CHAPTER

11

Imaging the delivery and behavior of cellulose synthases in *Arabidopsis thaliana* using confocal microscopy

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Chapter outline

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Abstract

Confocal microscopy has been a key tool for characterizing the behavior of cellulose synthase (CESA) proteins as they extrude cellulose into the apoplast to help construct plant cell walls. While other microscopy techniques like electron microscopy can achieve higher resolution images of CESAs, confocal microscopy is still the most accessible way to image these proteins in living plants as they are trafficked to and from the cell surface and move through the plasma membrane. Here, we describe a method for imaging fluorescently tagged CESA proteins in seedlings of *Arabidopsis thaliana* using spinning disk confocal microscopy, with a focus on quantifying the speed, density, and delivery rate of CESA particles. Many of these techniques can be adapted and applied to imaging other membrane-localized proteins and other plant species. In addition to imaging techniques, we describe several options for image analysis that can be optimized for different datasets.

1 Introduction

As an economically and biologically significant polymer, cellulose has long been a subject of intensive research. However, studying the biosynthesis and structure of cellulose has proven to be difficult. The structures and molecular interactions of the proteins that produce cellulose in plants, called cellulose synthases (CESAs), and the structures of the cellulose they generate are still undefined. CESAs are believed to function in a complex consisting of a hexamer of trimers, with multiple CESA isoforms contributing to the formation of each complex (Desprez et al., 2007; Hill, Hammudi, & Tien, 2014; Nixon et al., 2016; Persson et al., 2007). By tagging the N-terminus of a CESA with a fluorescent protein such as Green Fluorescent Protein (GFP), the movements of small CESA-containing particles can be tracked through the plasma membrane (Paredez, Somerville, & Ehrhardt, 2006; Sampathkumar et al., 2013). It is thought that the linear movements of these particles, which often co-align with cortical microtubules and might represent individual Cellulose Synthase Complexes (CSCs) of ~25 nm in size (Nixon et al., 2016), are driven by the polymerization of cellulose. CESA proteins are delivered to the plasma membrane from the Golgi by post-Golgi vesicles, and are cycled between the plasma membrane and intracellular vesicles that are variously called Microtubule Associated Cellulose Synthase Compartments (MASCs) or Small CESA Compartments (SmaCCs) (Crowell et al., 2009; Gutierrez, Lindeboom, Paredez, Emons, & Ehrhardt, 2009). Although FP-CESA imaging has been performed in moss and a monocot (Liu et al., 2017; Tran et al., 2018), most CESA imaging has been performed in *Arabidopsis thaliana* (Arabidopsis).

Imaging CESAs in living plants by confocal microscopy can be complicated by both the physiological status of the sample and the imaging conditions. In order to image a membrane-localized protein, the excitation laser needs to penetrate through the coverslip, plant cuticle, and cell wall before it reaches the protein of interest.

Not only does this add distance between the coverslip and the desired focal plane, but it also introduces several layers with unique refractive indexes that must be accounted for when optimizing the microscope setup (Shaw & Ehrhardt, 2013). Coverslip thickness and objective type (e.g., water vs. oil immersion) can both be adjusted to acquire the best images for the sample.

2 Collecting time-lapses of cellulose synthase particles

Imaging CESA particles with confocal microscopy can provide a plethora of data on their behavior while still being accessible to most researchers. Despite the diffraction-limited resolution of conventional light microscopy, confocal images can allow for quantification of the speed, directionality, density, and delivery rate of CESA particles. The use of small plants like Arabidopsis seedlings also facilitates the imaging of tissues within a living, intact seedling with functional CESAs rather than those from excised tissues that experience stress, which is often associated with internalization of CESA particles from the plasma membrane (Gutierrez et al., 2009). In this chapter, we describe methods for imaging CESA particles by spinning disk confocal microscopy in the hypocotyls of dark-grown Arabidopsis seedlings. Dark-grown hypocotyls represent a plant tissue with active cell wall synthesis, wall remodeling, and anisotropic cell elongation, and thus are a facile system in which to monitor the dynamics of cellulose synthesis and CESA trafficking. Furthermore, dark-grown seedlings are ideal for live-cell imaging due to their simple morphology and anatomy, and lack of auto-fluorescent chlorophyll.

2.1 Materials, equipment, and reagents

- Arabidopsis seeds expressing *GFP-CESA3* or another FP-CESA marker
- Microscope slides (e.g., VWR 16005-106)
- Corning glass coverslips, 24 × 40 mm, thickness 1.5
- Scotch 3 M permanent double-sided tape
- Square Petri dishes (e.g., VWR 60872-310) filled with ½ MS agar
- Growth chamber (e.g., Percival model CU36L5)
- Forceps (e.g., VWR 25607-856)
- Liquid ½ MS media
- P200 pipette + sterile pipette tips
- 3 M Micropore tape (VWR 56222-182)
- Spinning disk confocal microscope (or equivalent), preferentially equipped with a high-sensitivity CCD or CMOS camera
  - For reference, we use a Zeiss Cell Observer SD spinning disk confocal microscope (inverted) with a CSU-X1 spinning head (Yokogawa) equipped with a 100 ×/1.4 NA oil-immersion objective (Zeiss) and a QuantEM 512SC EM-CCD camera (Photometrics).

  - Excitation laser(s) of the appropriate wavelength(s) (be sure that your laser wavelength overlaps with your fluorescent protein's excitation peak; we use a 488 nm laser when imaging GFP.)
- Emission filter (make sure your emission filter matches your fluorescent protein's emission spectrum; we use a 525/50 nm emission filter for GFP)
  - fpbase.org (Lambert, 2019), a very useful resource for determining the optimal excitation laser and emission filter of fluorescent proteins
- mounted samples (see "Slide setup")

2.2 Plant preparation

1. In a sterile hood, sterilize seeds in aqueous 30% bleach with 0.1% SDS for 20 min before rinsing them four times in sterile water. Store the seeds in the dark in sterile, aqueous 0.15% (w/v) agar for 3 days at 4 °C before sowing.
2. In a sterile hood, sow sterilized seeds in two rows on ½ MS plates (see Section 4 for details) using a pipette and sterile pipette tips. The last 2 mm of the pipette tip can be excised using an ethanol-sterilized razor blade to allow seeds to pass through. Be careful when sowing to leave 2 cm above the seeds for hypocotyl growth; seeds should be spaced at least 2 mm apart (Fig. 1).
3. Allow seeds to dry on plates in the sterile hood with a light on for 2–4 h before closing the lids and sealing the edges with Micropore tape to prevent contamination.
4. Wrap the plates in 2 layers of aluminum foil to block out any light.
5. Place plates upright in a growth chamber with controlled light and temperature (we use 24 h light at 22 °C) for 3 days.

2.3 Slide setup

1. Place two pieces of 3 M double-sided tape ~15 mm apart on a glass slide (Fig. 2A). The tape prevents the sample from being crushed by the coverslip, but keeps the sample in contact with the coverslip.
2. Remove the seed coat from a seedling. Using forceps, gently remove the seedling from the plate by digging the open forceps under the seedling and lifting it off of the plate in a scooping motion. Do not over-bend or crush the seedling in the process. Excessive handling of the seedling will stress the seedling, inhibiting cytoplasmic streaming and Golgi movement.
3. Gently place the seedling on the glass slide between the two pieces of tape.
4. Add 60 µL liquid ½ MS media to the seedling to keep it from drying out during imaging.
5. Lower a coverslip at an angle onto the seedling and tape, avoiding bubble formation and creating a chamber that does not crush the seedling.

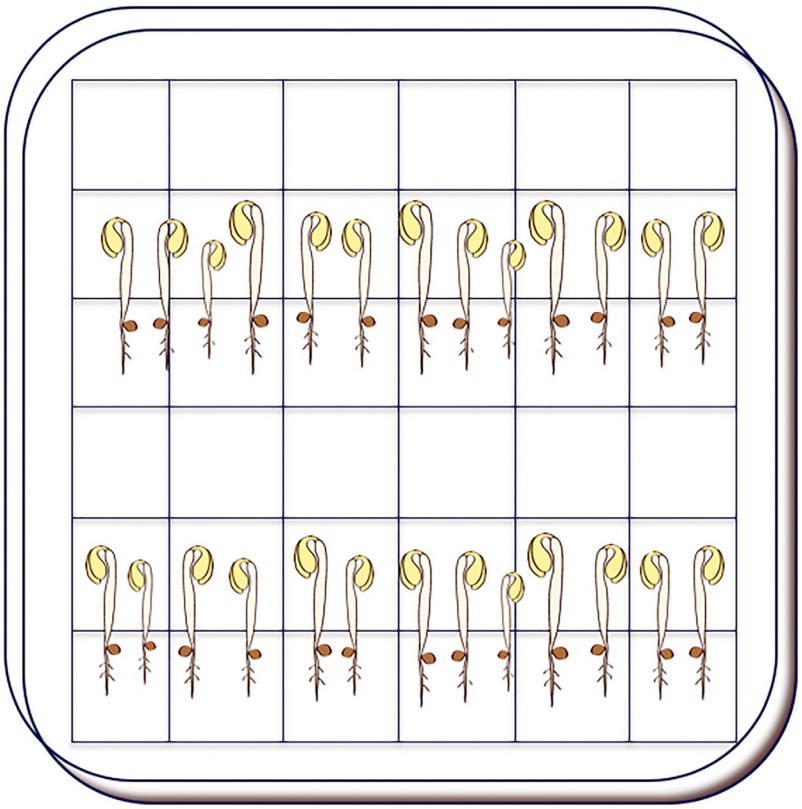

FIG. 1

Dark grown seedlings on a ½ MS plate.

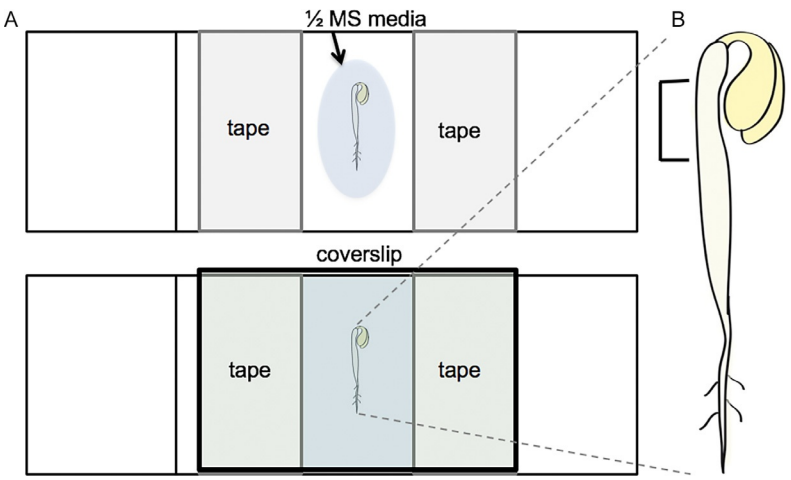

FIG. 2

(A) Tape chamber slide setup of a dark grown seedling. (B) Diagram of the optimal CESA imaging region (bracket) in a dark grown seedling.

6. Gently press along the tape to adhere the coverslip to the tape, and blot away any excess media that exits the chamber.
7. Add immersion oil (or water for water-immersion objectives) to the coverslip or objective before mounting the slide on the microscope.

2.4 Imaging protocol

1. Mount the slide on the microscope with the seedling centered over the objective.
2. Focus the objective until it contacts the immersion oil, and locate the seedling in brightfield mode using the eyepieces.
3. Focus until the cell outlines of the epidermis are visible.
4. For optimal CESA imaging, locate the region of the hypocotyl 1–2 mm below the apical hook across from the cotyledons (Fig. 2B).
5. Once the optimal region is in focus, the plants can now be imaged using the camera with the appropriate excitation laser (Fig. 3).
6. When using a spinning disk confocal microscope, set the laser power to 50%, EM gain to 1000, exposure time to 200 ms, no frame averaging, and readout gain to 1. Adjust these settings as needed to suit the sample and microscope setup, especially if extensive photobleaching occurs.
7. To ensure accurate particle tracking while minimizing photobleaching, images can be collected every 5 or 10 s for 5 min to create time-lapses of CESA particles.

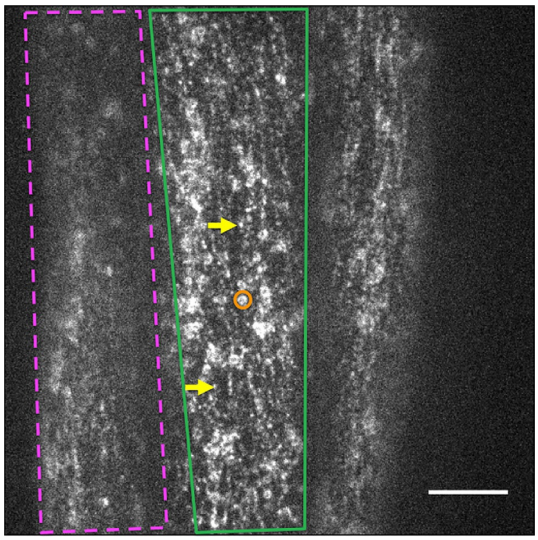

FIG. 3

Cell with CESA particles in focus (green outline) next to cell with CESAs out of focus (dashed magenta outline). Yellow arrows indicate CESA particles, orange circle indicates a Golgi. Scale bar = 10 μm.

2.5 Analysis

2.5.1 Speed analysis

Kymographs or particle tracking algorithms can both be used to collect data on particle speed. When using kymographs, average projections of each time-lapse should be created to identify CESA trajectories using ImageJ. These trajectories can be traced using the segmented line tool to create a region of interest (ROI) that can be overlaid onto the original time-lapse stack. With this ROI in place, use the kymograph plugin in ImageJ to collect a kymograph of particle trajectories. The slope of the kymograph provides particle speed, which can be quantified quickly using the online platform KymoButler (Jakobs, Dimitracopoulos, & Franze, 2019). Several other particle tracking tools are available to gather data on particle speed. These tools can collect particle speed data for a large movie dataset quickly, but potentially lose some accuracy in the process. ImageJ provides access to several free plugins such as TrackMate, which will track the trajectory of all of the particles in a time-lapse (Tinevez et al., 2017). Other free programs such as FIESTA can be easier to use, but cannot track a large number of particles at once so are best used for smaller datasets (Ruhnow, Zwicker, & Diez, 2011). If available, Imaris (Bitplane) is a useful software for tracking CESAs, but it does require the purchase of a license. We use Imaris for the majority of our particle analyses. Regardless of which software is used, setting the correct parameters is critical for the accuracy of particle detection and tracking. For CESAs, we use an average particle diameter to select CESAs over the Golgi that carry them intracellularly, as well as Connected Components as the motion detection algorithm to reduce the detection of faster-moving, intracellular compartments that contain CESAs (e.g., MASCs or SmaCCs). Compared to plasma membrane-localized FP-CESAs that move in slow, linear trajectories for extended durations, MASCs/SmaCCs can be distinguished by high-speed, erratic, non-linear motions. Thus, when tracking FP-CESA particles, it is important to monitor the behaviors of candidate particles; we count only those that appear to move in a slow, linear fashion, and stay in the focal plane for at least 1 min total as active, plasma membrane-localized CESA particles.

2.5.2 Density analysis

Density data can be collected from any frame of a time-lapse dataset, although the first frame is usually the frame that is the brightest and the most in focus. In ImageJ, use the Threshold tool (Image → Adjust → Threshold) to select only the CESA particles present in the frame, removing both background noise and Golgi. Once the threshold has been optimized, use "Analyze particles" under Analyze to quantify the number of particles present in the thresholded image. Neighboring cells that are not in focus can result in artifacts during the thresholding process that are spuriously counted as particles, so it can be useful to crop these cells out of the image before thresholding. Imaris is also an option for acquiring particle count information. Once the total particle count is measured, divide the number of detected particles by the area in which the particles were quantified to obtain the particle density.

3 Using FRAP to analyze newly delivered cellulose synthase particles

With the use of a Fluorescence Recover After Photobleaching (FRAP) module on a confocal microscope, one can bleach existing CESAs from a particular area of the plasma membrane, providing a dark region in which to image newly delivered CESA particles.

3.1 Materials, equipment, and reagents

- Materials from 2.1, plus:
- Spinning disk confocal microscope (or equivalent) equipped with a Fluorescence Recovery After Photobleaching (FRAP) or targeted bleaching module, preferentially equipped with a CCD camera. For reference, we use a Zeiss Axio Observer SD spinning disk confocal microscope with a CSU-X1 spinning head (Yokogawa) equipped with a 100× /1.4 NA oil-immersion objective (Zeiss) and a Laser Manipulation Module (Observer.Z1; Zeiss). Alternatively, bleaching can be achieved simply by maximizing the laser power in imaging mode in a single field of view for a long period, but this can lead to photodamage in the cells.
- Nunc® Lab-Tek™ II Chamber Slide™ System, Sterile (VWR 62407-290)
- Low melt agarose (DOT Scientific DSA20025-500)

3.2 Slide setup

1. Seedlings can be mounted on slides as previously described (Section 2.3). However, particle delivery is tracked over the course of 10 min, and the use of a chamber slide and agar cushion (see below) is helpful to maintain a hydrated environment for longer-term imaging and reduces drift in the Z direction.
2. Prepare 2% (w/v) low melt agarose in ½ MS liquid and melt agarose using a microwave or hot plate. Pipette hot agarose over the surface of several slides to create flat agar cushions. Let agar cushions solidify at room temperature, then cover in sterile water to keep hydrated.
3. Following the procedure in Section 2.3 step 2, place a seedling in the middle of a chamber slide rather than a regular slide.
4. When ready to use, cut an agar cushion that is slightly smaller than the area of the slide chamber using a razor blade, and scoop the agar cushion off of the surface of the slide. Gently place the agar cushion on top of the seedling. Prevent air pockets from forming by lowering the cushion onto one end of the seedling and slowly laying the remaining portions of the cushion over the exposed seedling.
5. Place the chamber slide lid on top of the chamber slide. This is optional as the agar pad is sufficiently moist to maintain seedling hydration for the duration of imaging.
6. Add immersion fluid to the objective before mounting the chamber slide on the microscope.

3.3 Imaging newly delivered particles using FRAP

1. Mount the chamber slide on the microscope, focus the objective, and locate the proper region of the hypocotyl (Fig. 2B).
2. When using a spinning disk confocal microscope, set the laser power to 50%, EM gain to 1000, exposure time to 200 ms, no frame averaging, and readout gain to 1. Adjust these settings as needed to suit the sample and microscope setup, especially if extensive photobleaching begins to occur (see Section 8 for troubleshooting tips).
3. Before photobleaching plasma membrane-localized CESA particles, collect a snapshot of the plasma membrane-localized CSCs for comparisons of pre- and post-photobleaching CSC density.
4. Using the FRAP module available on the imaging software and microscope, bleach a region of the plasma membrane occupied by CSCs for 4 s with 100% 488 nm laser intensity. We typically bleach a square area of roughly $12 \times 12\,\mu m$ to minimize phototoxicity but still allow for sufficient particle detection.
5. Immediately begin tracking the recovery of individual CSC particles by collecting a time-lapse of images every 5 or 10 s for 10 min (Fig. 4).

3.4 Delivery rate analysis

When determining the rate of FP-CESA particle delivery, it is important to verify that every fluorescent particle that appears in the focal plane is a bona fide plasma membrane-localized FP-CESA based on their smaller size and lower fluorescence intensity than larger, brighter particles such as MASCs or SmaCCs. Similar to the CESA speed analysis described above, particle behaviors need to be monitored to verify their identity as CESA particles and must stay in the focal plane for at least 1 min total. It is recommended that particle speeds be verified by kymograph analysis or particle tracking software as described above to avoid including transient MASCs/SmaCCs in particle delivery counts.

Following acquisition of FP-CESA time-lapse images after photobleaching, background subtraction (35 pixel rolling ball radius) and contrast enhancement

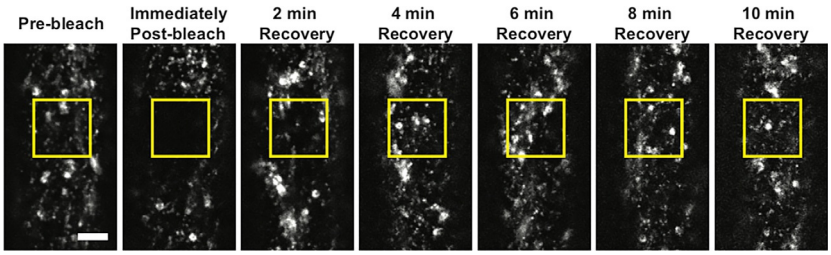

FIG. 4

Recovery of CESA particles to the plasma membrane after photobleaching. Scale bar = 5 μm.

(Saturated Pixels = 0.4%) in ImageJ can provide images with reduced background for analysis. If drift in the X or Y planes is prominent, an ImageJ plugin such as Stackreg can be applied. CESA particle delivery rate can be estimated using the following analysis protocol: make average projections for the images acquired from 0–2, 2–4, 4–6, 6–8 to 8–10 min to identify putative new particles and their corresponding tracks. Each new particle or track that is apparent in the average projections is then scrutinized in full time-lapse images to assess whether new particles are bona fide FP-CESA particles embedded in the plasma membrane exhibiting normal motility patterns. The ROI used for detection of new particles lies ~3 μm within the borders of the photobleached region to avoid misconstruing unbleached particles that have traveled into the ROI as new insertion events. After counting the number of insertion events and determining the density of new FP-CESAs at 2, 4, 6, and 8 min, a linear regression can be produced to calculate the estimated particle delivery rate for each ROI. As the rate of new FP-CESA particle delivery is typically linear until the plasma membrane is re-populated, a time point at 6 min can be used to infer the delivery rate. If we assume that a steady-state density of FP-CESAs in the plasma membrane is a result of an equilibrium of FP-CESA delivery and endocytosis, then we can infer FP-CESA residence time in the plasma membrane by dividing FP-CESA density by the delivery rate to provide additional information on CESA dynamics.

4 Precursor techniques

Half strength Murashige and Skoog medium (½ MS) plates:

1. Add 2.2 g Murashige and Skoog salts (Caisson Labs MSP-01) and 0.6 g MES (3 (N-morpholino)propanesulfonic acid) (VWR 97062-640) to a 2 L Pyrex bottle
2. Fill the bottle with purified, deionized water up to 1 L and dissolve the salts
3. Add 8 g Agar Type A, plant cell culture tested (Sigma-Aldrich A4550)
4. Adjust the pH to 5.6 with 1 N KOH
5. Autoclave to sterilize
6. Let cool to 65 °C (we use a water bath to control the temperature)
7. Pour into square petri plates (VWR 60872-310)
8. Let plates cool with open lids before sealing back into bags
9. Store at 4 °C

- To make ½ MS liquid media, omit the agar
- When growing light grown seedlings, add 25 mL of filter sterilized 40% sucrose to 975 mL autoclaved ½ MS media before pouring to make 1% sucrose (w/v) ½ MS plates

5 Safety considerations and standards

- Imaging fluorescent proteins with a confocal microscope can require Class 3B lasers, which can be harmful to the eyes if exposed. Ensure that all laser safety interlocks are operational on the microscope, and never stare at deflected laser light.
- Strong bases are needed to adjust the pH of ½ MS media and should be handled with the appropriate personal protective equipment (PPE) and stored in isolation from other chemicals, especially acids.
- Single-sided razor blades should be handled with care and disposed of in a sharps disposal container.

6 Pros and cons

Pros	Cons
Allows for behavioral analysis of CESA proteins *in vivo*	GFP tag could be affecting the natural state of the CSC
Dark grown Arabidopsis seedlings are an easy system to observe CESAs with minimal challenges or autofluorescent interference	Photobleaching limits imaging times
Spinning disk confocal microscopy can image quickly to allow for smaller time intervals between frames	Images taken with a spinning disk confocal microscope have lower resolution compared to a laser scanning confocal microscope

7 Alternative methods/procedures

Although dark grown hypocotyls are favored due to their lack of chlorophyll, CESAs can be imaged in light grown cotyledons as well. To alter this protocol for light grown cotyledons, adjust these steps:

- Use 1% sucrose ½ MS plates instead of the 0% sucrose ½ MS plates
- Sow seeds with 2 cm of space below rather than above to allow for root growth
- Let plants grow for 4–5 days instead of 3 days
- When imaging, find a pavement cell that is relatively flat against the cover slip to ensure that CESAs stay in focus in all regions of the frame

If a spinning disk confocal microscope cannot be obtained, a laser scanning confocal microscope can potentially work as well, especially if fast scanning is an option. Adjust the imaging parameters as needed.

8 Troubleshooting and optimization

Problem	Solution
CESA density is low, or particles are not moving	Mount a new seedling, don't press down so hard on the coverslip; the seedling was likely crushed and displaying a stress response
Photobleaching	Reduce laser power, increase time interval between frames, decrease exposure time (in difficult samples, we found that using 20% 488 nm laser intensity, 200 ms exposure time, and frame averaging produced images with minimal photobleaching)
Sample drift	Allow samples to equilibrate for 10 min after mounting, or use the agar chamber slide setup described in Section 3.2
CESAs are not in focus	Seedling might be too far from the coverslip
Some seedlings are not expressing GFP	Marker may be segregating; you can prescreen your seedlings for fluorescence on a dissecting scope to save time

9 Conclusion

Although this protocol has been optimized for CESA imaging, the key microscopy concepts can easily be applied to imaging other membrane-based proteins in plants. Understanding the appropriate sample requirements, challenges, and microscopy concepts allows for modification of this protocol for successful fluorescence-based imaging. For data collection, the refractive index of the sample as well as the requirements of the selected fluorophore must be considered when designing the experimental set up and choosing imaging parameters. For image analysis, the frame interval and movement patterns are important when deciding which algorithm to use for particle tracking. As with any protocol, adjustments can be made to optimize the imaging conditions and data analysis to best suit the sample.

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