Chapter 25

Live-Imaging of the Arabidopsis Inflorescence Meristem

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Abstract

The aboveground tissues of higher plants are derived from a small population of stem cells located at the shoot apex within a structure called the shoot apical meristem (SAM). The SAM not only includes the stem cells but also incorporates a region from which lateral organs arise. The SAM is therefore of prime interest for understanding plant growth and development. In this chapter we outline methods for using confocal microscopy to image the Arabidopsis inflorescence SAM. This method enables detailed examination of cell division and growth patterns (Reddy et al., Development 131:4225–4237, 2004) as well as gene expression and protein localization patterns over time (Heisler et al. Curr Biol 15:1899–1911, 2005). When combined with perturbation approaches, the method offers an extremely powerful system for investigating SAM function in great detail.

Key words Arabidopsis, Confocal, Imaging, Shoot meristem, Flowers

1 Introduction

Development is a dynamic process involving the growth and proliferation of cells to form distinct tissues. In plants new tissues arise continuously from apical meristems located at the tips of the root and shoot. The shoot apical meristem (SAM) not only contains stem cells from which all aboveground tissues are derived but also contributes to the continuous formation of lateral organs on its flanks. To fully understand these developmental processes it is important to be able to examine gene expression and protein localization within the SAM with adequate spatial and temporal resolution.

During the reproductive phase of the model species *Arabidopsis thaliana*, the stem elongates and the SAM gives rise to flowers rather than leaves. Due to changes in the shape of the meristem and the different morphology of the lateral organs, the inflorescence shoot apex and early flower buds become visible from above and therefore accessible for microscopy. The most important limitations, due to the morphology of the apex, are that a cover slip

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cannot easily be positioned, and that a relatively large distance between the meristem surface and objective lens needs to be maintained due to the presence of the floral buds. These challenges are easily met, however, by a family of objective lenses initially designed for electrophysiology called dipping lenses. Dipping lenses are immersed in water but unlike typical water immersion lenses, they do not require a cover slip and feature large working distances.

Two approaches were initially developed to utilize dipping lenses and confocal microscopy to monitor SAM cell division dynamics and gene expression patterns. The difference between the approaches was the use of either an upright or inverted microscope. In the upright case the plant is mounted in a small plastic container filled with water and observed from above [1]. This is a relatively simple set up compared to imaging the SAM using an inverted microscope since in this case either the plant has to be mounted upside down or the stem needs to be bent such that the apex is inverted [3]. Another difference between those first two studies was the use of the auxin transport inhibitor NPA to temporarily inhibit organ formation so that better access to the meristem is possible. However, adequate access is also possible without such treatment and this avoids any potential NPA associated artifacts.

In this chapter, a detailed description of the method developed for imaging inflorescence SAMs using an upright confocal microscope [1] is provided. The chapter covers both preparation of the plants for mounting on the microscope, as well as important points and tips for imaging. The points on imaging are brief; however, as a general description of confocal imaging is covered elsewhere (e.g., *see* ref. 4), As to the choice of GFPs, it is best to learn from examples in the plant imaging literature.

2 Materials

1. Confocal microscope: As mentioned above, for the method described here an upright confocal is required. More recent models are generally more sensitive and able to distinguish a greater variety of fluorophores compared to older models. However, no specific manufacturer of microscopes is recommended.
2. Dipping lens: These lenses are available from most microscope manufacturers. They typically have magnifications of 20, 25, 40, and 63×. Most microscope manufacturers now make low-power dipping lenses that have numerical apertures (NAs) between 0.95 and 1.1. These lenses are preferable since they provide great flexibility for looking at samples of varying size at high resolution. However, some of them require specialized turrets associated with fixed-stage microscopes. The more typical 40× 0.8NA and 63× 0.9NA lenses are also very useful.

3. Boxes: For mounting plant apices on the microscope stage small transparent plastic boxes are required (e.g., Clear boxes 2 7/8″ length × 2″ width × 1 ¼″ height, Durphy Packaging Co. Ivyland, PA, USA).
4. GM medium: 1 % sucrose, 1× Murashige and Skoog basal salt mixture, MES 2-(MN-morpholino)-ethane sulfonic acid, 0.8 % Bacto Agar, 1 % MS vitamins, pH 5.7 with 1 M potassium hydroxide solution.
5. Plants for imaging. These can express fluorescent markers, or fluorescent stains can be used for cell membranes.
6. Sterile and desalted water.
7. Large clear plastic boxes to store and help keep sterile the small boxes containing the seedlings during their growth.
8. Ethanol.
9. Large beakers (2–5 L) for submerging the imaging boxes in ethanol for sterilization.
10. Tweezers (e.g., Dumont 5.5 INOX).
11. Sterile hood.
12. Air permeable tape (e.g., 3M Scotch Filter Tape, from Carolina Biologicals).

3 Methods

Our general approach to imaging plant inflorescence apices is to submerge them under water while utilizing dipping lenses to observe the meristems and young floral buds from above. Specimens are mounted in small plastic containers, usually boxes, which can be filled up with water. The apex is anchored within the box by having either the plant root system (in the case of time-lapse imaging) or just the stem dissected below the apex (for one-time snapshots) embedded in solid growth medium or agarose, respectively. Furthermore, plants can either be grown in the boxes from the seedling stage or transplated into the box (including roots) from soil. The procedure for all three cases is described in detail below.

3.1 One-Time Snapshots of Dissected Apices

1. Prepare boxes for sample mounting: pour 1 % molten agarose (in water) into each box to a depth of approximately 1 cm. Leave to cool and solidify (*see* **Note 1**).
2. Prepare an apex for mounting: dissect the inflorescence apex from the growing plant, leaving approximately 1 cm of stem under the apex. Place the apex within a dissecting dish filled with water (*see* **Note 2**).
3. Dissect away the larger flowers and any siliques attached to the stem (Fig. 1c, d) (*see* **Note 3**).

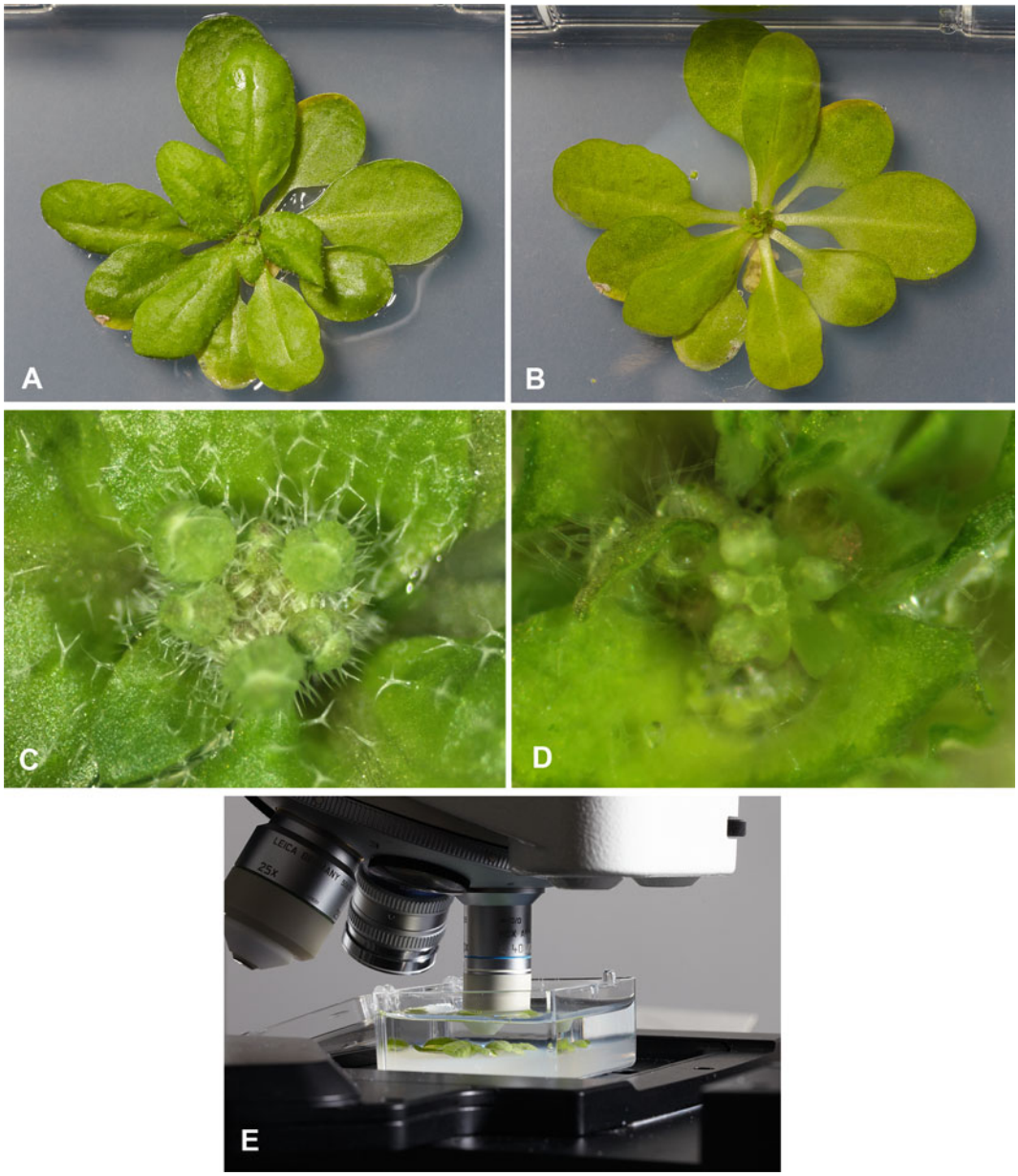

Fig. 1 Plant preparation for imaging. (**a**) Shows a view before dissection of a whole Arabidopsis plant that has been transplanted into a mounting box. (**b**) View of same plant as in (**a**) but after dissection of several leaves and flowers that either obstructed a view of the meristem or impeded the microscope objective lens from coming into close proximity with the SAM. (**c**) Close-up view of the un-dissected inflorescence meristem shown in (**a**). (**d**) Close-up view of the dissected inflorescence meristem shown in (**b**). Note the clear view of the meristem in the middle of the picture. (**e**) View of plant mounted within its box on the microscope stage. A 40× dipping lens is positioned above the apex, within the water

4. Mount the apex within the box: first use the fine point of the forceps to make vertical incisions or holes into the agar down to the bottom (*see* **Note 4**). Next gently transfer the apex from the dissecting dish into the box and drop it somewhere near

the newly created hole. Do not put water in the box yet since this makes it difficult to insert and mount the specimen. Although the hole may be difficult to see, with the help of a dissecting scope, position the apex such that the bottom of the stem is as close as possible to the hole. Then try to push the bottom of the stem gently, directly down, into the hole. Once it goes partway in, change the grip on the apex to more easily push the stem downward into the agar such that the apex is positioned rigidly above the agar surface.

5. After mounting the apex in the agarose, water can be added to the box to prevent dehydration. The apex should be completely submerged but the water should not be at a level such that it easily spills out of the box.
6. Next, use a 100 μL pipette to create jets of water aimed at the meristem and young flower buds to dislodge trapped air (*see* **Note 5**). Once most of the trapped air has been removed, use a dissecting microscope and forceps to make fine adjustments to the angle of the stem in the agar so that the meristem is maximally visible from directly above (repeat the procedure for the removal of trapped air whenever necessary). Lastly, again using fine forceps, dissect away any remaining flower buds that still obscure the inflorescence meristem by pinching where the pedicel meets the stem.
7. When there is a clear view of the shoot apical meristem from above (e.g., compare Fig. 1c, d), the box can be placed carefully (without spilling the water still in it) on the microscope stage (Fig. 1e) (*see* **Note 6**).
8. Once the plant apex within the box is roughly positioned under the objective lens, raise the microscope stage (or lower the objective) until the tip of the objective touches the water. Then, make sure there are no bubbles under the objective by looking from the side. If there are bubbles raise the objective out of the water and then repeat the immersion. If bubbles persist use a pasteur pipet to blow away any remaining bubbles with water.
9. Next, while using epifluorescence illumination (fluorescence filters work well), position the meristem within the microscope field of view by manually using the X–Y controls. Still looking from the side, position the plant apex at the focal point by moving the stage in all three dimensions until there is a maximum of deflected epifluorescence light coming from the apex (Fig. 1e).
10. Finally, look down the eye-pieces while controlling the X, Y, and Z controls until a bright signal is seen. In general the meristem is located centrally within the floral buds but it will take some practice to be able to recognize it reliably and quickly. A low power (e.g., 20–40×) objective will facilitate this. Proceed to Subheading 3.4 for tips on confocal imaging of the inflorescence meristem.

3.2 Growing in Boxes

In this approach, seedlings are first grown on plates and then transplanted into boxes where they grow until the transition to flowering. This approach seems to work well for keeping plants healthy during time-lapse imaging as the root system is fully developed within the box. The down side is that it can be difficult to maintain sterility and so typically many more plants need to be prepared than would actually be used for imaging to compensate for the loss due to contamination. However, the use of antibiotics and fungicides in the growth medium can reduce this problem.

1. Sow sterilized seed onto GM plates and allow to germinate.
2. Sterilize boxes by soaking, immersing them in 70 % ethanol briefly. Allow to dry within a flow hood with lids open. Expose to UV light from tissue culture flow hood for a few hours to further sterilize (*see* **Note 7**).
3. Fill each box with GM medium (containing 300 mg/L carbenicillin) to a depth of around 1 cm.
4. Using sterile conditions, transplant one seedling from the plates into each box. Position the plant centrally and push the root into them medium.
5. Close the box and seal with air permeable tape. Repeat for all plants/boxes.
6. To try to further prevent contamination, the boxes can be enclosed within a larger transparent box that has been surface sterilized with ethanol and also sealed with air permeable tape.
7. Place boxes in growth cabinet or growth room in a position that minimizes condensation.
8. Grow plants until flower buds are visible at the center of the rosette prior to bolting (Fig. 1a, c).
9. Next, proceed to **step 11** in Subheading 3.3 below.

3.3 Transplanting from Soil

This method is a convenient way to do short term (1–2 days) time-lapse imaging with a minimum of preparation. Plants are simply transferred from soil at the right stage directly into boxes without sterilization. It does help, however, to have antibiotics such as carbenicillin in the solid medium.

1. Grow plants in soil until flower buds are visible at the center of the rosette prior to bolting (Fig. 1a, c).
2. Prepare boxes as described in Subheading 3.2, **step 3**.
3. Remove the solid growth medium from a central area (1–2 cm^2) within the box for insertion of the plant root system.
4. Using forceps, dig into the soil surrounding the plant to be transplanted so that the plant (including much of the root system) can be lifted out of the pot (*see* **Note 8**).
5. Place plant into petri dish containing water.

6. Using forceps gently agitate the roots together with clumped soil under water so as to wash away much of the dirt.
7. Again using forceps, very gently try to dislodge away into the water any large clump of dirt or vermiculite that is still clinging to the roots. However, avoid breaking the roots. Removal of all soil or vermiculite particles will not be possible without great damage so the aim here is just to reduce the size of the root system plus soil such that it can fit into the hole previously created within the mounting box growth medium (*see* **step 3**, above).
8. Next pick up the plant and gently insert the root system into the hole within the mounting box growth medium. The plant should be oriented such that the inflorescence is facing directly upward to expose the meristem optimally later on.
9. Now pour some nutrient concentrate (e.g., Miracle grow) into the hole to partially cover the roots (*see* **Note 9**).
10. Finally, using a 1 mL pipette, squirt molten 1 % agarose gradually into the hole on top of the roots so as to solidify the root system. This should be done gradually so as to minimize heat damage.
11. To anchor and position the plant for subsequent imaging, more 1 % molten agarose is used. This molten agarose is applied to the leaves where they contact the solid agarose medium and around the petioles so as to rigidify the plant. Again, the agarose should be applied gradually and care should be taken not to immerse the meristem itself in agarose. While applying the agarose, the plant should be held in an orientation compatible with subsequent imaging. This can either mean trying to keep the plane of the rosette leaves horizontal, or if there are only a few leaves, trying to keep the inflorescence meristem facing upwards with the help of direct observation using a dissecting microscope. If necessary, after solidification the agarose can be broken up with tweezers to reposition. Ultimately, the orientation should be such that the inflorescence meristem or young buds are visible through a dissecting microscope looking down directly from above (Fig. 1a, c).
12. Fill the box with water to a level just above the inflorescence meristem (Fig. 1e).
13. Remove air bubbles as described in Subheading 3.1, **step 6**.
14. With a razor blade dissect away both leaves and flowers that obscure the meristem. Also remove leaves that will prevent the objective lens from approaching the meristem from above (Fig. 1b, d).
15. Again as described in Subheading 3.1, **step 6**, remove any air bubbles from the meristem region and proceed as described in **steps 7** and **8** of that section.

3.4 Points for Consideration When Imaging the Inflorescence Apex

This section does not describe confocal imaging in detail since such information can be obtained elsewhere. Rather, it provides some general bullet points for live-imaging the inflorescence meristem.

1. Minimize photodamage by using the lowest laser power that still gives adequate signal. If necessary, the pinhole aperture can also be increased to collect more light, although Z resolution will suffer. For gaining a general impression of the morphology and cellular structure optical, sections can be up to 2 μm apart (we usually use 1 μm). Although a smaller spacing between slices may better match the optical slice thickness, smaller spacing also means more optical slices and therefore more photodamage for time-lapse experiments.
2. Realize that the stem below the apex may be growing rapidly. This means that as you image the tissue, the tissue may be moving upwards in the *Z* direction. Also, be aware that growth may have to be taken into account when setting the top section of a Z-stack. These complications can be avoided if the plant has not bolted yet or if a dissected apex has been left underwater for 20–30 min before imaging. Also, an intact plant can be immersed under ice-cold water within its mounting box for 30 min before imaging to stop growth temporarily.
3. For time-lapse imaging, the time intervals chosen should reflect both the biological question being asked as well as the risk of photodamage. The settings required to avoid exposure of the plant to excessive photodamage depends on many factors, including the sensitivity of the microscope, the brightness of the signal and the number of optical sections acquired at each time-interval. Typically, a feel for these parameters must be gained through trial and error; however, for bright signals, intervals of 3 h over 5 day is possible [1].
4. To gain an intuitive feel for the morphology, 3D animations of volume rendered Z stacks are most useful. Maximum intensity projections are also helpful.

4 Notes

1. Although one box can accommodate many specimens, it is usually best to prepare multiple boxes since any boxes not used immediately can be stored at 4 °C for future use. Boxes to be stored should be kept in plastic wrap to prevent dehydration of the agarose.
2. Submersion under water soon after separating the apex from the plant helps prevent dehydration of the tissue, especially

during the subsequent dissection of flowers (even though initially the apex will float).

3. Successful imaging of the inflorescence meristem depends on making sure that no flowers obstruct a view of the meristem from above. Also, to mount the apex the stem must be inserted into the agar. Therefore any siliques as well as older flowers need to be removed. However, care must be taken not to use forceps to hold the apex by the stem, thereby crushing it. Instead, one pair of forceps can be used to hold the flower or silique that is to be removed, while the other pair of forceps is used to pinch the corresponding pedicel at a point adjacent to the stem. Then, by simultaneously pinching and pulling the two points of contact apart the silique or flower can be removed without further damage. As these dissections are taking place, an effort should be made to keep the apex submerged as much as possible, especially the wounded regions. Roughly, all the flowers that can easily be removed in the dissecting dish should be removed. In practice, this usually means at least all flowers older than stage 12 [5]. Any smaller flower buds that obscure the apex can more easily be dissected once the apex is mounted in the agar within the box.
4. In general the incisions should be made towards the center of the box since if the specimen is placed too close to the wall of the box, the objective lens may not gain access to it.
5. Trapped air is visible under the dissecting microscope as a silvery region usually obscuring the smaller flower buds and meristem.
6. This can be a little tricky. Firstly the microscope stage is lowered to its extreme bottom position. Often the objective lens will also have to be moved out of the way by rotating the microscope turret. Also, any objective lens in positions adjacent to the dipping lens will have to be removed. Lastly, the box itself may need to be tilted temporarily to enable the dipping lens to move back into position above the sample.
7. Every effort must be made to keep the boxes sterile throughout this procedure, otherwise the plants will become contaminated and die.
8. This digging should occur at a distance from the plant in order to leave the soil directly contacting the bulk of the root system intact. Dig around and then under the plant and try to minimize damage to the roots themselves. Do not try to strip away dirt from the roots at this stage.
9. The aim here is to both provide some nutrients for growth as well as reduce the heat damage caused by the subsequent addition of molten agarose on top of the roots.

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References

1. Reddy GV, Heisler MG, Ehrhardt DW, Meyerowitz EM (2004) Real-time lineage analysis reveals oriented cell divisions associated with morphogenesis at the shoot apex of *Arabidopsis thaliana*. Development 131:4225–4237
2. Heisler MG, Ohno C, Das P, Sieber P, Reddy GV, Long JA, Meyerowitz EM (2005) Patterns of auxin transport and gene expression during primordium development revealed by live imaging of the Arabidopsis inflorescence meristem. Curr Biol 15:1899–1911
3. Grandjean O, Vernoux T, Laufs P, Belcram K, Mizukami Y, Traas J (2004) In vivo analysis of cell division, cell growth, and differentiation at the shoot apical meristem in Arabidopsis. Plant Cell 16:74–87
4. Pawley J (ed) (2006) Handbook of biological confocal microscopy. Springer, New York
5. Smyth DR, Bowman JL, Meyerowitz EM (1990) Early flower development in Arabidopsis. Plant Cell 2:755–767