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November 06, 2018

Re: *JoVE* Invitation to Publish

Dear Editor,

I am writing to submit for your consideration the manuscript titled, "Confocal live imaging of shoot apical meristems from different plant species", by Yuan Geng and myself.

In this manuscript, we described in detail protocols used to live image the vegetative shoot apical meristems and inflorescence shoot apical meristems from different plant species, including the model plant Arabidopsis and crops, using laser scanning confocal microscopy. The method that we described in the manuscript will enable researchers to accomplish the whole experimental procedure from sample preparation to high resolution image acquisition within as short as twenty minutes. We also introduce the simple imaging processing method for analyzing and comparing different meristems in 3D.

This method provides the key results in our previous studies, and it potentially can be applied to study the meristem structure and development across many different plant species. We believe this protocol is of board interest in plant cell and developmental biology research fields, and thus, it is suitable for publication in *JoVE*.

We wish to thank you and your selected reviewers in advance for the efforts they expend to assure our work is carefully and expertly reviewed.

Sincerely,

Yun Zhou, Ph.D.
Assistant Professor
Department of Botany and Plant Pathology
Center for Plant Biology

TITLE:

Confocal Live Imaging of Shoot Apical Meristems from Different Plant Species

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KEYWORDS:

Confocal live imaging, shoot apical meristem, SAM, vegetative meristem, inflorescence meristem, tomato, soybean, *Arabidopsis*

SUMMARY:

This protocol presents how to live image and analyze the shoot apical meristems from different plant species using laser scanning confocal microscopy.

ABSTRACT:

The shoot apical meristem (SAM) functions as a conserved stem cell reservoir and it generates almost all aboveground tissues during the postembryonic development. The activity and morphology of SAMs determine important agronomic traits, such as shoot architecture, size and number of reproductive organs, and most importantly, grain yield. Here, we provide a detailed protocol for analyzing both the surface morphology and the internal cellular structure of the living SAMs from different species through laser scanning confocal microscope. The whole procedure from the sample preparation to the acquisition of high resolution three-dimensional (3D) images can be accomplished within as short as 20 minutes. We demonstrate that this protocol is highly efficient for studying not only the inflorescence SAMs of the model species but also the vegetative meristems from different crops, providing a simple but powerful tool to study the organization and development of meristems across different plant species.

INTRODUCTION:

The plant meristem contains a pool of undifferentiated stem cells and continuously sustains the plant organ growth and development[1]. During the postembryonic development, almost all aboveground tissues of a plant are derived from the shoot apical meristem (SAM). In crops, the activity and size of the SAM and its derived floral meristems are tightly associated with many agronomic traits such as shoot architecture, fruit production, and seed yield. For example, in tomato, an enlarged SAM causes an increase in the shoot and inflorescence branching, and thus results in generating extra flower and fruit organs[2]. In maize, an increase in SAM size leads to a

higher seed number and total yield[3,4]. In soybean, the meristem indeterminacy is also closely associated with the shoot architecture and yield[5].

The morphology and anatomy of SAMs can be characterized by several different methods, including histological sectioning/staining and scanning electron microscopy (SEM)[6], both of which have greatly advanced the meristem research through providing either the sectional view or a three-dimensional (3D) surface view of SAMs. However, both methods are time consuming, involving several experimental steps from the sample preparation to the data acquisition, and these methods mainly depend on fixed samples. Recent advances in laser scanning confocal microscopy technique have overcome these limitations and provide us with a powerful tool to investigate the cellular structure and developmental process of plant tissues and organs[7,8]. Through optical rather than physical tissue sectioning, confocal microscopy allows the collection of a series of z-stack images and the subsequent 3D reconstruction of the sample through image analysis software.

Here, we describe an efficient procedure for investigating both inside and surface structures of the living SAMs from different plant species using laser scanning confocal microscopy, which potentially allows researchers to accomplish all the experimental process within as short as 20 minutes. Different from other published methods for live confocal imaging of *Arabidopsis* inflorescence SAMs[9-15] and *Arabidopsis* flowers[12-13], here we demonstrate that this protocol is highly efficient for studying not only the inflorescence meristems of the model species but also the vegetative shoot apical meristems from different crops, such as tomato and soybean. This method does not rely on transgenic fluorescent markers, and potentially can be applied to study the shoot meristems from many different species and cultivars. In addition, we also introduce the simple image processing steps for viewing and analyzing different SAMs in 3D. Taken together, this simple method will facilitate researchers better understanding both the structure and developmental process of the meristems from both model organisms and crops.

PROTOCOL:

1. Media and imaging dishes preparation

1.1 MS plates: add 0.5x Murashige & Skoog MS medium, 1% Agar into deionized water and then adjust pH to 5.8 using potassium hydroxide solution (Optional: Add 1% sucrose for the long-term plant growing). Autoclave and pour plates.

1.2 Imaging dishes: Fill plastic Petri dishes (6 cm wide, 1.5 cm depth) to 0.5-8 cm with 1.5% molten agarose.

2. Plant growth

2.1 *Arabidopsis* growth

2.1.1 Sow sterilized seeds on MS plates and place plates under 4 °C for two days. Then, move

MS plates to a short day (8 h light/ 16 h dark), at 22 °C for two weeks.

2.1.2 Transplant seedlings to soil and grow them in a short day (8 h light/ 16 h dark) at 22 °C for four weeks.

2.1.3 Transfer plants to continuous light, at 22 °C to induce the transition from the vegetative to the reproductive stage and for imaging the inflorescence shoot apical meristems.

2.2 Tomato and soybean growth

2.2.1 Incubate the seeds with wet filter paper under 28 °C till they germinate.

2.2.2 Transplant seedlings to soil and grow them in a long day (16 h light/ 8 h dark) at 25 °C for one week or longer for imaging the vegetative shoot apical meristems.

3. Dissection of the shoot apex

3.1 Dissection of the inflorescence shoot apex

3.1.1 Cut the inflorescence shoot apex together with 1-2 cm main stem from the bolted *Arabidopsis* plants with a razor blade. Hold the basal part of the main stem and remove as many older flower organs as possible from the main stem by dissecting out the peduncles with forceps.

CAUTION: Avoid cutting fingers when using a razor blade. Dispose of the used razor blades to a proper sharps' container.

3.1.2 Hold the attached stem of the shoot apex with forceps or fingers in the field of the stereomicroscope, continue removing the rest of flowers till nearly the whole SAM can be viewed from the eyepieces. Remove peduncles clearly at the junction of the main stem.

3.2 Dissection of the vegetative shoot apex

3.2.1 To view the vegetative SAMs from either tomato or soybean, dissect out the cotyledons, leaves, and roots from the plants.

3.2.2 Hold the hypocotyls of the plants under the stereomicroscope and further dissect out the leaf primordia covering the vegetative SAMs using jewelry forceps.

4. Staining

4.1 To directly visualize cells in SAMs, use freshly prepared propidium iodide (PI) solution to stain cell walls. Dissolve PI powder in sterile, deionized water, make 1mL PI staining solution at the concentration of 1 mg/mL and store the PI solution in microcentrifuge tube covered with aluminum foil.

4.2 Pipette 50 µL PI solution in a clean and empty petri dish and dip the whole dissected shoot apex into dye for 2 min. Rinse the stained shoot apex twice in sterile, deionized water. During the staining process, immerse the whole inflorescence SAM or vegetative SAM into the PI solution to achieve the uniform staining.

5. Image collection

NOTE: For this method, all the SAMs are imaged using an upright confocal microscope and with a 20x water-dipping lens. As described in other protocols[9,12-13,15], it is also feasible to image SAMs using an inverted microscope. In addition, the live imaging can be achieved using different brands of confocal microscopes, with the same sample preparation steps. In this study, the imaging steps are described in detail as an example.

5.1 Pierce a hole at the center of an imaging dish using forceps and stick the stained shoot apex upright in the medium.

5.2 Fill the imaging dish with sterile, deionized water to completely immerse the sample. Viewing from the stereo microscope, pipette up and down to remove air bubbles trapped around the meristem. Then, adjust the angle of the stem in the agar to make sure that the SAM is fully visible from directly above.

5.3 Place the imaging dish on the sample stage of the confocal microscope. Lower water-dipping lens and raise the microscope sample stage to let the tip of lens dip into the water.

5.4 Open the confocal microscope software and locate the SAM in the brightfield in the eyepieces. Move the SAM sample right below the objective lens through adjusting the XY controller, then focus on the SAM from eyepieces through cautiously adjusting the Z controller.

5.5 Operate the acquisition function in the confocal microscope software (see **Table of Materials**), start the **Live mode** to view the sample from the computer screen, and set up all parameters for the laser scanning experiment.

5.5.1 When adjusting the parameters, use **Range Indicator** function to define whether the signal is saturated or not.

5.5.2 Optionally, apply the **Reuse** function to reload all the parameter settings from the selected confocal file.

NOTE: Suggested imaging parameters: Laser line (excitation): 515nm or 561nm; Emission 570-650nm; pinhole: 1 airy unit (AU); Gain: 600-750, scan mode: Frame; Frame size: either 512 X 512 or 1024 X 1024; scanning speed: from 7 to Max; Scanning direction: bi-direction; Averaging number: 2-4; Averaging method: mean; Bit depth: 16 Bit; Scanning Interval: 0.5-1 µM. Further, optimize all these parameters based on the nature of different plant samples and the specific

imaging needs.

6. Image processing

6.1 For visualizing optical orthogonal and transverse section views, use the same commercial software for the imaging acquisition. Open the original confocal file, click ortho menu, select ortho. Then select either x position, y position and z position of the image, and save the images as the tiff files.

6.1.1 Optionally select **3D distance** function to define the physical distance between two points that have been selected from the stack of the confocal images.

6.1.2 Alternatively, use Fiji/Image J, the open resource image processing package to visualize the orthogonal and transverse section views.

6.1.3 Open the original confocal file with Fiji, click image menu, select **Stacks** and then select **Orthogonal views**.

6.1.4 Select **XY, YZ**, and **XZ** planes in the middle position and save as Tiff format images.

6.2 For visualizing a 3D transparent projection, use the same software.

6.2.1 Open the original confocal file, click **3D menu**, and select **Transparent** to generate a 3D projection view.

6.2.2 Optionally click **3D menu**, select **Appearance**, and then select **Transparency** to adjust three parameters of the projection including **Threshold**, **Ramp** and **Maximum** for the transparency of the 3D image.

6.2.3 Click **3D menu**, select **Appearance,** and select **Light** to adjust the brightness of the 3D image.

6.2.4 Export the projected images and save them as the Tiff files.

6.3 For visualizing a 3D maximum intensity projection, open the confocal files with the same software, and click the **3D menu**.

6.3.1 Select **3D menu** and select **Maximum**.

6.3.2 Alternatively, use Fiji/Image J to visualize a 3D maximum intensity projection.

6.3.3 Open the original confocal file with Fiji, click **Image menu** and select **stacks**.

6.3.4 Select **3D project** and save as Tiff format images.

6.4 For visualizing the depth coding view of the 3D images, use the same software.

6.4.1 Click **3D menu** and select **Appearance**.

6.4.2 Select **Special** and select **Depth Coding**.

6.4.3 Alternatively, use Fiji/Image J to visualize the depth coding view.

6.4.4 Open the confocal files, click **image menu**, select **Hyperstack**.

6.4.5 Select **Temporal-Color code** and save as Tiff format images.

NOTE: The depth coding z-stack also can be achieved through the plugin *Z Code Stack* for Fiji.

6.5 For visualizing the 3D rotation view as shown (**Movie 1** and **Movie 2**), use the same software (See the **Table of Materials**).

6.5.1 Open the confocal files, click **3D menu**, and select **Series**.

6.5.2 Select **Render series**, and select one of the four options including **Turn around x**, **Turn around y**, **Start and end**, and **Position list**.

6.5.3 Save the render series as the AVI files.

6.5.4 Alternatively, use Fiji/Image J to visualize the 3D rotation view.

6.5.5 Open the original confocal file with Fiji, click **Image menu**, and select **Stacks**.

6.5.6 Select **3D project** and save as AVI format videos.

REPRESENTATIVE RESULTS:

To evaluate the efficiency of our protocol and to explore the morphology of the meristems from different species, we have performed the confocal live imaging experiments on the inflorescence meristem from *Arabidopsis* and the vegetative meristems from both tomato and soybean. In this study, *Arabidopsis* ecotype Landsberg *erecta*, tomato cultivar Micro-Tom and soybean cultivar Williams 82 have been used as examples.

Seen from the orthogonal section through the middle of the *Arabidopsis* SAM, it is clear that PI was able to stain the horizontal walls from almost all the cells at the multiple cell layers (**Figure 1A**). Shown from one transverse section through the corpus of the inflorescence SAM, the cells from the XY plane are also clearly imaged (**Figure 1B**). In the 3D projection view, the inflorescence meristem forms a dome like structure and is surrounded by the developing flower primordia, where the cells are also stained and imaged (**Figure 1 C-D**) (**Movie 1**).

Seen from the orthogonal section through the middle of the tomato SAM, it is clear that PI was able to stain the horizontal walls from cells at the multiple cell layers, although the PI signal from the deep interior region is slightly lower. From one transverse section through the deep layers of the vegetative SAM, the cells from the XY planes are also clearly imaged, and the boundary formed between the vegetative meristem and leaf primordia is also imaged (**Figure 2B**). The 3D project view can further provide a comprehensive view of the shape and organization of the vegetative meristem from tomato (**Figure 2C-D**) (**Movie 2**).

In the orthogonal view through the middle of the soybean SAM, we can see the dome-like vegetative meristem and its derived new leaf primordia (**Figure 3A**). In the 3D projection view, both the soybean vegetative meristem and the tomato vegetative meristem form the dome like structure, however, the shape of the soybean vegetative meristem is different from that of the tomato meristem, and the organization and patterns of the leaf primordia surrounding these two SAMs are distinct (**Figure 3B-C**).

FIGURE AND TABLE LEGENDS:

Figure 1: Live imaging and analyzing the inflorescence SAM of *Arabidopsis*. A and **B**. optical orthogonal (Orth) and transverse (Trans) section views of middle plane of the same SAM, PI stain (purple). **C**. a 3D projection of the same SAM, PI stain (gray). **D**. depth color coding of the 3D projection, with blue indicating the top surface layer and red representing the deepest layer. Cell walls were stained with propidium iodide (PI). Scale bars: 20 μm (**A, B**); 50 μm (**C, D**).

Figure 2: Live imaging and analyzing the vegetative SAM of tomato. A and **B**. optical orthogonal (Orth) and transverse (Trans) section views of middle plane of the same SAM. **C**. a 3D projection of the same SAM. **D**. depth color coding of the 3D projection, with blue indicating the top surface layer and red representing the deepest layer. Cell walls were stained with propidium iodide (PI). Scale bars: 20 μm (**A, B**); 50 μm (**C, D**).

Figure 3: Live imaging and analyzing the vegetative SAM of soybean. A. an optical orthogonal (Orth) section view of the middle plane of the vegetative SAM. **B**. a 3D projection of the same SAM. **C**. depth color coding of the 3D projection, with blue indicating the top surface layer and red representing the deepest layer. Cell walls were stained with propidium iodide (PI). Scale bars: 20 μm (**A**); 50 μm (**B, C**). Arrow: leaf primordium.

Video 1: 3D rotation of the *Arabidopsis* inflorescence meristem in **Figure 1**.

Video 2: 3D rotation of the tomato vegetative meristem in **Figure 2**.

DISCUSSION:

Here, we describe a simple imaging method that can be applied to the study of shoot apical meristems from different plants with minor modification, opening a new avenue to study the meristem regulation at both vegetative and reproductive stages in model plants and crops. In contrast to the SEM and histological staining methods, this protocol can help reveal both surface

view and internal cellular structures of the SAMs, without the need for labor-intensive sample fixation and/or tissue sectioning steps. This protocol is also compatible to the established imaging method for the fluorescence based reporters in the SAMs, potentially providing a good cellular resolution which allows us to obtain a 3D view of expression patterns of key genes and proteins in the SAM[16-19]. In addition, the associated image processing procedure described here will be able to greatly help researchers analyze and compare the SAMs from different species in 3D, advancing both evolutionary-developmental biology studies and agricultural research.

There are a few critical steps in this simple protocol. First, sample preparation and dissection. A plant SAM is usually hidden by the developing primordia and young organs at the shoot tip, and it cannot be directly imaged under a confocal microscope. To image the vegetative SAM, it is necessary to remove all the leaves and older leaf primordia that cover on top of the SAM using fine jewelry forceps. To image the inflorescence SAM, it is necessary to carefully remove all the young flowers to expose the SAM, also using fine jewelry forceps.

Second, the live cell staining using the PI solution. In this protocol, we use freshly prepared PI solution (1 mg/mL) to stain cell walls to directly visualize live SAMs, which is fast, efficient, and easy to perform. Although the PI solution can be stored at 4 °C for several weeks, a freshly prepared solution usually gives the best staining result for the shoot apical meristems. Although PI mainly does not cross the membrane of living cells and it stains intact cells with the clear cellular outline, it can easily penetrate the damaged/dead cells and strongly stain the nuclei and other internal membrane systems in those damaged areas, potentially affecting the quality of the confocal images. Thus, it will be essential to avoid any physical damages while preparing the SAM samples for the staining and live imaging. On the other hand, PI at a high concentration shows a toxic effect to plant cells, and thus, FM4-64 can be used as a substitute for PI to stain the living SAM cells[9]. However, FM4-64 labels plasma membrane, which may potentially be taken up into cell interior through endocytosis, making it challenging to stain the samples with high cellular resolution.

Third, imaging acquisition. 1). High numerical apertures (NA), the water-dipping lens is critical for the live imaging of the SAMs. Water has a higher index of refraction than air and the high NA of the objective lens helps collect signal photons scattered through deep cell layers of SAMs. 2). The settings of laser power (watts or watts per surface) can be highly variable between different confocal microscopes. Higher laser power may allow better signal collection but leads to more photodamage/ photobleaching on the samples. There are trade-offs between resolution, bleaching and imaging time. In general, selecting a larger frame size leads to better XY resolution but more imaging time, and selecting a smaller scanning interval leads to a better Z resolution but also more imaging time. Longer imaging time potentially causes more photodamage/ photobleaching

With this method, sometimes it may not be easy to observe cellular details in deeper tissue layers, likely due to the limitation of the confocal detection and the inefficient PI staining in the internal tissues. Based on our experiences, increasing the concentration of PI solution and/or the staining time can help get a better stain. Alternatively, the modifications to the staining

procedure can be made. For example, the modified pseudo-Schiff propidium iodide (mPS-PI) method works well for the fixed tissues[20]. And it is still necessary to find alternative dyes with better staining for the living tissues in the future. In addition, it is also interesting to test whether the method described here can be generally applied to the study of SAMs from all the other flowering plants, considering the fact that some plant species have special cellular contents or different cell wall compositions.

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors have nothing to disclose.

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Figure1

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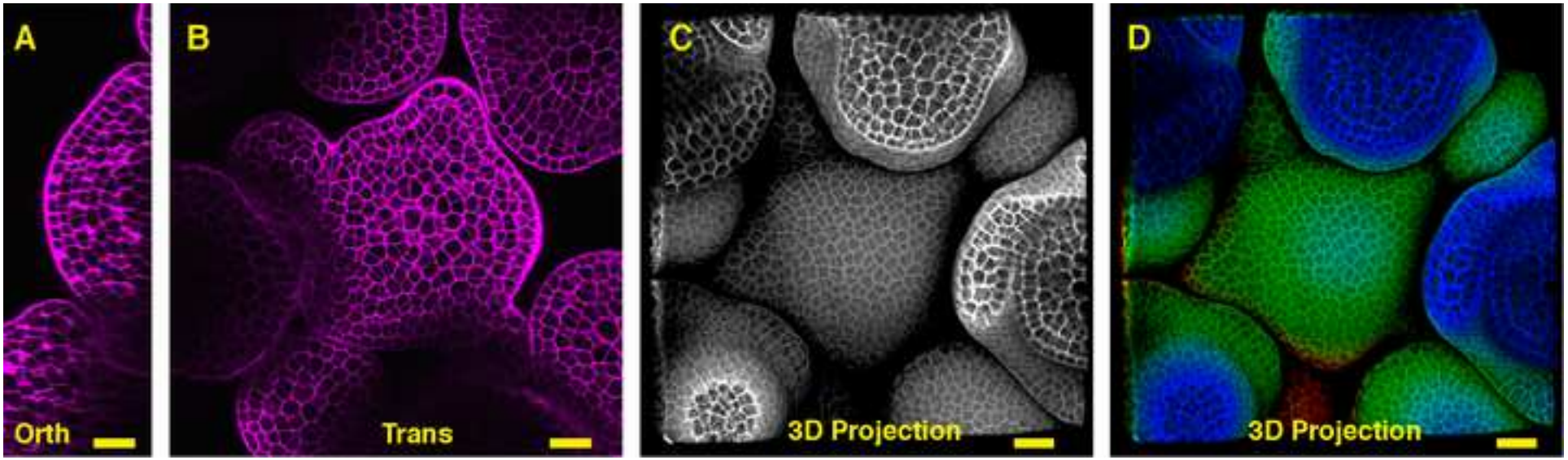

Figure2

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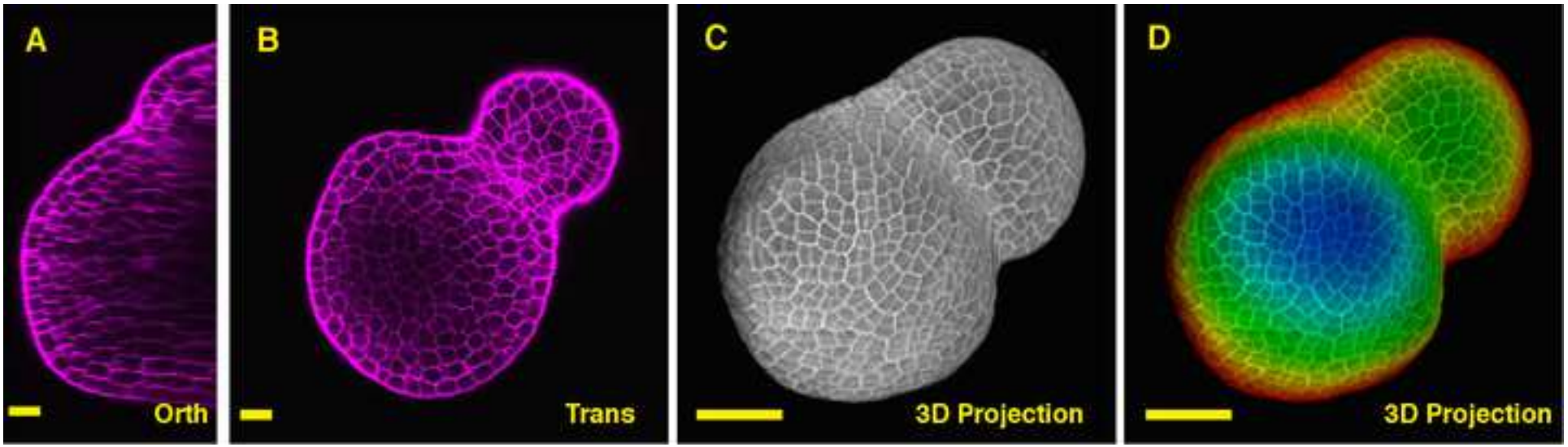
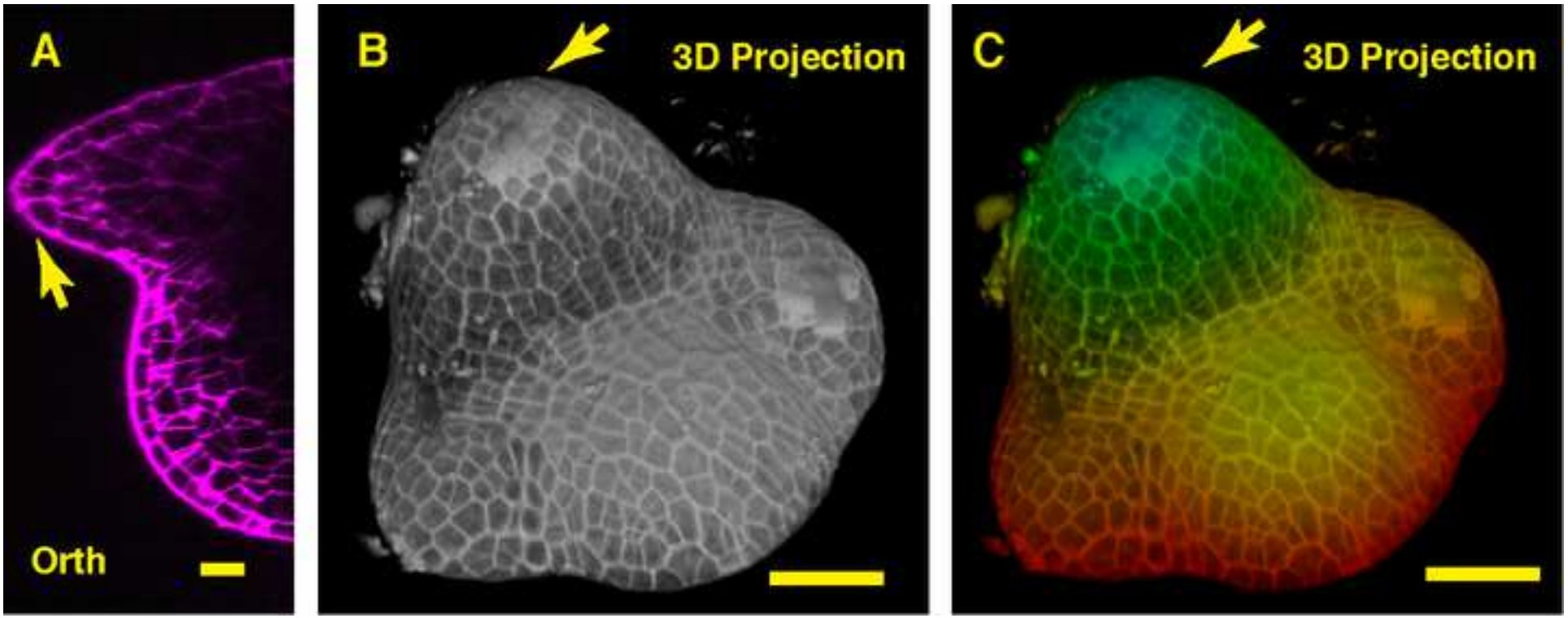


Figure3



Video1

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Video or Animated Figure

Movie 1.avi

Video2

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Name of Material/ Equipment	Company	Catalog Number
Agar Phyto	Dot Scientific Inc.	DSA20300-1000
Agarose	Dot Scientific Inc.	AGLE-500
Forceps	ROBOZ	RS-4955
LSM 880 Upright Confocal Microscope	Zeiss	
Murashige & Skoog MS medium	Dot Scientific Inc.	DSM10200-50
Plan APO 20x/1.1 water dipping lens	Zeiss	
Plastic petri dishes 100 mm X 15 mm	CELLTREAT Scientific Products	229694
Plastic petri dishes60 mm X 15	CELLTREAT Scientific Products	229665
Propagation Mix	Sungro Horticulture	
Propidium iodide	Acros Organics	440300250
Razor blade	PERSONNA	62-0179
Stereomicroscope	Nikon	SMZ1000
Tissue	VWR	82003-820
Zen black	Zeiss	

Comments/Description

Dumont #5SF Super Fine Forceps Inox Tip Size .025 X .005mm, for dissecting shoot apices.

Use as making MS plates

Use as imaging dishes

1 mg/mL solution in water, to stain the cell walls
For cutting shoot apex from plants

Image acquisition software

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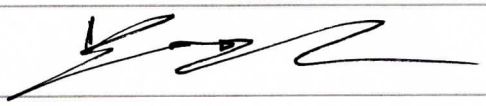
Signature:	 Date: 11/06/2018

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