Imaging and quantifying root behavior in their natural orientation using a vertical stage confocal microscope

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Introduction:

Plants are sessile organisms that are rooted in the soil. As such, they cannot physically move to find favorable growing conditions or escape adverse environmental factors such as abiotic stress, pathogen attack, or herbivory. Instead, plants evolved a different strategy by orienting their growth toward or away from specific stimuli. This oriented growth is called tropism and it comes in two flavors, positive tropism (growth toward a given stimulus) and negative tropism (growth away from a given stimulus) (1). For example, plant shoots are attracted by light, a type of positive tropism called phototropism. By contrast, high ionic stresses (i.e. salt stress) are detrimental to plant roots. As such, they trigger negative tropism away from the salt source. A very important tropism, which defines the architecture of plants, is called gravitropism. Gravitropism is the orientation of plant organs according to the gravity vector. Plant shoots have a negative gravitropism as they grow upward against the gravity vector. Plant roots grow aligned with gravity and thus have a positive gravitropism response.

Most microscopes have either an upright or inverted geometry, but in both cases, their stages are horizontal. This is problematic when analyzing plant growth since plant organs will respond to the gravity stimulus during any live imaging study (2). In this technical note, we describe how we can use a vertically mounted confocal microscope to study root growth in its natural orientation and analyze how roots respond to changes in gravity. In particular, we present techniques to study root growth and responses at the whole organ scale as well as at the cellular scale.

Quantifying root gravitropic angles typically involves growing seedlings in agar medium in plates and imaging them with an office scanner. While this method can detect strong gravitropic defects, it lacks the spatiotemporal resolution needed to identify subtle or delayed phenotypes. Vertical-stage microscopy addresses this limitation by providing high spatiotemporal resolution and fluorescence imaging capabilities. For instance, the auxin cytosolic receptor AFB1 mutant shows no visible phenotype in scanned images but exhibits a 10-minute delay in root gravitropic response. This delay highlights AFB1's role in the rapid auxin response, a phytohormone essential for root bending (3,4).

Experimental procedure:

In all the experiments, the *Arabidopsis thaliana* accession Columbia-0 (Col-0) was used as wild type. Seeds were surface sterilized using chlorine gas, vernalized at 4°C for 2 days and grown on solid half-strength Murashige and Skoog media supplemented with 1% sucrose and xmM MES buffer at pH 5.8 for 5 days at 21°C in a 16-h light/8-h dark cycle with 70% relative humidity and a light intensity of 200 µmol photons.$m^{-2}.s^{-1}$ prior experiment. Recently harvested, synchronized seeds were used. Five-day-old roots were imaged with a vertically mounted (see

results) LSM 980 Axio Observer 7 (Carl Zeiss group, http://www.zeiss.com/) equipped with an AiryScan 2 module, GaAsP-PMT detectors. A 10x objective (EC Plan-Neofluar 10x/0.3, serial number 420340-9901-000) was used for brightfield gravitropic imaging and a 20x objective (Plan-Apochromat 20x/0.8, serial number 420650-9903-000) for confocal imaging.

Results:

- **Vertically mounted LSM980**

An LSM 980 confocal microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) has been modified in factory before its installation. The microscope body is mounted on a vertical breadboard with a 90° rotation so that the motorized microscope stage ends in a vertical direction (**Figure 1**). However, the confocal scan head is not turned and has the same orientation as in a conventional set-up with an upright or inverted microscope, which allows fewer motorizations to be reoriented. In this way, the confocal head retains all its functionality and all the options of this model are available, as on a normal set-up. Specifically, this microscope is equipped with a spectral emission detector and an Airyscan 2 module, which provides improved resolution and signal-to-noise ratio. To accommodate this relative rotation between the microscope body and the scan head, the optical coupler and control software (Zen 3, Carl Zeiss) must be modified. Another noticeable change is the absence of eyepieces: as they would have been inaccessible to users, they have been replaced by a monochrome camera (Axiocam 503 mono), which can be used to locate the samples before imaging in confocal mode but also to take images in widefield mode, in epifluorescence or transmission. Lastly, the transmission arm cannot be tilted to load the samples on the stage but it is present with all the available options that the condenser can offer in the transmission mode. A multicolor LED light source (Colibri 7 model) is used for epifluorescence excitation.

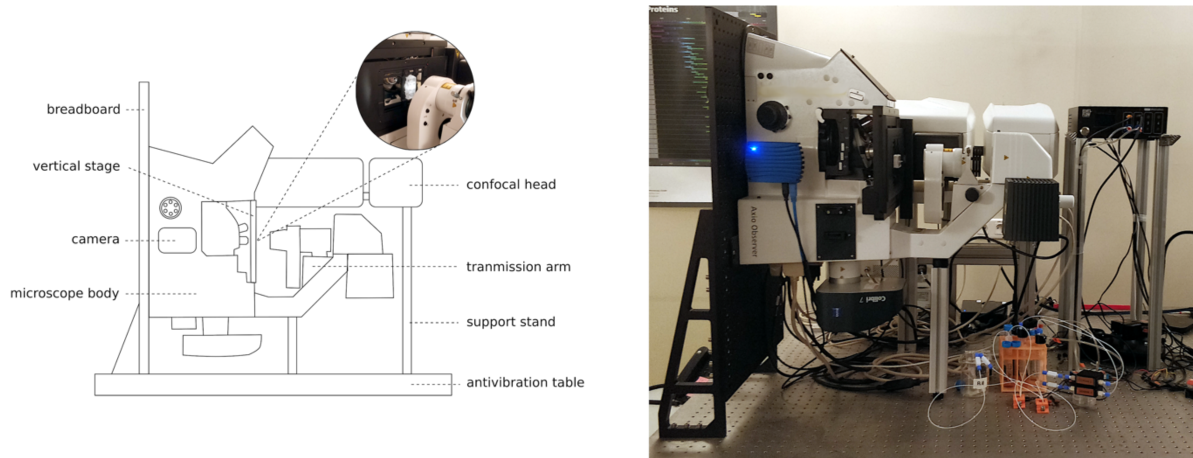

***Figure 1**: **Presentation of the vertical stage confocal microscope.** Schematic representation (left) and picture (right) of the vertical stage confocal microscope used in this technical note.*

- **Mounting seedlings for gravitropic imaging in brightfield**

A typical way of mounting *Arabidopsis thaliana* seedlings for microscopy is in liquid medium or water between a glass slide and coverslip. Another method is mounting the seedlings in a microscopy chamber between agar medium and coverslip. These mounting methods are suitable for most applications. However, having the root compressed between two materials is a source of mechanical stress which can perturb and/or slow down the establishment of the root gravitropic response. In the case of root bending angle measurements, only brightfield images are needed and thus, to overcome mechanical stress a new mounting method has been developed (5). In this method, the seedlings are mounted on top of a thin solidified agar layer in a microscopy chamber (e.g. Nunc or diy chambers). Imaging through agar medium decreases the focus quality and does not allow for fluorescence imaging. Nevertheless, simple brightfield widefield imaging does provide the necessary data to quantify root bending angle over time.
A thin layer (approx. 2mm) of non-solidified ½ MS, 1% sucrose, pH 5.8 MES buffered is deposited in a microscopy chamber. After solidification of the medium, 5-day-old seedlings are gently transferred on the medium with a sliding movement from tip to cotyledons to straighten the root. The chamber is then tape closed to maintain humidity and hold the microscopy chamber lid (**Figure 2**).

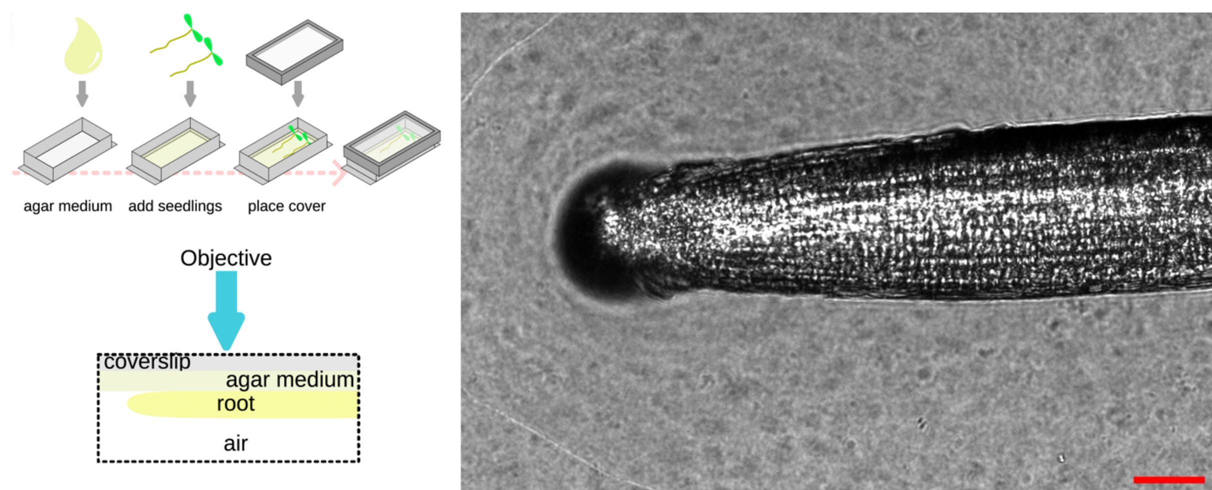

***Figure 2**: **Mounting seedlings for gravitropic imaging in brightfield through agar medium**. Left schematic representation of the mounting strategy and on the right bright field image of the root. Scale bar = 50 µm.*

- **Brightfield/Widefield imaging and quantification of the root gravitropic bending**

After allowing the seedlings to recover from the transfer vertically (minimum 30 minutes, see troubleshooting), the chamber is placed with a rotation of 90° on the microscope stage to induce a gravistimulation. Root tip positions are registered using the widefield camera, added in the “Tiles” window of Zen, and imaging is started using a brightfield setup such as the one presented in **Figure 3**. Diminishing the speed and acceleration of the stage motors to 10% allows to decrease unwanted acceleration/gravitropic stimulation. Following the same precaution, we recommend taking an image of each position every 2 minutes and limiting the

number of seedlings to a maximum of 6. It will also help reduce the position registering time and thus, start the imaging as soon as possible after gravistimulation. The time associated with stage position registration is measured to have the actual starting time of the imaging after gravistimulation. Images are typically recorded for about 40 minutes, a duration enough to detect small differences in the gravitropic response (4,5).

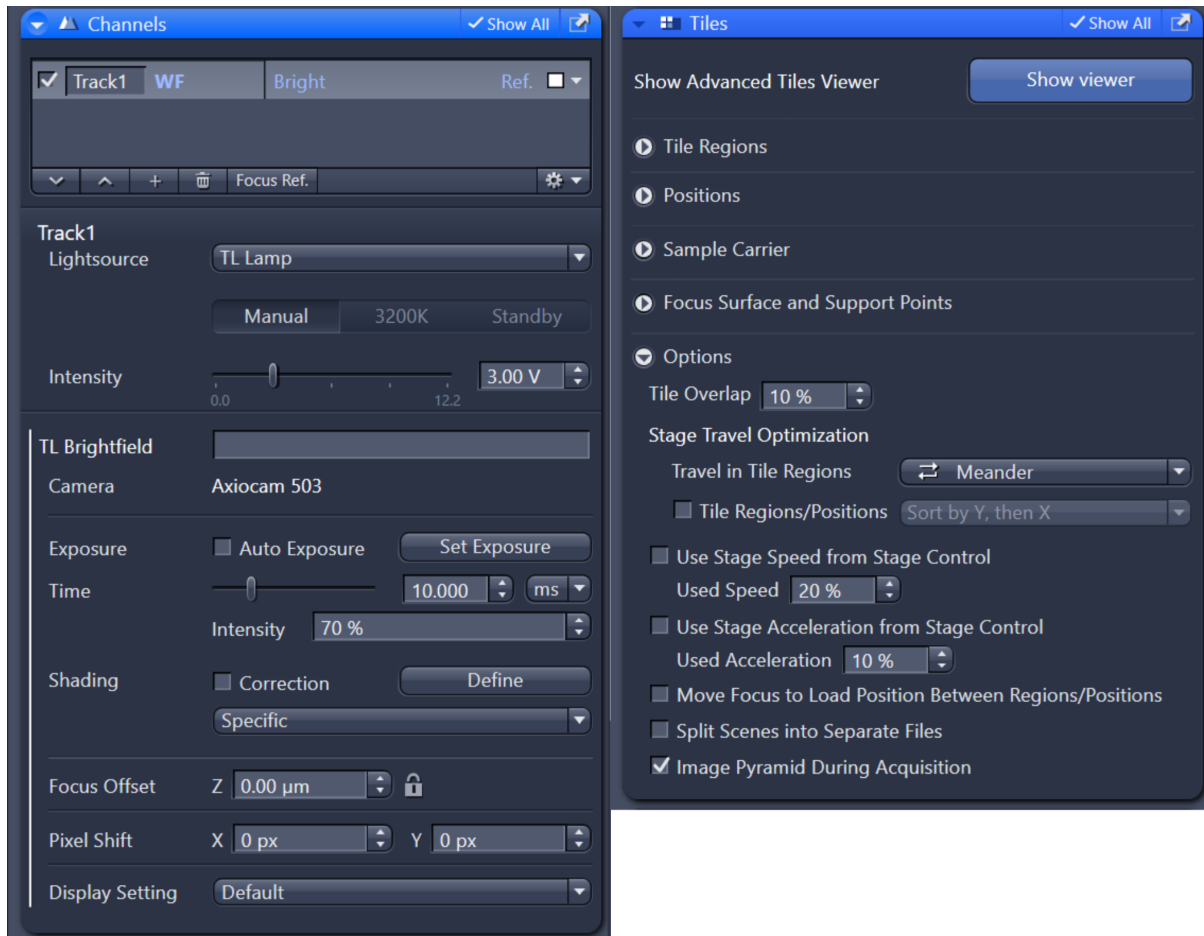

Figure 3: Example of light and stage settings for brightfield widefield gravitropic bending imaging.

Once the movies are acquired, they can be processed either manually using ImageJ/FIJI (https://imagej.net/) or using an automated software specifically developed for measuring root bending angle automatically, the ACORBA software. Use of the software is out of scope of this application note. However, all documentation can be found in the original publication (5) and in the dedicated repository (https://sourceforge.net/projects/acorba/). This software uses deep machine learning for image segmentation and has been trained on both images acquired through agar or between coverslip and agar medium (method described below). Quantifications allow quantifying small (<5°) and early bending angles as soon as 10 min after gravi-stimulation (**Figure 4 and movie 1**).

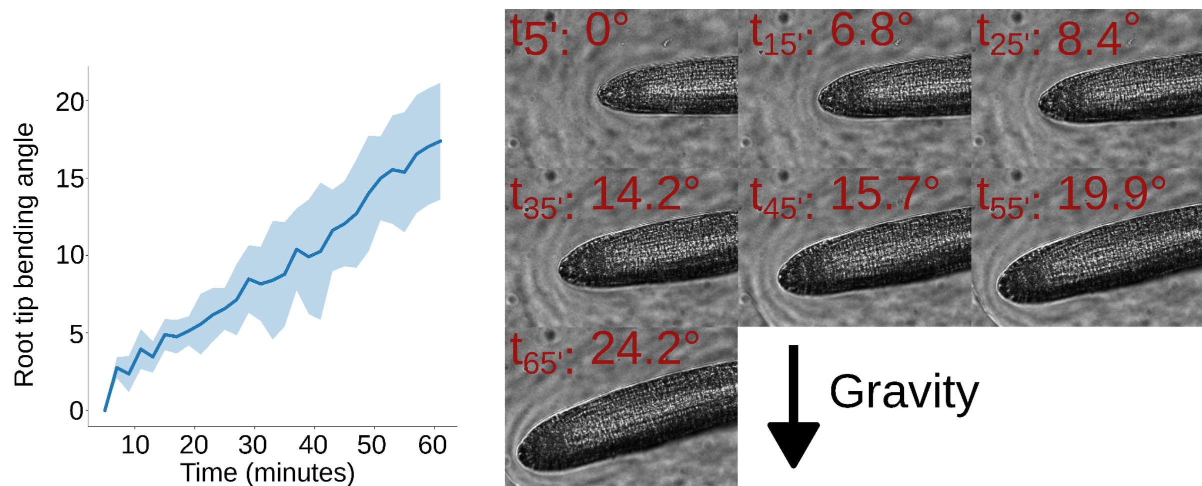

Figure 4: Analysis of root growth orientation in the first 60 minutes following gravistimulation. *Arabidopsis thaliana wild-type Col-0 primary root tip bending towards gravity after a 90° gravi-stimulation for 1 hour on a vertical stage microscope. Left panel: quantification of root tip bending angles over time (n = 5 individual seedlings). Right panel: Microscopy image mosaic displaying the root angle over time after gravi-stimulation.*

- **Mounting seedlings for gravitropic fluorescence imaging**

When fluorescence imaging of biosensors is needed, the through agar method described above is not applicable. In this case, a compromise between mechanical stress and imagery is to sandwich the seedling roots between the coverslip of a microscopy chamber and a patch of agar medium (less rigid than a microscope glass slide, **Figure 5**). In the plate where the seedlings grew or in a plate with freshly poured agar medium (approx. 5 mm thick), a rectangular patch of agar medium smaller than the microscopy chamber is cut in place. Then, seedlings are transferred to this patch. Finally, the patch with seedlings is transferred to the chamber (seedlings facing the coverslip). As for the previously described method, the chamber is closed with its lid taped and seedlings are left to recover vertically for at least 30 minutes before imaging.

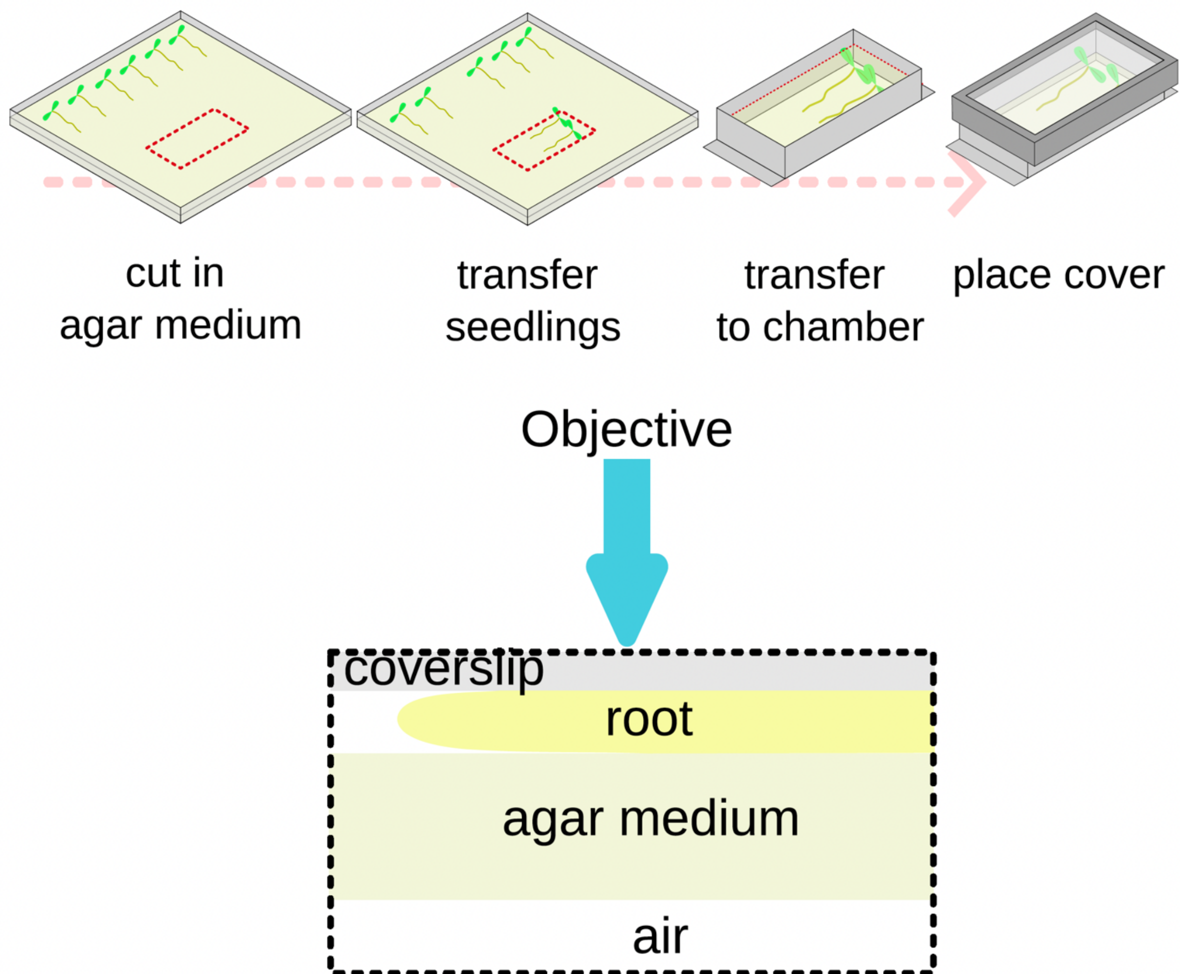

***Figure 5**: Schematic representation of the procedure for mounting seedlings for gravitropic and fluorescence imaging.*

- **Confocal imaging to track cellular growth and responses during gravitropism**

Countless lines expressing genetically encoded biosensors (e.g. Calcium, auxin reporters) or dyes (e.g. pH reporter) can be used in combination with gravi-stimulations to answer biological questions. In this context, the Airyscan Multiplex mode of the Zeiss LSM 980 allows fast acquisition with reduced bleaching of fluorescent molecules over large areas (e.g. the whole root tip zones using tiles) and reduced timeframes (here 1 minute at the minimum). These fast acquisitions allowed by the Multiplex mode also open the possibility of multichannel imaging or doing Z stack to observe signals in different tissues, for example.
After the seedlings are transferred to the microscopy chamber using the method described above, the chamber is placed at 90° (for the root) on the microscope stage. If several roots, the positions are recorded and a large tile area is created using the tile viewer.
In the context of this application note, we first chose to illustrate the method using p35S::GFP-Lti6b (6), a plasma membrane genetically encoded reporter to show the shape/length of the cells during the gravitropic response (**Figure 6 and movie 2**).

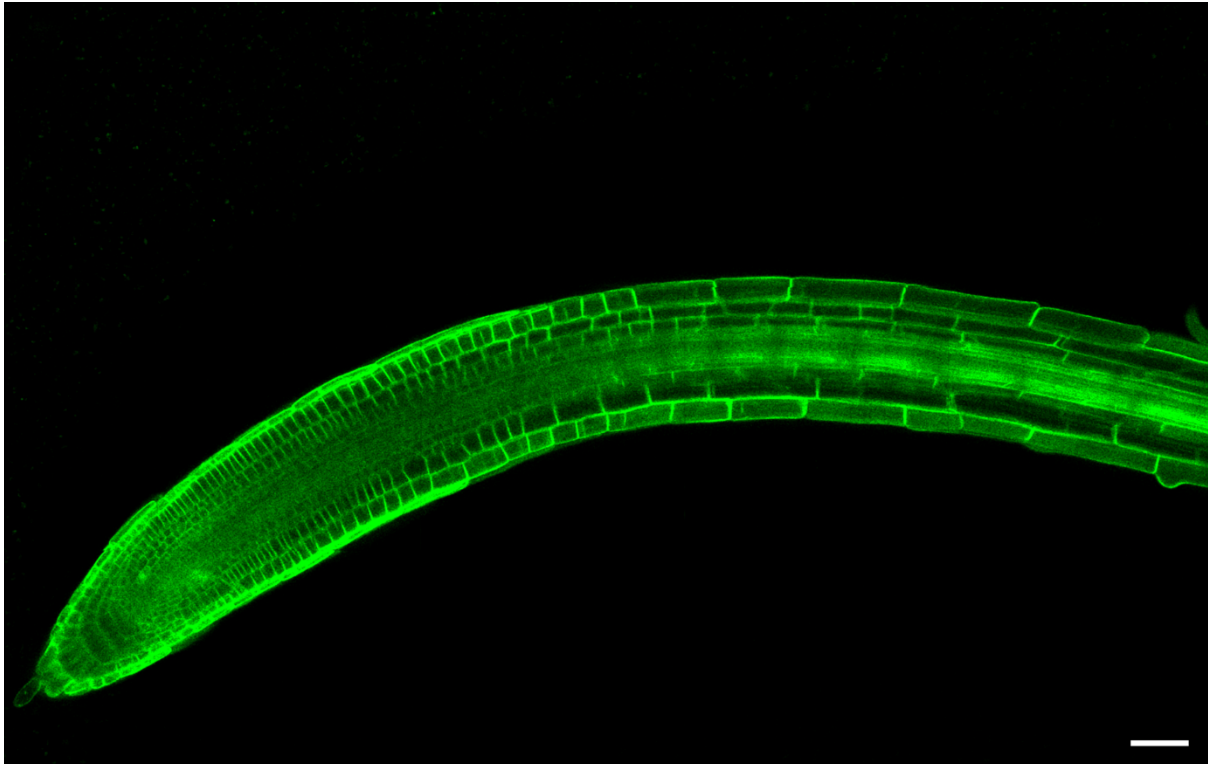

Figure 6: Gravitropic bending observed at the organ and cell scales using the plasma membrane reporter p35S::GFP-Lti6b.

Secondly, we wanted to display and quantify the movement of the hormone auxin during gravitropism using the auxin reporter qDII (7). This biosensor is a ratiometric reporter consisting of an auxin insensitive nuclear marker in blue (TagBFP) and the DII auxin reporter in yellow, which is degraded upon auxin perception. This sensor is nuclear and reports auxin activity. The reporter DII is degraded by auxin and thus, less signal means more auxin activity. During gravitropic bending, the hormone is accumulated in the lower side of the root, inhibiting root elongation and thus, creating a differential of growth between the upper side and the lower side (1). This elongation differential results in the actual bending. Here, we show that qDII monitoring during gravitropic bending can measure relative changes in auxin concentration (between the upper and lower part of the root) as soon as 5 to 10 minutes following gravistimulation (**Figure 7 and movie 3**), which is in agreement with models of auxin redistribution during gravitropism (8)

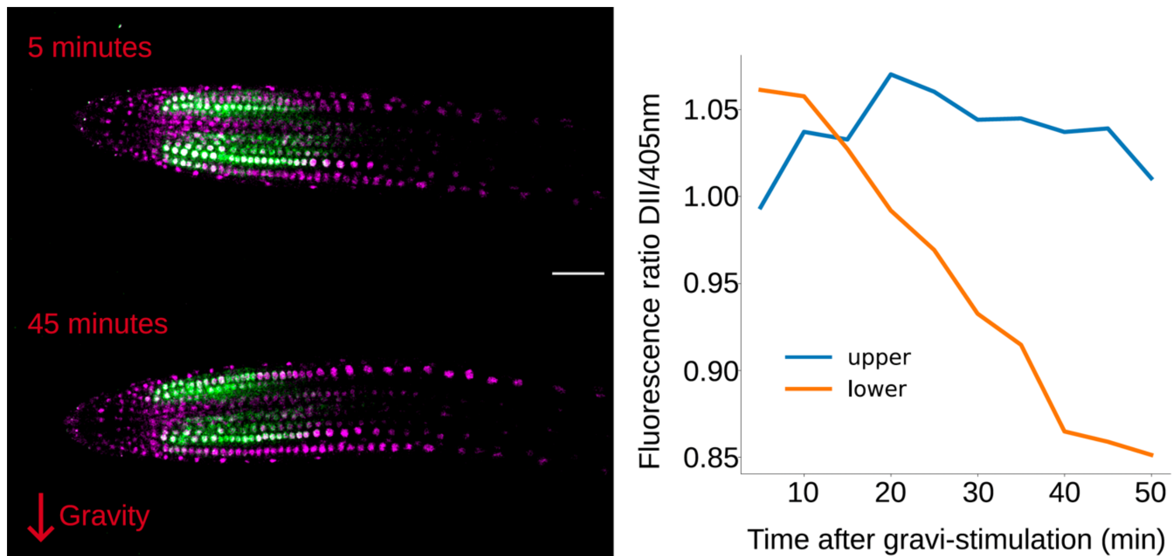

Figure 7: Auxin relocalization in the root tip during the gravitropic bending. *Left panel: qDII reporter line composite images after 5 and 45 minutes after gravi-stimulation. Magenta: TagBFP reference and green: DII auxin activity reporter. Scale bar = 50 µm. Right panel: Quantifications of auxin activity in the upper and lower side of the root meristem. The data represent the ratio of DII signal over TagBFP reference over time.*

- **Imaging root elongation with high spatiotemporal resolution**

Quantifying root elongation with seedlings growing in their natural orientation toward gravity using a vertical stage microscope allows one to measure precisely root elongation rate in µm/min. To demonstrate the kind of experiment that can be carried out with a vertical stage microscope we transferred wild type seedlings to agar medium patches containing or not 100 nM of the phytohormone auxin (IAA) known to rapidly inhibit root growth (9). The seedlings were mounted using the sandwich between agar and coverslip from a microscopy chamber (see above). Here, the seedlings were imaged after 25 minutes of treatment for 30 minutes on which the average elongation rate in µm/min was quantified. This experiment allowed us to demonstrate that adding 100 nM IAA reduced, by a factor 3, the average root elongation (**Figure 8 and movie 4)**

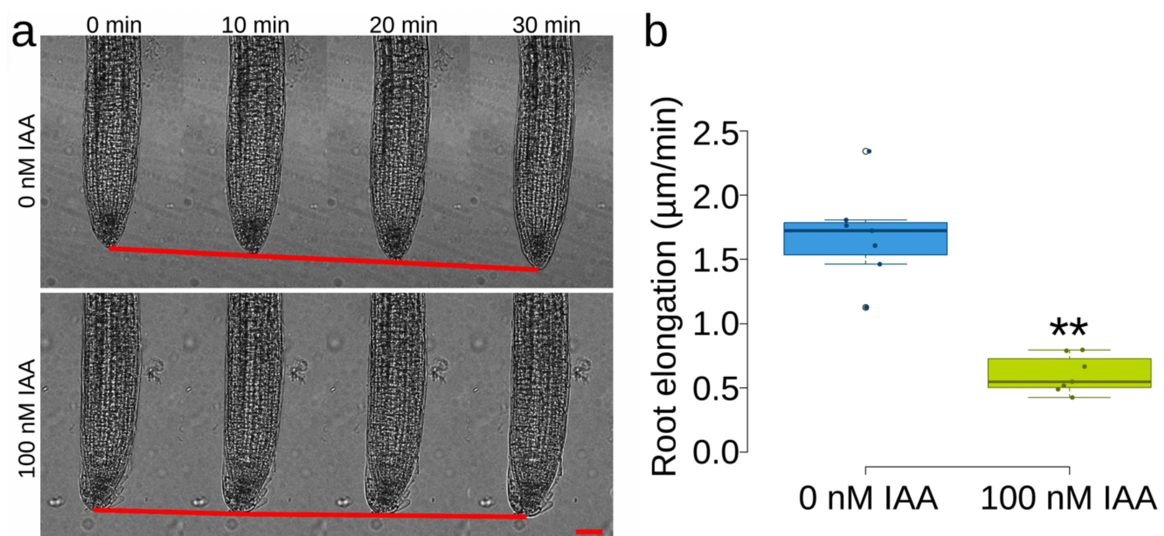

Figure 8: Quantifying the effect of the phytohormone auxin on root growth with high spatiotemporal resolution. *a) Arabidopsis thaliana primary root tip observed for 30 minutes +/- 100 nM IAA. Scale bar = 50 µm. Red line was added connecting the root tips between frames to visualize root elongation b) Quantification of the primary root elongation (µm/min) over 30 minutes of imaging (n=7 individual seedlings per condition). ***: p-value = 0.001.*

- **Observation and quantification of biosensors in seedlings growing in their natural orientation toward gravity**

These vertical observations can also be carried out using fluorescent reporters. Here, we reused the qDII line to demonstrate the disruption of the auxin gradient by the application of external auxin (7,10). Auxin is present in the root as a gradient also called the “inverted fountain” with a maxima in the quiescent center cells (1). Saturating the root with external auxin completely disrupts the root to maintain this gradient as illustrated on **figure 9**. In this experiment, we used propidium iodide (PI), which interact with pectins, to label the cell wall and visualize cell contour. PI is commonly used to label cell contour in the root and reveal root architecture. However, it should be used with caution as it may impact root elongation and gravitropism, depending on the concentration of PI or the media used during the experiment (see troubleshooting).

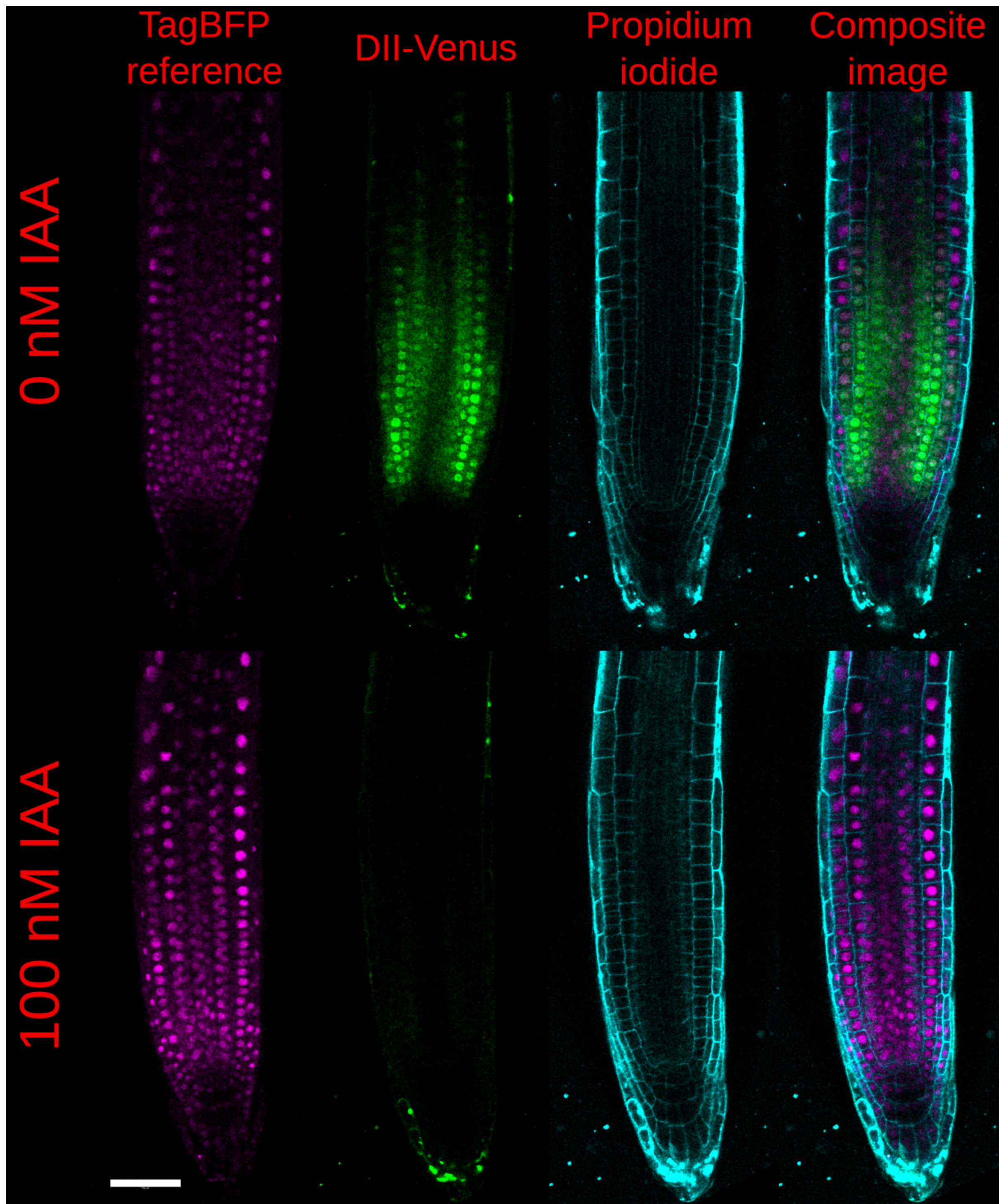

***Figure 9: Demonstration of the auxin gradient disruption by external auxin application**. qDII reporter line and cell wall staining (propidium iodide, PI) composite images after 25 minutes of treatment with or without 100 nM IAA. Magenta: TagBFP reference, green: DII auxin activity reporter and cyan: propidium iodide cell wall staining. Scale bar = 50 µm.*

Discussion and troubleshooting:

*Evaluation of recovery time after root mounting***.** Response to gravity and root elongation are very sensitive responses that can be impaired by other biological responses and/or by overstimulation. In order to limit these biases, seedlings must be transferred as gently as

possible. They should grow as vertically as possible and they should have sufficient time to recover. In general, 30 minutes of recovery time is sufficient for roots to reestablish a normal growth rate and bending capacity. However, this should be adapted to individual microscopy set-up and longer-recovery time may be required. The following criteria can be applied to evaluate root growth recovery:

- a quantifiable bending angle can and should be detected as soon as 5 minutes after gravistimulation
- root elongation of the *Arabidopsis thaliana* wild type Col-0 in control condition should be between 1.5 and 2.5 µm/min minimum.

Limiting variability in gravitopic bending. Gravitropism is influenced by many environmental factors. This is reflected in the variability often observed in gravitropic experiments. If experimental conditions allow for the use of sucrose we recommend using it (e.g. 1% m/v) for faster recovery and bending/elongation. In our experience, the age of the seedlings after germination is influencing the gravitropic response. We recommend using 4-5 days old plants. Agar thickness plays a role in the establishment of the response as a too-thick agar will tend to not bend around the root and thus, trap air. Furthermore, a thicker agar will contain less water. We recommend an agar content of 0.8 to 1% for easy agar patch manipulation and experimental conditions.

Use of propidium iodide to label the cell wall. Cell wall-stains such as propidium iodide (PI) are often used to label the cell contour. They enable the automatic segmentation of individual cells. In addition, PI offers several advantages as it can be applied to live samples and thus is compatible with live imaging. Furthermore, it also labels dead cells differentially and serves as a cell death marker. However, PI is labeling the cell wall by interacting with negatively charged pectins, which may interfere with root growth and the gravitropic response. This effect of PI is likely mediated by stiffening the cell walls. If PI is used in specific experimental setups, we recommend to first evaluate its effect on root growth rate or gravitropic bending.

Conclusion

Vertical stage microscopy opens a world of experimental possibilities and new biological questions to be asked by imaging seedlings in their natural orientation to gravity using different genetic backgrounds and/or reporter lines and/or fluorescent dyes. Furthermore, this experimental setup in combination with microfluidics (https://www.elveflow.com/microfluidic-applications/microfluidic-cell-culture/microfluidic-microscopy-imaging/) to observe the effect of a/biotic treatments in high spatiotemporal resolution has proven to be a powerful set of methods to observe and quantify biological processes *in vivo* (4,9,11). The need to study roots in their natural growing orientation is more and more recognized as a key experimental parameter to study root physiology, development, and interaction with their environment. As such, the use of vertical-staged microscopes is rapidly expanding and is becoming an essential tool for root biologists.

Appendix

The following lines were used, Col-0, 35S:EGFP-Lti6b (NASC ID: N84726 and ABCR ID: CS84726) (6) and qDII (7).

About the authors

Nelson BC Serre is a postdoctoral researcher working in the Cell Signaling and Endomembranes group at the RDP lab of ENS Lyon. His project is focused on quantifying lipid dynamics in cell membranes in response to signaling peptides. To do so, he uses vertical-stage microscopy coupled with microfluidics to image genetically encoded fluorescent lipid biosensors. Contact: nelson.serre@ens-lyon.fr

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