Imaging Calcium Oscillations in Arabidopsis Root Hairs with Light-Sheet Fluorescence Microscopy

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Abstract: The employment of light-sheet fluorescence microscopy together with the development of customized software for image registration enables high-resolution imaging and fast, reliable analysis of calcium oscillations in root hairs of *Arabidopsis thaliana* plants.

1. Introduction

Understanding gene function in plant growth helps identify traits that enhance robustness and promote faster development. Tip growth is a phenomenon which regulates the development of both root hairs and pollen tubes. It is characterized by a localized oscillatory tip calcium gradient, influenced by genetic and environmental factors. Prior studies have linked these oscillations to healthy root hair development [1]. Using fluorescent calcium indicators combined with fluorescence microscopy techniques, we can visualize and analyze calcium dynamics in different genetic backgrounds. Traditional microscopy methods have been applied to *Arabidopsis thaliana* for studying calcium dynamics, but they face challenges such as limited sectioning in wide-field microscopy, high photodamage, stress-induced root hair bursting due to horizontal mounting, and slow acquisition rates in confocal and multiphoton microscopy. To overcome these limitations, we adopted light sheet fluorescence microscopy (LSFM) and developed a protocol specifically designed for plant studies [2]. LSFM minimizes photodamage by illuminating a thin sample plane with a laser light sheet while capturing fluorescence orthogonally. This setup allows seedlings to remain vertically oriented in near-physiological conditions, with root hairs growing freely in 3D, enabling the visualization of natural growth patterns with minimal disturbance [3]. Here we present the implementation of this technique with a focus on the open-access *napari-roi-registration* software, which facilitates and accelerates the image analysis pipeline.

2. Materials and method

2.1. Sample preparation and mounting

Two sets of *Arabidopsis thaliana* specimens were measured. The first set represented the control group, also called wild type (WT), consisting of specimens with an unmodified genetic background. The second set consisted of Arabidopsis mutant seedlings with a specific gene knocked out (KO). Both sets were engineered to express the intensiometric genetically encoded calcium indicator (GECI) R-GECO1, which is excited at 561 nm and emits fluorescence at red wavelengths (575-625 nm). Samples were grown in fluorinated ethylene propylene (FEP) tubes filled with a gelled medium (Gelrite™ at 0.5% (v/v)) and positioned vertically inside a pipette box filled with a hydroponic solution for seven days [2]. This protocol allowed the plant's root to grow freely inside the tube under near-physiological conditions. Finally, during imaging, the tubes were transferred to an imaging chamber filled with the same hydroponic medium and positioned vertically to ensure minimal disturbance to the root hairs.

2.2. Light-sheet fluorescence microscope

The setup consisted of a single-side illumination LSFM, where a light sheet is generated by focusing the light from a single-mode fiber-coupled laser emitting at 561 nm through a cylindrical lens. A 10X water-dipping objective (UMPLFLN 10XW, Olympus) projects a 4.8 µm-thick light sheet onto the sample, while fluorescence signal is collected by a second objective (UMPLFLN 10XW Olympus) held orthogonally to the first one. In this way, only the volume that is observed by the detection unit is excited, providing sectioning capability and

limited phototoxicity. For detection, we used a fast sCMOS camera (Neo 5.5, ANDOR) with a large field of view (1656 × 1404 µm²), positioned immediately after a tube lens (UTLU-1-2, Olympus). A longpass emission filter blocks excitation light while allowing fluorescence detection.

Image acquisition is synchronized with a translation stage that moves the sample through the light sheet, enabling multi-plane imaging of the root at varying depths. A total volume of 45 µm is scanned by sampling every 3 µm. A volume stack is acquired every 3 seconds for a total acquisition time of 13.5 minutes. In each acquisition, we captured an average of five root hairs across a population of at least 10 plants per genotype.

2.3. Development of the open-access software napari-roi-registration for fast and easy analysis of time-lapse data

To reduce data volume, we save only the maximum intensity projection (MIP) of the ratio stack for each time point, creating a time-lapse dataset. Before extracting the fluorescent signal of interest from the tips of root hairs, a preliminary step is required. Since root hairs continue to grow during acquisition, their position in the field of view changes over time (Fig. 1A). Therefore, before extracting intensity signals, root hair tips must first be identified and re-aligned in each frame of the time-lapse. Given the large number of root hairs to analyze, performing this process one tip at a time would be extremely time-consuming.

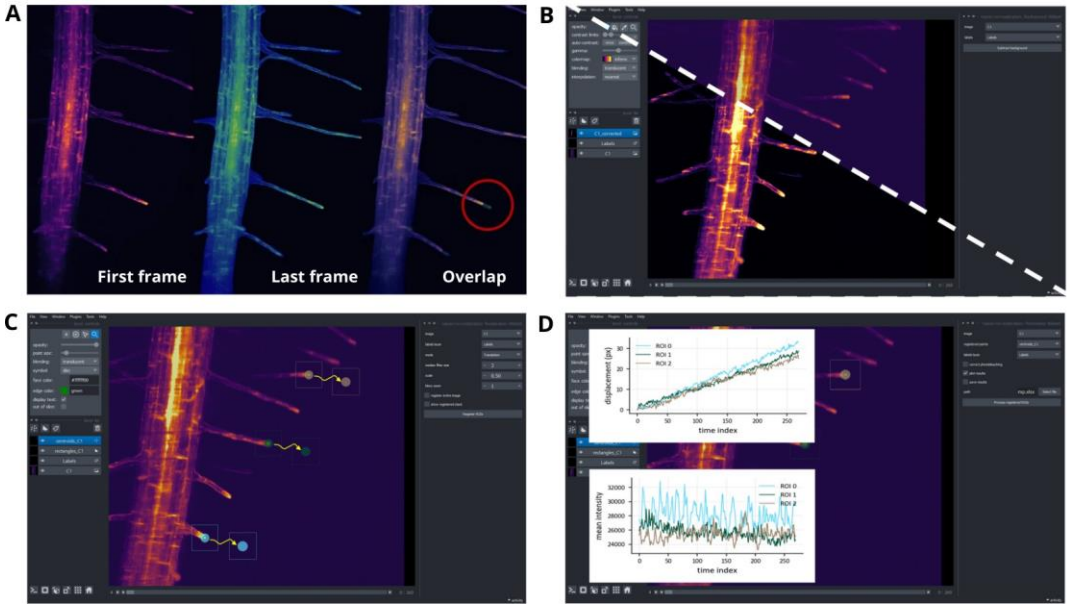

Fig. 1: Napari software. A) Frame comparrison. Overlapping the first and the last frame it is possible to visualize that the root hair grew and changed position in the FOV. B) Background Widget. Top right corner shows the image before the background subtraction, while bottom left corner shows the processed image. C) Registration Widget. Image after registration showing the registered paths and bounding boxes. D) Processing Widget. Plots obtained at the end of the processing step showing the extracted intensities and displacements of the registered ROIs.

To overcome this limitation, we developed a new tool for fast and efficient simultaneous registration of an unlimited number of user-defined regions of interest (ROIs). Specifically, we created an open-access, user-friendly plugin called *napari-roi-registration* [4] for napari, a multi-dimensional image viewer for Python designed for visualizing and analyzing large datasets which is organized into layers, offering various types (e.g., image, labels, shapes, points) to facilitate user interaction with images.

napari-roi-registration consists of three widgets that enable complete pre-processing of data to extract relevant information from acquired images. The first widget (Background widget) allows for background correction, which is particularly useful when ambient light affects measurements. The user creates a labels layer and manually draws a label on one frame of the dataset, selecting a region from which to calculate background intensity. The background is computed as the mean intensity of pixels under the drawn label. Pressing the ”Remove background” button automatically removes the background from all frames, generating a corrected image that is added to the viewer (Fig. 1B). The second widget (Registration widget) is the core of the plugin and enables ROI registration across all frames. The user creates a new labels layer and selects areas of interest by drawing labels. There is no limit to the number of labels that can be created. Each label color represents a different ROI, which is registered separately but simultaneously with the others. This allows for the identification of all root hair tips in a single step, significantly reducing pre-processing time (Fig. 1C). Finally, the Processing widget extracts information from the registered ROIs. Specifically, it quantifies intensity and displacement data (Fig. 1D), which can be saved in an Excel file for further analysis.

3. Results and discussion

We wanted to analyze calcium oscillations in WT plants and KO plants for a gene whose absence affects root hair development. To assess whether the mutant's oscillations remained periodic, we first computed the autocorrelation of the signal (Fig. 2A and Fig. 2B). Our analysis confirmed that the mutant still exhibited periodic tip cytosolic calcium oscillations. Additionally, we observed that in the mutant, the autocorrelation signal shape varied depending on root hair length (Fig. 2B).

To further analyze variability and perform statistical comparisons of oscillation frequencies between wild-type and mutant plants, we transitioned to the Fourier domain. A statistically significant difference in the Power Spectral Density (PSD) between the WT and the mutant was found (Fig. 2C). The high-frequency oscillation band characteristic of the WT is still visible in the mutant's PSD, even if slightly shifted. In addition, a low-frequency band appears, supporting the hypothesis of a disruption in calcium dynamics in the mutant as a consequence of the gene mutation.

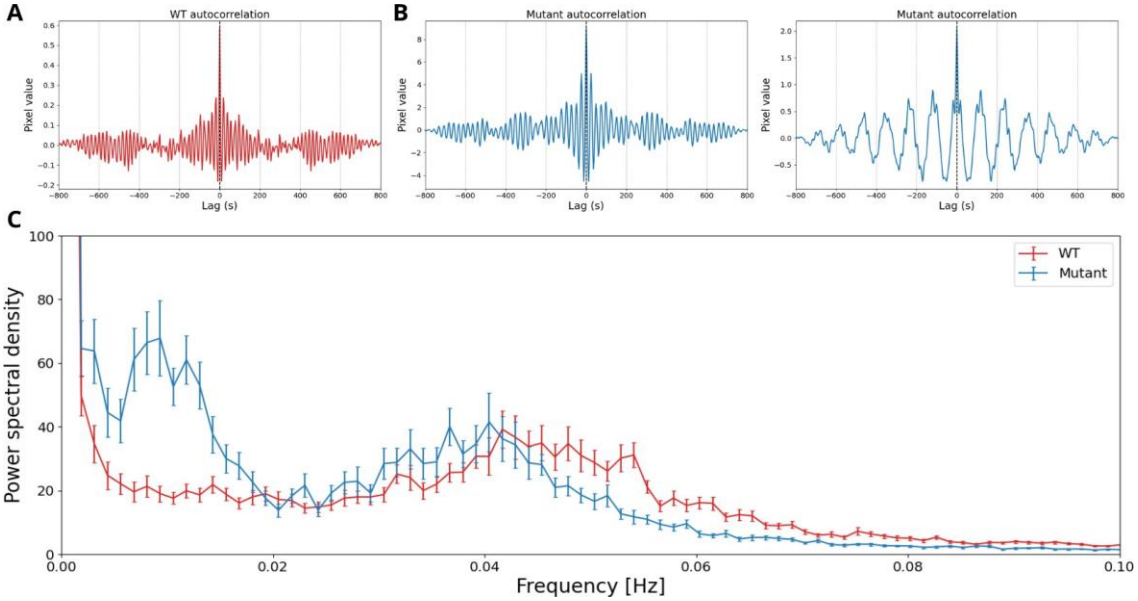

Fig. 2: A) Typical root hair calcium signal autocorrelation of a wild type seedling. B) Examples of calcium signal autocorrelation from root hairs with different length of a mutant seedling. C) Power Spectral Density of the average of all the calcium signals obtained from root hairs of wild type (WT) and mutant plants with standard deviation ($n_{WT} = 105$, $n_{Mut} = 79$).

In conclusion, the employment of LSFM in combination with the developed napari-roi-registration software enabled the precise visualization and extraction of calcium signals at the root hair tip, revealing their oscillatory dynamics over time. Fourier analysis identified a dominant high-frequency oscillation band in wild-type plants, while mutant plants, where the target gene was knocked out, exhibited a disrupted calcium dynamics. A lower frequency band appeared while retaining the high-frequency component (Fig. 2C). This suggests that the knocked- out gene plays a crucial role in the regulation of cytosolic calcium oscillations in root hairs. These results underscore LSFM as a powerful tool for studying gene function in plants, particularly for identifying key regulators of growth and development.

4. Acknowledgements

We acknowledge financial support by the Chan Zuckerberg Initiative for the project "Registration of Regions of Interest in Time-Lapse Data" supported by the napari Ecosystem Grants.

5. References

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