detects different spectral characteristics from a special point in the sample whereas each column represents one focus. The eight exciting foci are scanned in the region of interest inside the sample by the two scanning mirrors in x- and y-direction. As a result of this imaging technique eight spectrally resolved images of slightly shifted sample regions are generated simultaneously and added up after the measurement, maintaining the spectral information. We present spectrally resolved 3D-data of various biological samples like pollen grains, tobacco cells and orange peel cells.

Keywords: TPLSM, multifocal multispectral descanned detection, fluorescence microscopy

1. INTRODUCTION

Two-photon laser scanning microscopy (TPLSM)[1] has become a powerful tool in biological applications as it allows contact free, 3-dimensional analysis of biological material with high resolution. In contrast to conventional brightfield microscopy, where whole samples are illuminated, TPLSM generates a fluorescence image by scanning the sample point by point. This is done by focused, ultrashort near infrared (NIR) laserpulses. The light intensity in the focus is high enough for a simultaneous absorption of two photons. The quadratic dependence of the two-photon absorption on the laser intensity causes only fluorophores in the very small focal volume (< 1 fL) to emit fluorescence after two-photon excitation. The energy gap between the fluorophores' ground- and excited states corresponds the energy sum of both photons. NIR two-photon excitation commonly results in fluorescence emission in the visible (VIS) wavelength range, as the energy of the two absorbed NIR photons is equal to the energy of one photon in the ultraviolet (UV) or low VIS wavelength regime. As biological samples are less affected by NIR than by UV light, photo toxicity and out-of-focus bleaching are reduced in TPLSM compared to one-photon excitation techniques. Moreover, the wavelength range between 700 nm and 1100 nm is particularly suitable for biological microscopy, because water has a low absorption coefficient in this regime and light scattering is reduced. Therefore, a deeper penetration into the investigated biological sample is possible.

As sensitive CCD-chips or photo multiplier tubes (PMT) do not record the spectral characteristic of fluorescence emission, filters or diffractive elements (i.e. gratings or prisms) are commonly used in fluorescence microscopy to differentiate between different spectral emission characteristics and therefore fluorophores. The use of emission filters is especially compromised in laser scanning microscopy, because the sample has to be imaged subsequently with several filters to acquire a spectrally resolved image. This results in prolonged image acquisition times as well as in possible

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image shifts due to mechanical drift or sample movement. Therefore, a simultaneous detection of several wavelength regions is desirable.

When a two-dimensional picture is imaged by using a diffractive element, spatial and spectral information are mixed. To avoid this problem and to detect several wavelength regions simultaneously, it is necessary to utilize a cascade of dichroic mirrors and multiple CCD-cameras. In a laser scanning microscope it is however possible to generate a stationary identical detection beam path for each sampling point. This allows for a defined separation between spatial and spectral information, because image generation is performed by correlating the fluorescence intensity with the position of the exciting laser focus in the sample.

The basic idea of the descanned detection is to generate this stationary image of the focus. The laser beam scans the sample by using two scanning mirrors. The fluorescence life time typically spans a few ns. During this period, the scanning mirrors practically remain in the same scanning position. Therefore, the fluorescence light takes the same optical way as the exciting laser light. By the use of a dichroic beamsplitter the incoming laser pulses and the outgoing fluorescence photons can be separated. The optical way of the fluorescence light after the scanning mirrors is independent of their position and thus the focus is stationary for a detector. Hence it is possible to apply a prism to split up the fluorescence light spectrally without the problem of mixing spectral and spacial information.

The advantage of fast recording times in simultaneous multispectral detection can be strengthened by using more than one excitation focus, resulting in an excellent photon statistics of the generated image, while keeping the excitation intensity and therefore photo-toxicity and bleaching minimal.

2. MICROSCOPE DESIGN FOR MULTISPECTRAL IMAGING

2.1 Experimental setup

The NIR laser light is generated by a modelocked femtosecond Ti:Sa Laser (Tsunami, Spectra Physics) pumped by a solid state laser (Millenia X, Spectra Physics).

The laser beam is coupled into a multi beam scanning unit (TriM-Scope, La Vision BioTec). This unit is equipped with a step motor driven polarizer and a fixed analyser for beam intensity attenuation. The beam is directed through a beam expander into a pre-chirp section which compensates the pulse dispersion of the following optics, particularly in the beam multiplexing section. It consists of a central 50% mirror and a set of ten 100% mirrors which multiplex the incident laser beam into up to 64 beamlets, each of them carrying the same average intensity. By adjusting the number of passages through the 50% mirror of the incident laser the number of beams (1, 2, 4, 8, 16, 32, 64) is selectable. In this particular case, eight beams are used. The beamlets are directed through a shutter section onto a dichroic mirror (680 dcspxr, Chroma Technology Corp.). In descanned detection the exciting laser light and the fluorescence light of the sample are separated by this mirror. The last optical components of the scanning unit are two silver coated scanning mirrors.

The beams are imaged onto the back aperture of the objective lens by a telecentric lens combination, consisting of a scan lens (f=50mm, LaVision BioTec) and a tube lens (f=180mm, LaVision BioTec). Two objective lenses could be used (UP-LAPO60XW3/IR with WD = 0.28 mm and NA 0 1.2; XLUMPLFL20XW with WD = 2 mm and NA = 0.95, both Olympus). The exciting laser beam is reflected by a 100% mirror, installed in the turret of the inverted microscope (IX 71, Olympus). Inside the sample eight two-photon excited fluorescence foci can be scanned in the focal plane of the objective lens. Just like the exciting beam, the sample's fluorescence light is reflected by the 100% mirror via the tube and scan lens onto the scanning mirrors. Therefore, the fluorescence light is guided into the descanned detection path after passing the two scanning mirrors again. The detection beam is now stationary or descanned. The descanned detection path consists of two lenses (f=160mm, f=-50mm, both Linos Photonics) and the eight fluorescence foci pass a straight vision prism (331120, 71 mm, Linos Photonics). This prism spectrally splits up the fluorescence beamlets, resulting in eight parallel spectral fluorescence lines. These lines are imaged onto a slit block array in front of a 8x8 multi anode PMT (H7546B-20, Hamamatsu). At each mirror position all 64 PMT channels are recorded, generating 64 complete images after scanning the complete region of interest. As a result eight spectrally resolved images of eight slightly shifted sample regions are generated. Therefore, this measurement mode enables a simultaneous detection of eight different joining spectral integrals for each of the eight fluorescence foci without the need to perform sample

scanning or turn a filterwheel. The result of one measurement are eight stacks each containing eight images. An individual stack represents one focus, while each image of the stack represents one spectral integral.

The detected spectral range depends on the relative position of the PMT with respect to the beam path and prism position. The pixelation of the image as well as the frame time depend on the scanning properties. Especially the frame size and the incremental mirror movement are the important parameters for the acquisition time which commonly amounts to 2-6 seconds.

Figure 1 shows the described experimental setup.

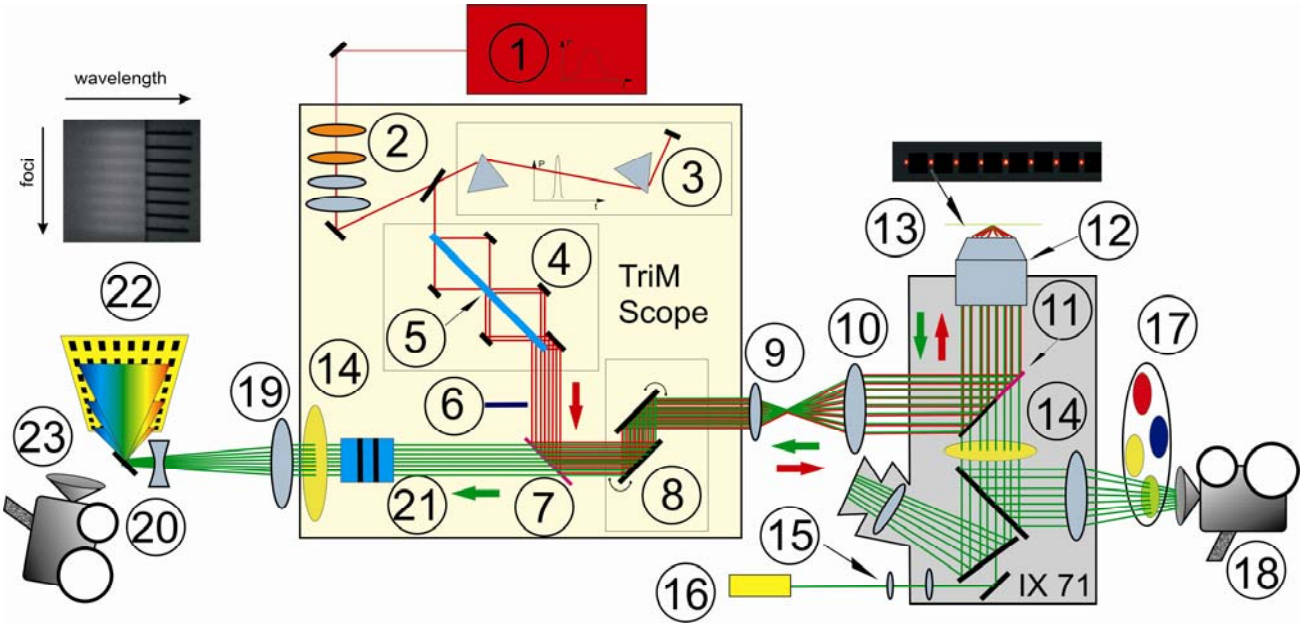

Fig 1. Descanned multifocal multispectral detection: 1) Ti:Sa Laser; 2) polarizer/analyzer and beam expansion; 3) pre-chirp section; 4) beam multiplexing section; 5) 50% mirror; 6) laser shutter; 7) dichroic mirror; 8) scanning unit; 9) scan lens; 10) tube lens; 11) 100% dichroic mirror; 12) objective lens; 13) sample; 14) short pass filter; 15) focusing lens; 16) PMT; 17) filter wheel; 18) imaging camera; 19) descanned tube lens #1; 20) descanned tube lens #2; 21) straight vision prism; 22) multi anode PMT, picture above shows split up fluorescence light on slit block (left hand side is covered to show the fluorescence lines); 23) control camera; unspecified black bars indicate 100 % mirrors; additional (tube) lenses inside the microscope are indicated but not specified[2]

2.2 Multi anode PMT electronics

The used multi anode PMT (H7546B-20, Hamamatsu) requires a suitable amplification and sample-and-hold electronics, as the individual channels are sampled by a multiplexed AD-conversion board (DAQ 2204, Adlink). The first component of the detection electronics is an inverting transimpedanz two stage amplifier for each individual channel of the PMT. All 64 amplifiers are located directly on the PMT`s connector circuit board. The operational amplifiers (AD8620) used for these circuits have particularly low offset voltages and low thermal drift. This is an important feature, as the second stage of the complete detection electronics consists of a dual integration electronics, which integrates the amplified PMT anode's currents during two read-outs of the AD-conversion board. This integration has to be performed, because during the delay times between two read-outs, i.e. the time while the other 63 channels are being sampled, fluorescence photons can not contribute to the generation of a fluorescence image, although they are being detected. The selection of the different integrators is performed by digital counter electronics, the timebase of which is the AD-conversion trigger from the AD-conversion board. Therefore, the integration electronic is automatically triggered by software.[2]

2.3 Image processing

2.3.1 Software

Measurement control and data analysis like spectral unmixing are performed with Imspector(LaVision BioTec). Image addition and shifting are performed with ImageJ. 4-dimensional representations (including spectral axis) are calculated with Imaris 4 (Bitplane).

2.3.2 Determination of the translation vector

The spatial translation vector represents the relative shift of the eight exciting foci. For an experimental determination of this vector the same point in each of the eight slightly shifted sample images has to be chosen.

Therefore, fluorescence beads are particularly suitable samples due to their small size. Thus, the position of an individual bead can be determined with an accuracy of few pixels in each stack representing one focus. After determining the position of the same fluorescence bead in each of the eight images their relative spatial translation vector can be calculated.

2.3.3 Image shift

The spatial misalignment of the eight image stacks has to be compensated for by shifting the stacks according to the translation vector. Therefore all stacks, which consist of eight images containing the spectral information for one focus, have to be superposed with respect to the translation vector. The utilization of the translation vector overlaps all eight image stacks, in such way that they all exhibit the same sample region in the same position of the image. Afterwards the same spectral images of all eight foci are added up now generating one 8-fold oversampled image of one spectral channel. This results in one stack composed of eight spectrally neighboring images.

It has to be noted that only in the (spatially) overlapping region of all eight foci the full 8-fold oversampling is achieved. Sample regions that have been probed by less than eight foci appear in the image as small stripes in the right-most position (see Figure 6) of the complete image.

The image shift is performed by using a self-written macro in *ImageJ*. Three-dimensional images require an image shift for every step in z-direction. However, the macro is written in such a way that the handling of three-dimensional data is also automatically possible.

2.3.4 Spectral unmixing

Spectral unmixing is a powerful tool to differentiate between fluorophores/components on the basis of their emission characteristics. The basic idea of spectral unmixing is a test on linear dependence. Because emission spectra of different fluorophores often do overlap, for a certain wavelength (-range) it can not be decided to which fluorophore the emission intensity has to be assigned to. In spectral unmixing the spectral emission characteristics of every pixel in an image is compared to example spectra by determining the proportional share of all example spectra to the particular pixel's spectra. After determining a best fit, the coefficients of contribution are assigned to corresponding pixels in images that represent the different example spectra. For the fundamental idea it does not matter whether the spectral differentiation is based on emission spectra or just on different filtered images. The advantage of this tool is its relative intensitivity towards overlapping emission characteristics, even if they differ considerably in intensity.

The Imspector (LaVision BioTec) software package is equipped with this image processing option.[2]

An image generated with the new multifocal multispectral descanned detection mode can be unmixed with this tool. The software averages the intensity of a chosen region of interest and displays the intensity for the eight wavelength channels in a graph. Figure 2b) shows this graph, generated from the red marked regions of interest in a) and c), with the eight spectral channels on the x-axis and the integrated intensity on the y-axis as well as the images of channel 8 and channel 3. These two channels are characteristic for the two different component parts of the pollen grain. Image a) shows the outer membrane whereas image c) displays the inner segment. Accordingly, the spectra differ exceptionally at channels 3 and 8. The spectrum of image a) has a characteristic peak at channel 7 and 8 in contrast to the spectrum of image c) which features a peak at channel 3.

The usage of the spectral unmixing option within the Imspector software generates two images for each of the spectra. They are displayed in figure 2d). It becomes clear, that the two components of the pollen grain are seperated very clearly. The colors red and green are selected to illustrate the differentiated components in one image.

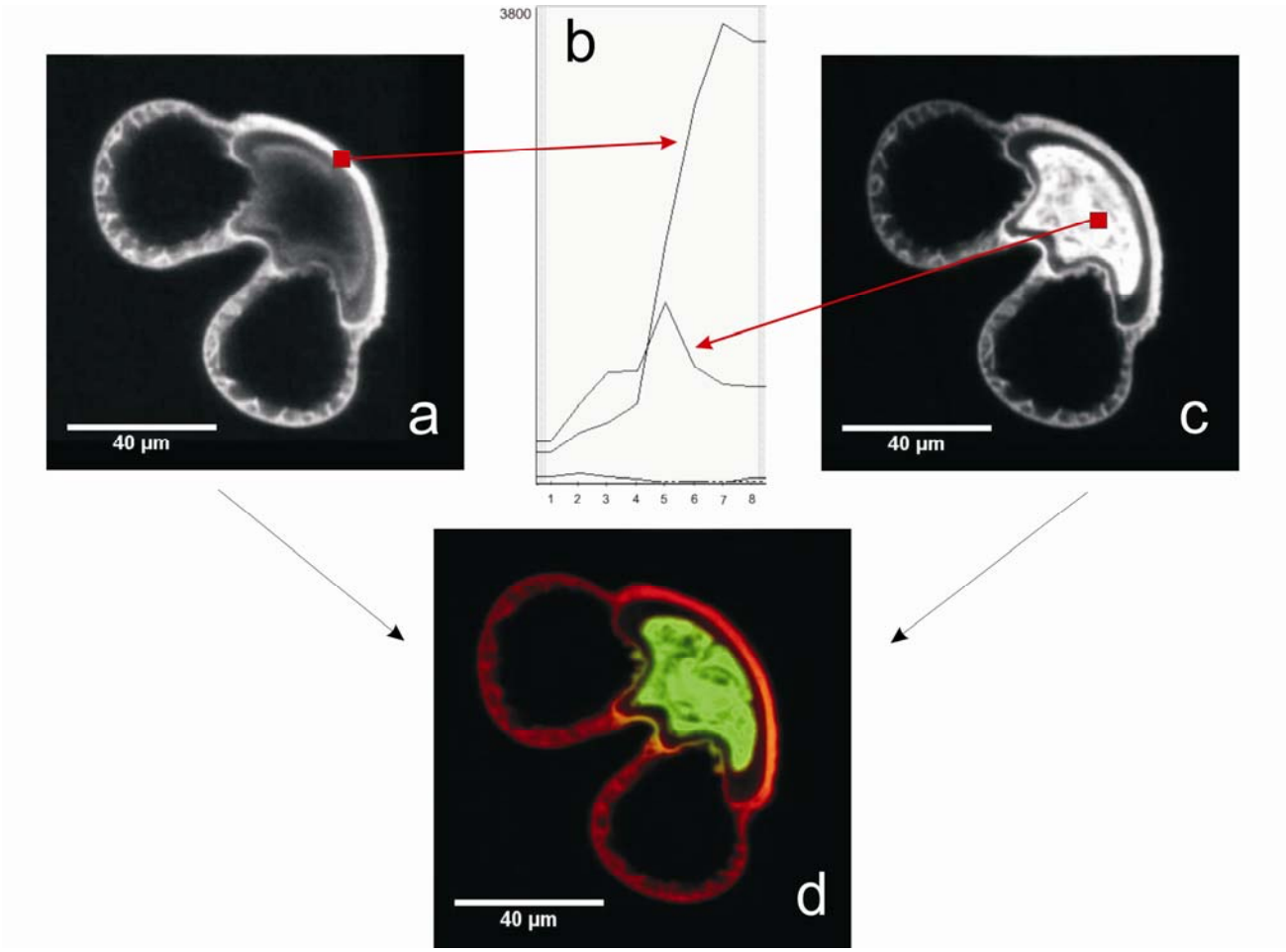

Fig. 2. Image of a spectrally unmixed pollen. a) and c) display the intensity images of channels 8 (a) and 3 (c). The red squares indicate the regions of interest for the measurement of spectra presented in b). The abscissa displays the number of the spectral channel while the ordinate shows the relative fluorescence intensity. d) shows the spectrally unmixed image according to the fluorescence spectra in b). Pixels with high similarity to the emission spectrum a) are colored red whereas pixels with high similarity to the emission spectrum c) are colored green.

2.4 Spectral calibration

A calibration of this measurement method is necessary to determine the detected wavelength range of the fluorescence light for each spectral channel. The 8x8-fold PMT can be shifted vertically, allowing a shift of the spectral detection range. To calibrate a current setup the multi anode PMT has to be illuminated with a set of known and defined wavelengths. By analyzing the detected signal of a single wavelength, respectively a small band, this wavelength's detection channel can be determined. Roughly, the overall spectral detection range covers a wavelength range of 110 nm in the present setup.

A small wavelength band is generated by using small bandpass filters, positioned in front of the PMT. The fluorescence light is produced by a pollen grain because its fluorescence intensity compensates the low transmission of the bandpass filters (peak: 40% to 50%, FWHM: 6 to 8 nm) and it additionally provides a broad emission spectrum.

The presented calibration was performed with filters from 512 nm to 630 nm. The pollen was imaged with each of the available filters on the multi anode PMT. Afterwards, the detected wavelengths have been correlated with their spectral detection channels. The illuminated channels exhibited a significant high overall intensity compared to the other

channels which only detected background of low intensity. Some filters allowed fluorescence to be detected by more than one channel, because the filter's peak transmission has been positioned between two channels or because their real FWHM values exceeded the specifications.

Figure 3 shows the resulting calibration curve. It consists of the number of channels on the abscissa and the wavelength on the ordinate. As the prism's dispersion is nonlinear, a calibration is required when changing the experimental setup.

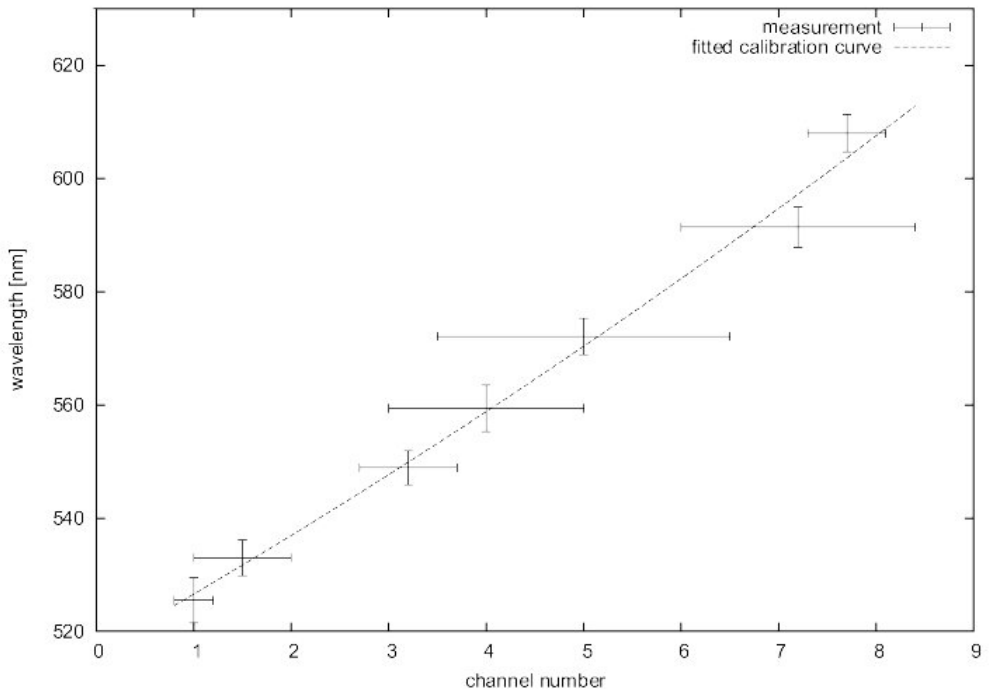

Fig. 3. Example of a calibration curve. The abscissa shows the channel number and the ordinate the wavelength. At higher wavelengths the prism's nonlinear dispersion becomes obvious. Y-error bars indicate the used filters' FWHM which vary between 6 and 8 nm. X-error bars indicate number of channels on which the pollen could be detected.

3. RESULTS

3.1 Tobacco protoplasts

The examined tobacco BY-2 protoplasts have been transfected with two different reporter proteins. One is GFP fused to LCL1, the other one is a dsRed nucleus marker, called "NLS-CHS-dsRed". LCL1 is a nucleocytoplasmatic shuttle protein which possess a nuclear export signal (NES) as well as an nuclear localization signal (NLS).[3]

The dsRed marker is fused to the chalcone synthase (CHS).

Figure 4 shows a spectrally resolved image of a transfected tobacco protoplast, which has been two-photon excited at 800 nm and detected with the multifocal multispectral descanned detection made. The red color in Figure 4 represents the fluorescence of dsRed located only within the protoplast's nucleus.

LCL1 on the other hand is actively transported between the nucleus and cytosol. Therefore, the green fluorescence of GFP tagged to LCL1 is detected in the cytosol as well as in the nucleus.

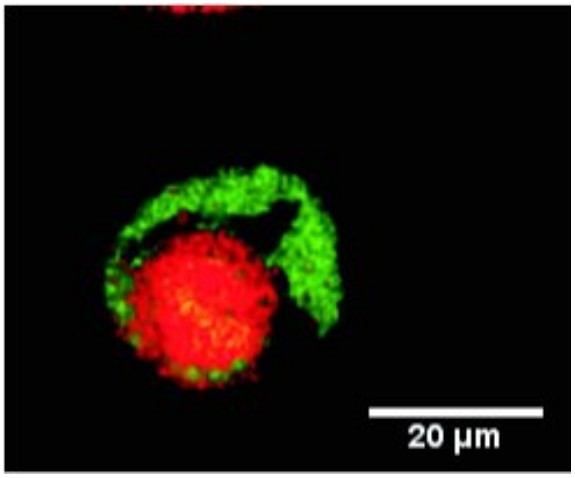

Fig. 4. Spectrally resolved tobacco protoplast imaged in the multifocal multispectral descanned detection mode. The red color represents dsRed-transfected CHS located in the nucleus, green represents the GFP-LCL1 complex shuttling between cytosol and nucleus.

3.2 Pollen

We analyzed fixed pollen grains which have been purchased from Carolina Biological Supply Company.

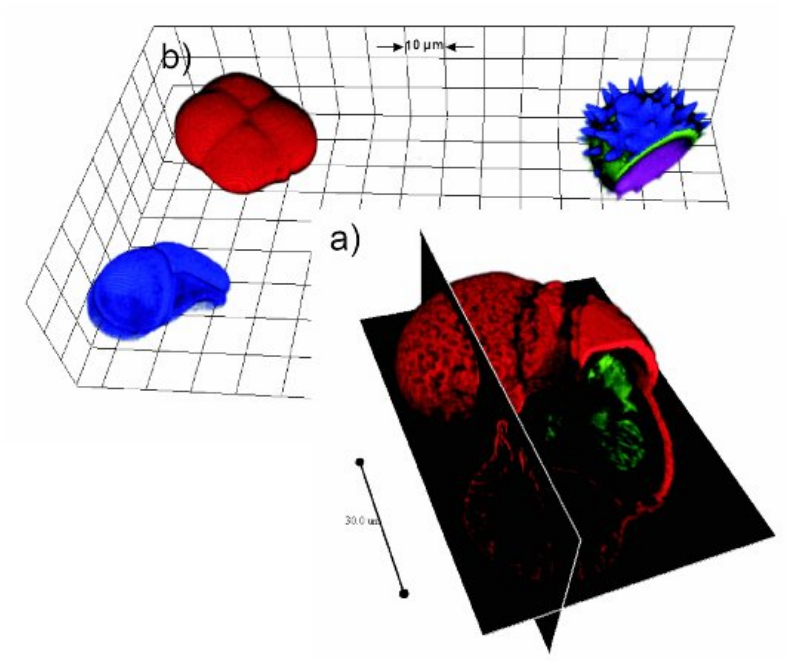

Fig. 5. a) 3-dimensional image of a spectrally resolved pollen grain. The spectral unmixing is based on two different spectral emission characteristics (red, green). The pollen is digitally cut open to show its inside. b) Spectral unmixed result of three simultaneously imaged pollen. Spectral unmixing which was based on four different emission characteristics, reveals four different spectral characteristics in the sample. The upper right pollen has been digitally cut open to present its inside with an additional emission characteristic.[2]

Figure 5a) shows a spectrally resolved 3-dimensional *Abies pectinata* pollen grain.[4] It is obvious that the grain mainly consists of two different constituents the outer membrane colored red and the protoplasm colored green. The latter basically consists of starch.

Figure 5b) shows a voxel representation of three imaged pollen. It reveals four different emission characteristics for the three pollen. As these four characteristics have been imaged simultaneously, it is a good example of the capability of multifocal multispectral descanned detection.

3.3 Orange

We analyzed orange peel to determine its cell composition.

Figure 6 shows the cells of an orange peel. The green color represents the cell walls which consist of cellulose, while the cells' cytosol is colored red. The apparent stripes on the right edge of Figure 6 are the typical artifact of superposing eight shifted image layers. These layers correspond to the eight exciting foci used in multifocal two-photon excitation.

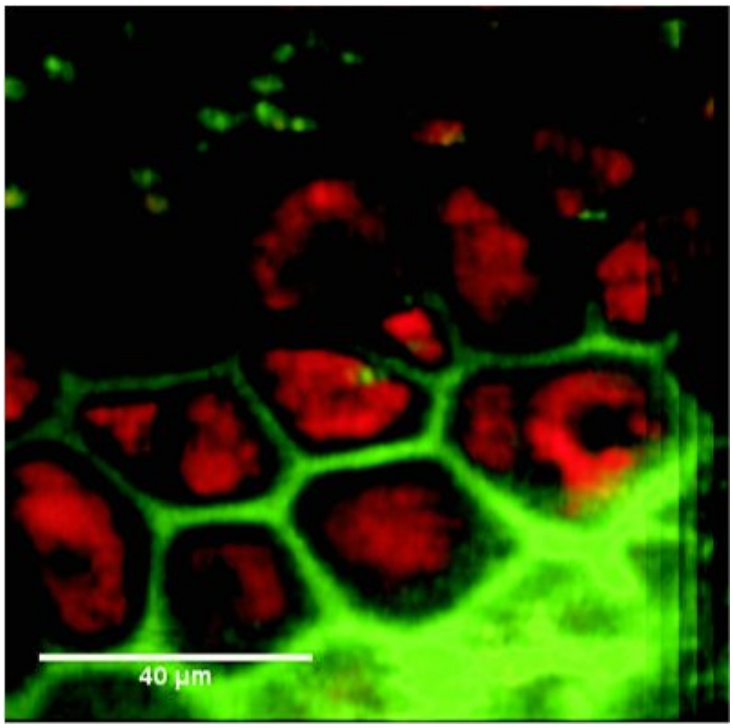

Fig. 6. Spectrally resolved image of an orange peel. The cell walls (green) and the cytoplasm (red) are differentiated from each other. The right-most area results from the shifting process (2.3.3).

4. SUMMARY AND OUTLOOK

We have presented the basic concept of multifocal multispectral descanned detection in TPLSM as well as its application to various biological samples. The simultaneous detection of eight fluorescent foci leads to a significantly improved photon statistics compared to single focus excitation. This enables the simultaneous detection of eight individual neighboring fluorescence wavelength ranges. The complete spectral detection range can be adjusted by varying the multianode PMT position with respect to the spectrally dispersing prism. With these detection options at hand we

presented the simultaneous detection of up to four different emission characteristics in one sample. Therefore, speeding up 3-dimensionally resolved fluorescence image generation of multiple fluorescent markers is now possible because subsequent filtered measurements of the same sample region can now be omitted. This results in an overall decreased image acquisition time and less image distortion due to mechanical drift and sample movement.

Due to the flexibility of the spectral detection region it is furthermore possible to differentiate between fluorophores which show a strong overlap in their spectral emission characteristic. By choosing small spectral detection ranges and applying a spectral unmixing algorithm to the detected data set it will be possible to differentiate between these overlapping emission spectra which is commonly a problem when using wide emission filters.

Multifocal multispectral descanned measurements therefore offer a powerful new detection tool for analyzing biological samples by means of TPLSM.

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