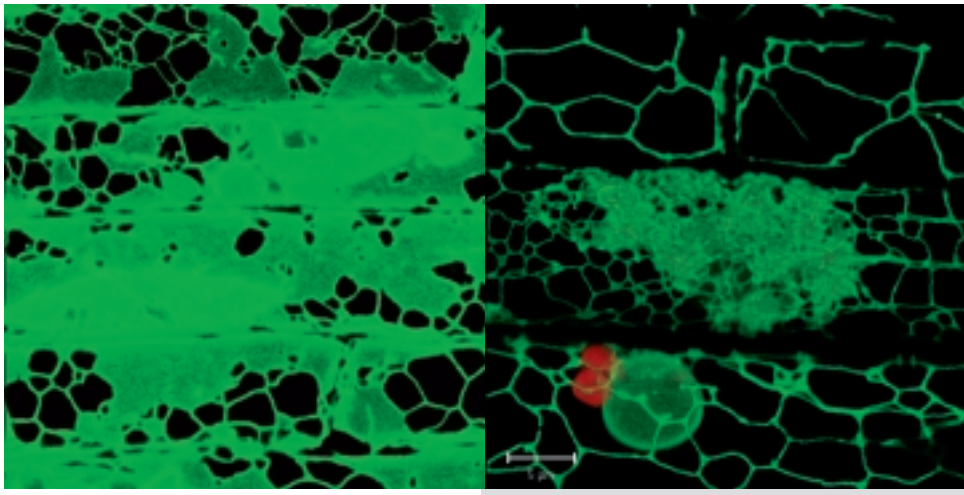
Imaging GFP and plant autofluorescence – Multitracking

Introduction

Plant tissues often show strong autofluorescent signals. These signals may be used to visualize, e.g., plastids or chloroplasts. Hence, in combination with fluorescent labels like GFP, autofluorescence often interferes, or the excitation of the fluorescent label introduces additional autofluorescent signals. This short application note shows how the LSM510 can be used to separate the GFP signal and detect the autofluorescence of plastids by means of Multitracking, without being influenced by the GFP excitation.

Application

The images were taken from petals of a Nicotiana benthamiana plant (a kind of tobacco) in which the GFP-gene is present as a transgene.
The GFP contains an ER-retention signal, which means that the entire endoplasmic reticulum network in the plant is fluorescing. These cells contain a large vacuole (seen as a black area), surrounded by cytoplasm containing the fluorescing ER network.



An extended focus image of a complete confocal stack is used to visualize the entire plant cell.

Viral infection of plant tissue

Upon infection with cowpea mosaic virus (CPMV), the ER network changes drastically. An image of the dilated ER network in a virus-infected cell is shown on the right. The image on the left shows the ER network in an uninfected cell. The observation of these living cells in the context of intact tissue provides new possibilities to follow the development of an infection as a function of infection time.

Nicotiana benthamiana:

Left: without infection
Right: with infection

Green: GFP; excitation: 488 nm, detection: 505–530 nm

Red: autofluorescence; excitation: 543 nm, detection: LP 560 nm

Setup:
2-channel fast line-by-line Multitracking

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LSM 510: Eliminating Crosstalk by Multitracking

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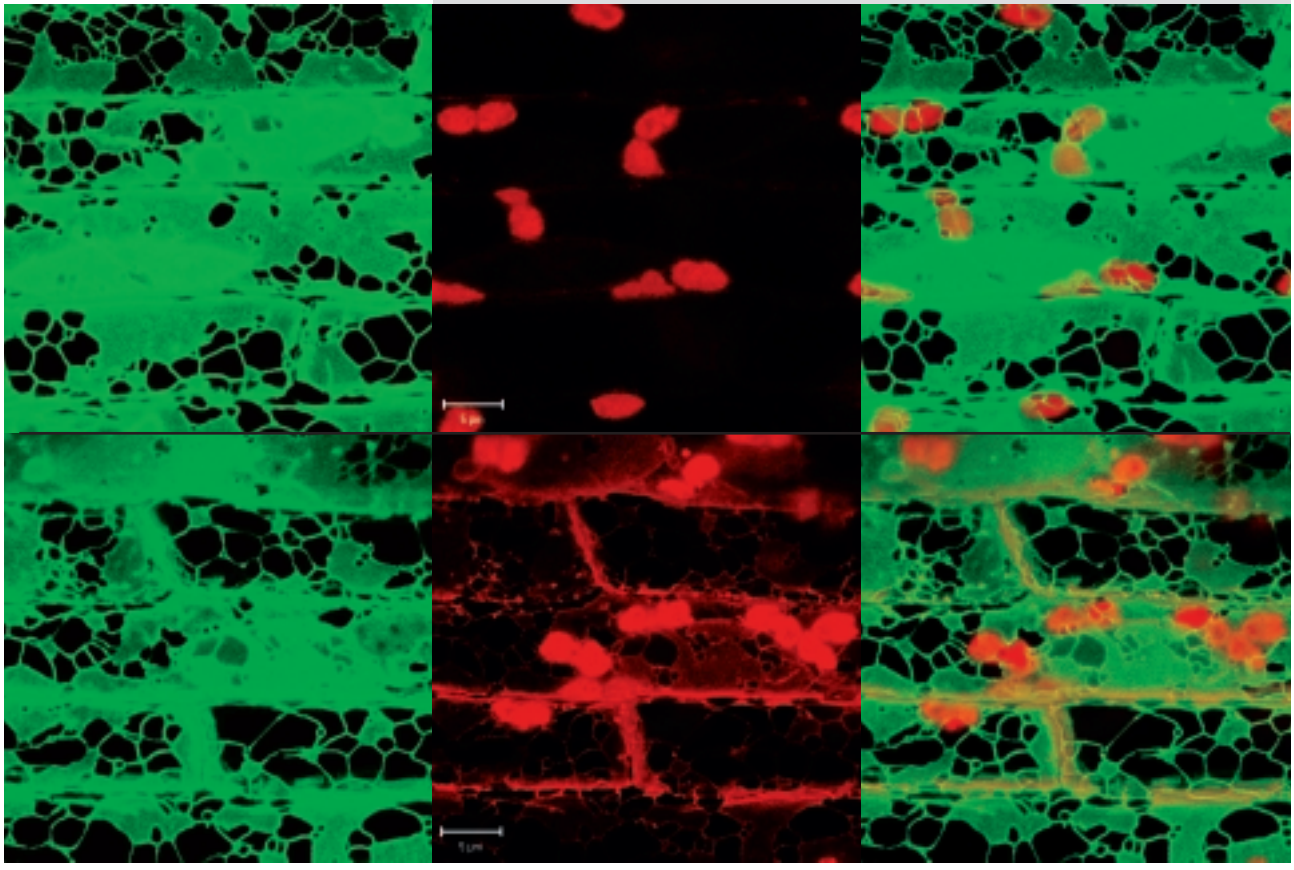
Crosstalk and Background

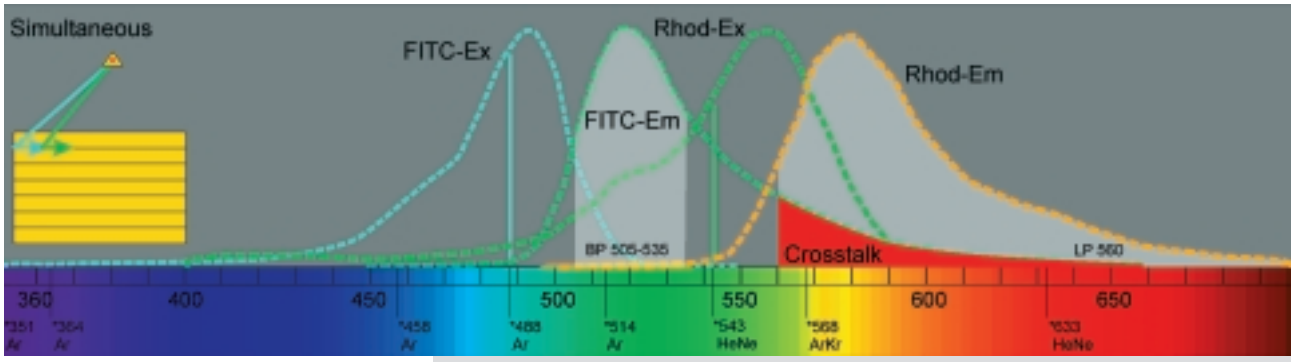
Comparison of simultaneously collected, images and images taken by multitracking (quasi simultaneous)
Top: line-wise multitrackin (GFP, autofluorescence); Bottom: simultaneous (exactly the same imaging conditions, but a different region of the sample)



Crosstalk/Background elimination

In In both cases, GFP was excited with the 488 nm line of an Ar+ laser and detected via a 505-530 nm band pass filter. The plastids were excited by the 543 nm laser line of a HeNe laser and detected with a 560 nm long pass filter.
To visualize the difference between the classical way of acquiring the fluorescent signal for both channels simultaneously and the new Multi-tracking technique, we separated the images into the two channels: Both rows of images show the single GFP image on the left (green), the autofluorescence in the middle (red), and the overlay of both channels on the right.
The upper row shows the images collected with the Multitracking feature, whereas the lower row shows the images recorded in the classical simultaneous way.
Imaging conditions were the same for both cases, but different regions of the sample were imaged.
The difference is quite obvious: there is a strong autofluorescent signal of the membranes excited by the 488 nm laser line, which leads to a strong crosstalk signal in the red channel. This can be seen in the overlay image on the right as well; here of course the red and green mix into yellow.

Multitracking procedure



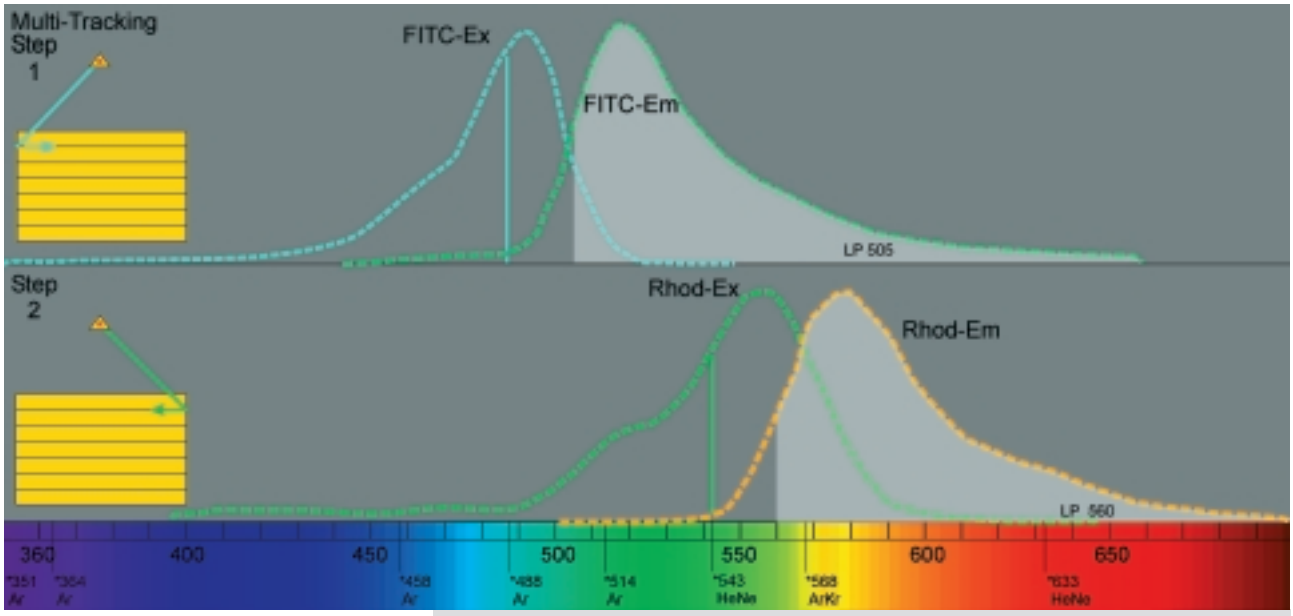
Classical simultaneous excitation and detection of fluorescence FITC/Rhodamine

The excitation of fluorescent dyes in the LSM510 is accomplished in a very fast and accurate way by means of a DSP (digital signal processor) and an AOTF (acousto-optical tunable filter). During scanning of the lines the intensity of the laser beam and the way of the detection can be switched. This procedure, called multitracking, offers several advantages compared to the classical detection, where the excitation and the detection of the fluorescent signals is done simultaneously.

Elimination of crosstalk

Due to the spectral properties of fluorescence, the emission of one dye typically overlaps into the emission of the second dye. This effect is present in all combinations of fluorescent dyes, even for the classical combination of FITC and Rhodamine.
Simultaneous excitation and detection of both dyes leads to crosstalk where the emission of the green fluorescence contributes to the detection of the yellow (red) dye (indicated in red).

To eliminate this effect the multi-tracking procedure does not scan both laser lines simultaneously, but in two steps. In the first step the system scans the cyan laser from the left to the right and collects the green fluorescence (track 1); within the second step the system scans the same line from the right to the left (green laser) and collects the yellow fluorescence (track 2).
The yellow (red) channel therefore cannot contain emissions of the green dye. By this procedure the crosstalk is not just minimized but completely eliminated.



Multi-Tracking: Separate excitations – separate detection
Step 1: Left to right: cyan excitation
Step 2: Right to left (on the way back): red excitation

For time dependent preparations

The multitracking procedure might be implemented by frame-by-frame switching as well.
But for moving or, in general, time-dependent preparations this makes limited sense only. Here the sample has moved during the time between the two single-channel images. The same applies for time series, where a temporal or spatial shift between the different channels will occur. This problem can be overcome by using line-wise switching (Fast-Multitrack). The switching takes place after about every 750 µs (Speed 10, 512 pixels per image line).

Compared to frame-wise switching (every 0.4 s for 512 image lines), this is about 500 times faster. For bigger formats (2k x 2k) the factor will even increase.

Improvement in sensitivity

Applying the multitracking procedure one can use long-pass filters instead of band-pass filters for the detection of the fluorescent light even in cases of double or multiple stainings. This leads to an improvement of sensitivity and can be applied for weak stainings very well.

Different staining levels

The multitracking procedure makes sense, in particular, in the detection of different staining levels. The overlapping spectral regions will be blocked very efficiently.
As an example, imagine the detection of a weak rhodamine signal in a quite strongly Dapi-stained nucleus. The broad Dapi-fluorescence typically covers the complete spectral range of the rhodamine staining. Only the multitracking technique provides the possibility of separating both dyes.

Combination with ROIs

Finally, the multitracking procedure can be combined with any real „Regions Of Interest" (ROI) which again increases the applicability and flexibility of the technique.

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