Calcium Imaging in Plants

LSFM Allows Rapid and Long-Term Acquisition of Calcium Dynamics

One of the challenges of modern plant biology is to understand the specific functions of plant tissues in close-to-physiological conditions. In order to understand how plant perceive and transduce environmental and developmental signals we use Light Sheet Fluorescence Microscopy. This method allows one to observe the plant growth in vivo, follow cellular differentiation processes and link these events with the dynamics of signaling molecules, including calcium.

Light Sheet Fluorescence Microscopy (LSFM) is a fluorescence imaging technique highly suited to study living samples, particularly developing embryos, tissue cultures and plants [1]. In LSFM a biological specimen is illuminated in a single plane (light sheet) and the fluorescence emitted by the sample is collected along the direction perpendicular to the illumination plane. This approach offers two major advantages compared to conventional and confocal microscopy techniques. First, fluorescence images are collected using common widefield optics and CCD or CMOS cameras, that offer high acquisition rates: more than 100 frames per second are acquired routinely with LSFM. Second, only the plane of the specimen under observation is illuminated. This configuration greatly reduces the light dose and therefore photo-bleaching effects and photo-toxicity.

When imaging plants, such as Arabidopsis thaliana, LSFM offers a third advantage that makes it ideally suited for long-term time-lapse experiments. The sample can be placed in vertical position, the plant root can be grown directly in an agar based medium, eventually placed in a hydroponic solution and the leaves can be illuminated with an artificial light: this makes the measurement as close as possible to physiological. In these conditions, plant development can be recorded over several hours of growth [2] and the plant function, such as the calcium dynamics occurring in response to internal and external stimuli can be studied over large tissue volumes [3].

Working Principles of LSFM-FRET
Calcium imaging in plants can be achieved using several different fluorescent labels
(e.g. Fluo-3) but for subcellular imaging, genetically encoded Ca\textsuperscript{2+} indicators (aequorin, GCaMP, Cameleon) are the tool of choice.

Cameleons are Förster Resonance Energy Transfer (FRET) based calcium indicators in which two fluorescent proteins, CFP and YFP (or circularly permuted variants of YFP, such as cpVenus), are linked together by the calcium-binding protein calmodulin and a calmodulin binding peptide. Binding of Ca\textsuperscript{2+} to these calcium-responsive elements alters the distance between the two fluorophores. Hence, the efficiency of FRET varies, allowing a quantitative assessment of calcium dynamics.

Using LSFM one can image Arabidopsis transgenic plants expressing, for example, the nuclear or cytosolic-targeted Cameleon [3]. A typical setup consists in an illumination path to create the light sheet and a widefield detection path (fig. 1a), where two wavelength channels are simultaneously acquired for ratiometric FRET imaging. A laser at 442 nm coupled to a single mode fiber is collimated and used for excitation. A cylindrical lens focuses the light in a horizontal plane and a telescope (f1, f2) images the focal plane of the cylindrical lens in the back focal plane of a water dipping microscope objective (10X). As a result, a vertical light-sheet is created on the sample. The fluorescence is collected by a 20X water dipping microscope objective which, in combination with a lens (f3), creates an intermediate image. Calcium imaging with FRET requires the detection of the signal emitted by the two fluorescent molecules CFP and cpVenus. This task is achieved using two separate cameras or an image splitter placed in front of a single detector. Here, the intermediate image is split in two channels (fig. 1b) on the CMOS camera by a custom beam splitter. The plant root is mounted on a motorized stage for accurate and automatic scanning of the sample.

**Live Imaging of Arabidopsis Development and Function**

By moving the sample back and forth through the light sheet, the system creates three-dimensional recordings of the sample fluorescence and FRET signal, with an acquisition time of one or few seconds per volume. This rate is suited to study a large number of physiological processes in different plant cell types (and tissues) that occur with seconds or minute time scales, such as the outgrowth of root hairs or the growth of primary and later roots. Looking at the fluorescence, either in the CFP or in the cpVenus channel, one can observe the phenotype of the root and, even more importantly, follow the growth of the sample (fig. 2) under the microscope, for minutes, hours or even days. At the same time, FRET signals can be acquired and analyzed in real-time, in order to estimate the calcium concentration in the different compartments of the cells within the root cells or in root hairs. In these experiments, normally the ratio (R) between the signals emitted by the two
fluorescent proteins is measured and eventually normalized to its initial value (R0), calculated at the beginning of the experiment (fig. 3).

Looking at the outgrowth of root hairs, spontaneous calcium oscillations appear specifically at their tips, being characterized by a period that ranges between few and tens of seconds.

Are these oscillations related to the growth of the root hairs? We think that LSFM can help to finally answer this question as many other questions related to the function, development and gene expression of plants.

Conclusions
LSFM combines the advantages of different microscopy approaches in a single technique: it provides single cell resolution, typical of confocal laser scanning microscopy and is fast enough to observe systemic responses, characteristic of wide field microscopy. Calcium signaling can be monitored in vivo, at high resolution, with negligible photo-bleaching over a large portion of the root tip, including a large number of root hairs, thus proving statistically relevant information for plant biology studies.

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References
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