Chapter 22

Live Fluorescence Visualization of Cellulose and Pectin in Plant Cell Walls

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Abstract

The plant cell wall comprises various types of macromolecules whose abundance and spatial distribution change dynamically and are crucial for plant architecture. High-resolution live cell imaging of plant cell wall components is, therefore, a powerful tool for plant cell biology and plant developmental biology. To acquire suitable data, the experimental setup for staining and imaging of non-fixed samples must be straightforward and avoid creating stress-induced artifacts. We present a detailed sample preparation and live image acquisition protocol for fluorescence visualization of cell wall components using commercially available probes and stains.

Key words *Arabidopsis*, Cell wall, Cellulose, Confocal laser scanning microscope, Fluorescence, Live cell imaging, Onion, Pectin, Plant development, Polysaccharides, Staining

1 Introduction

The plant cell wall is a complex matrix located outside of the plasma membrane. It encapsulates the cell, determines its shape, and connects it to its neighbors. The primary cell wall is established in undifferentiated cells and is continuously built in dividing and growing cells; it is composed of a network of cellulose microfibrils, hemicellulose, pectins, glycoproteins, and structural proteins. The roles of the primary cell wall are to protect the cells, mediate cell–cell interactions, and provide mechanical flexibility and structural support for growing cells, tissues, and organs [1]. Organogenesis in plants is regulated by the local mechanical properties of the cell wall that are in turn modulated by the spatial and temporal distribution of the individual components, their intermolecular linkages and their chemical configuration [2–7]. Being able to visualize the different cell wall macromolecules over time is therefore important to understand how these components regulate cell growth and morphogenesis.

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Since most of the cell wall macromolecules are polysaccharides, the option of generating fluorescent protein chimeras for labeling purposes is not available. Instead, the spatiotemporal distribution of the cell wall components can be visualized by labeling individual polysaccharide types using fluorescence probes with specific binding affinity. Many labeling protocols, such as the ones involving antibodies, require fixation of the sample prior to administration of the label. The fixation serves to stabilize cellular structures during washing and labeling steps as well as imaging. The fixation thus facilitates intense visualization protocols and repeated imaging without the risk of altering the structure of the specimen during the process. However, by virtue of its mechanism, chemical fixation is irreversible and lethal for the specimen. Therefore, a given sample represents only a snapshot in time, and documenting developmental processes requires fixing multiple specimens at different developmental stages. The continuous observation of a given specimen as it develops and undergoes morphogenesis is thus impossible. Another drawback of fixation-based labeling methods is the fact that these protocols are both time- and resource-consuming, and the many steps involved in such an experimental protocol pose multiple risks for introducing artifacts. To circumvent these shortcomings, labeling protocols compatible with live samples are desired, but must be carefully optimized and tailored to ensure specimens stay alive and unaffected by the experimental protocol over the course of the experiment.

To provide plant developmental and plant cell wall researchers with experimental protocols that accomplish live imaging while also minimizing the other shortcomings of chemical fixation, we developed a live labeling and imaging protocol that yields results of similar quality to conventionally fixed samples [8]. This protocol is faster and more cost-effective than conventional fixation-based methods, and crucially, it allows for time-lapse imaging for time periods as long as several days. In the following, we provide two detailed step-by-step procedures to label both thin plant tissues such as onion epidermis and whole organisms such as *Arabidopsis thaliana* seedlings. Using different commercially available dyes and probes, we stained both the pectin and cellulose/hemicellulose networks in living tissues. Pectins were stained with propidium iodide and COS^{488} (very low de-esterified homogalacturonans) [9]. Cellulose was stained with Pontamine Fast Scarlet 4B. β-Glucans were stained with Calcofluor White. We also provide tips and tricks when acquiring images with a confocal laser scanning microscope and show examples of artifacts that can result from imaging samples over an extended period.

2 Material

All solutions should be prepared in deionized water (sensitivity of 18 MΩ/cm at 25 °C).

2.1 Material and Solutions

1. Onion bulbs purchased from the market (*see* **Note 1**).
2. *Arabidopsis thaliana* seedlings grown on half-MS medium (*see* **Note 2**).
3. 0.075-mM propidium iodide (*see* **Note 3**): dissolve 0.5 mg in 10 mL of water.
4. 0.52-mM Calcofluor White: dissolve 5 mg in 10 mL of water.
5. 6.15-mM Pontamine Fast Scarlet 4B (S4B): dissolve 50 mg in 10 mL of water.
6. MES buffer: 25-mM 2-(N-morpholino)ethanesulfonic acid, pH 5.7.
7. COS^{488} [9]: dissolve 0.04 mg of COS^{488} in 10 mL of MES buffer (dilution of 1:250 of the delivered stock at 1 mg/mL).

2.2 Equipment

1. Orbital shaker.
2. Laser scanning confocal microscope (here we used a Zeiss LSM710 confocal laser scanning microscope).

3 Methods

Prepare all the solutions before starting the experiment. Excised plant tissues and seedlings should never be left exposed to air for any significant amount of time and should always be in contact with a solution.

Keep all solutions on ice prior to usage.

Prepare a negative control sample for each experiment: the negative control consists of a sample treated the same way as the stained sample but replacing the staining dye with water or buffer.

3.1 Staining of Onion Epidermal Leaves

1. Remove the outer dead scales of the onion bulb.
2. Remove the first two live leaves (scales) to expose the third one.
3. Using a sharp scalpel or razor blade, cut a 1 cm^2 rectangular window into the third leaf (Fig. 1a) (*see* **Note 4**).
4. Retrieve the cut section with the regular forceps, and place it on its abaxial epidermis. The adaxial epidermis should be facing up (*see* **Notes 5** and **6**) (Fig. 1b).
5. With the fine forceps, pinch a corner of the section, lift, and gently peel off the adaxial epidermis (Fig. 1c).

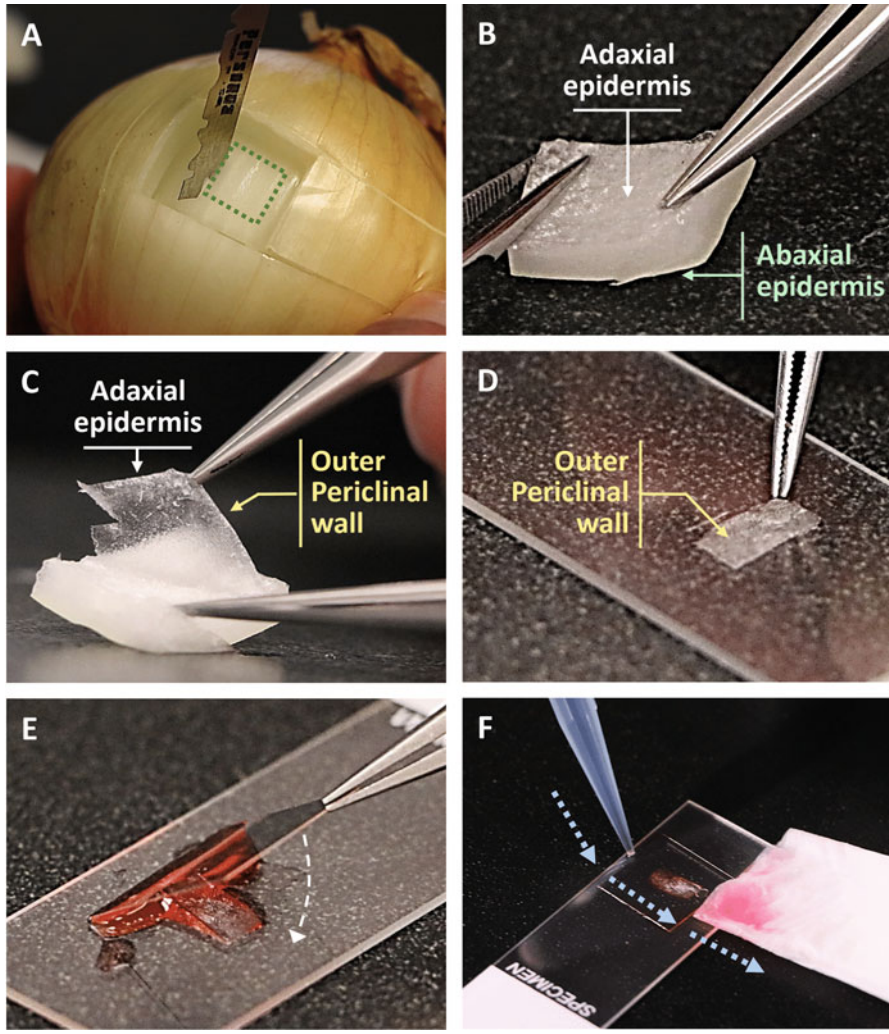

Fig. 1 Setup for staining onion epidermal leaves. Cut a rectangular window of 1 cm^2 into the third leaf of the bulb (dashed square) (**a**). Place the excised tissue block with the adaxial epidermis facing up (**b**). Detach the adaxial epidermis with fine forceps (**c**), and place it on the slide, with the outer periclinal wall facing up (**d**). Cover the section with 150 μL of the desired staining solution. To avoid the formation of air bubbles, place one end of the coverslip on the slide, and slowly lower the other end using a forceps to cover the section (arrow) (**e**). Place a folded task wipe or filter paper at the edge of the coverslip to absorb the staining solution. Simultaneously, inject deionized water between the slide and coverslip from the opposite side. The blue arrows indicate the direction of the water flow from the pipette tip to the task wipe (**f**)

6. Detach the entire adaxial epidermis from the block of tissue and place it on the slide with the outer periclinal wall facing up (*see* **Note 7**) (Fig. 1d).
7. With the forceps, apply a gentle pressure on the margins of the epidermal layer to make the epidermis adhere to the slide.
8. Cover the section with 150 μL of staining solution.

9. Cover the section with a coverslip (*see* **Note 8**). Do not apply pressure on the sample (Fig. 1e).
10. Place the slide on ice in the dark. Wait 30 min (*see* **Note 9**).
11. Place a folded task wipe or filter paper at the edge of the coverslip to absorb the staining solution. At the same time, inject deionized water between the slide and coverslip from the opposite side using a pipette (*see* **Note 10**) (Fig. 1f).
12. Using this technique, rinse each specimen with 15 mL of water.
13. Place the slides on ice until ready to observe (*see* **Note 11**).

3.2 Staining of Arabidopsis thaliana Seedlings

1. Fill a 1.5-mL Eppendorf tube with 0.7 mL of staining solution.
2. Delicately retrieve an *Arabidopsis* seedling from the Petri dish (*see* **Note 12**), and plunge it into the staining solution (*see* **Note 13**).
3. Add another 0.7 mL of staining solution to the tube and close it.
4. Cover the tube with aluminum foil (or any opaque material).
5. Place the tube on an orbital shaker at 175 rpm for 40 min.
6. Gently remove the staining solution with a disposable pipette without touching the seedlings.
7. Add 1 mL of water.
8. Slowly inverse the tube thrice and remove the water.
9. Add 1 mL of water, cover the tube with aluminum foil, and place on an orbital shaker at 175 rpm for 5 min.
10. Repeat the last step twice (*see* **Note 14**).
11. Put 150 μL of water on a slide.
12. Place the entire seedling or the organ of interest in the water with the area of interest facing up (*see* **Notes 15** and **16**).
13. Cover the sample with a coverslip (*see* **Note 8**).
14. Observe the sample immediately (*see* **Notes 11** and **17**).

3.3 Tips for Imaging Using a Confocal Laser Scanning Microscope

The purpose of this sub-section is to provide ways of minimizing stress caused by prolonged exposure to the excitation light of the fluorescence microscope. As a general rule, use the lowest light intensity possible and image as fast as possible. Figure 2 shows examples of stained leaves of *Arabidopsis* and onion with the above-mentioned dyes. Figure 3 shows examples of artifacts indicative of stress through suboptimal imaging conditions.

1. Turn on and prepare the microscope system at least 30 min prior to your samples being ready to image (*see* **Note 18**) (Table 1).

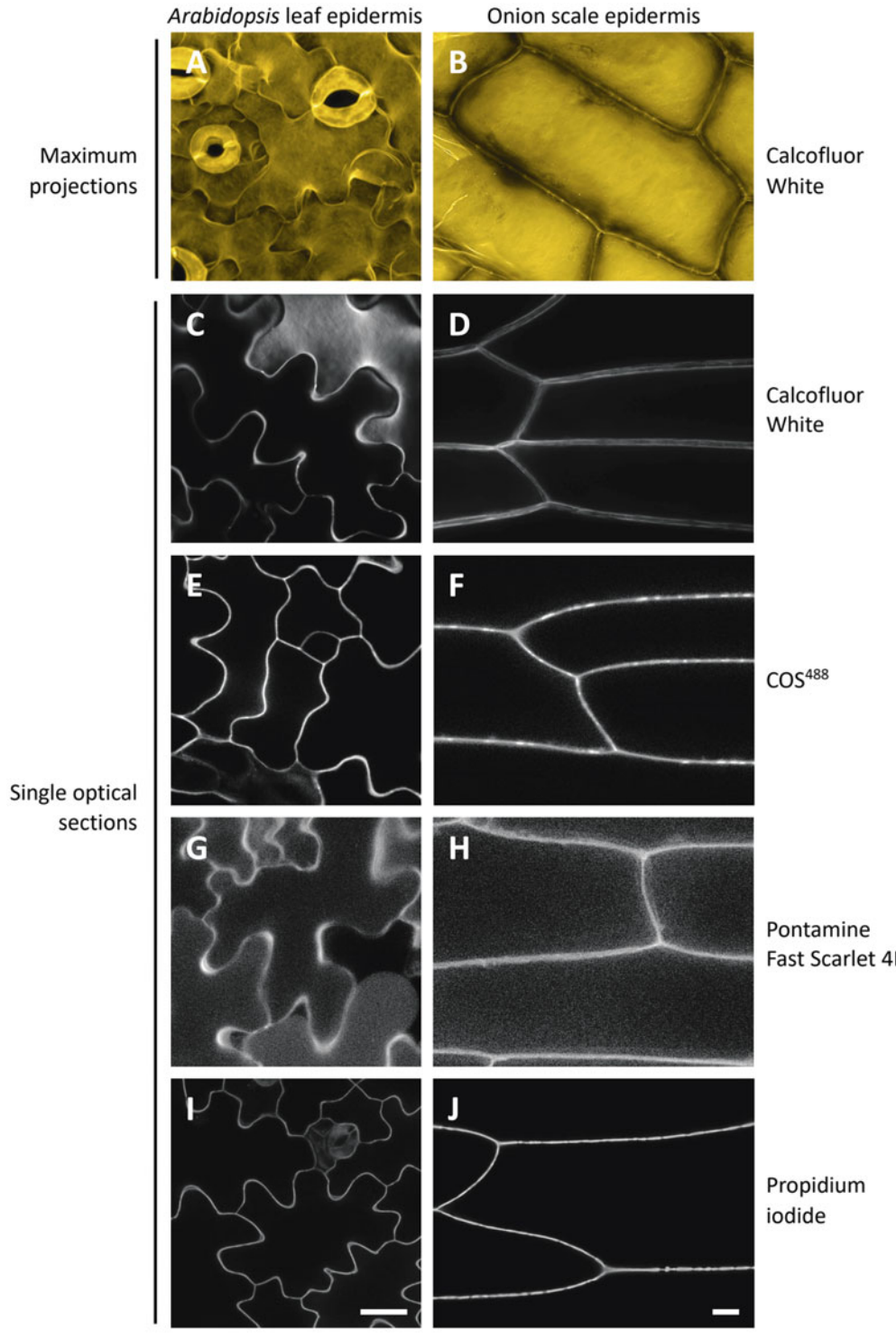

Fig. 2 Maximum intensity projections (**a**, **b**; false colored) and single optical sections (**b**–**j**) of *Arabidopsis* (**a**, **c**, **e**, **g**, **i**) and onion (**b**, **d**, **f**, **h**, **j**) leaf epidermal cells stained with Calcofluor White (**a**–**d**), COS^{488} (**e**, **f**), Pontamine Fast Scarlet 4B (**g**, **h**), and propidium iodide (**i**, **j**). Scale bars = 20 μm. Bar in (**i**) applies to left column, bar in (**j**) to right column

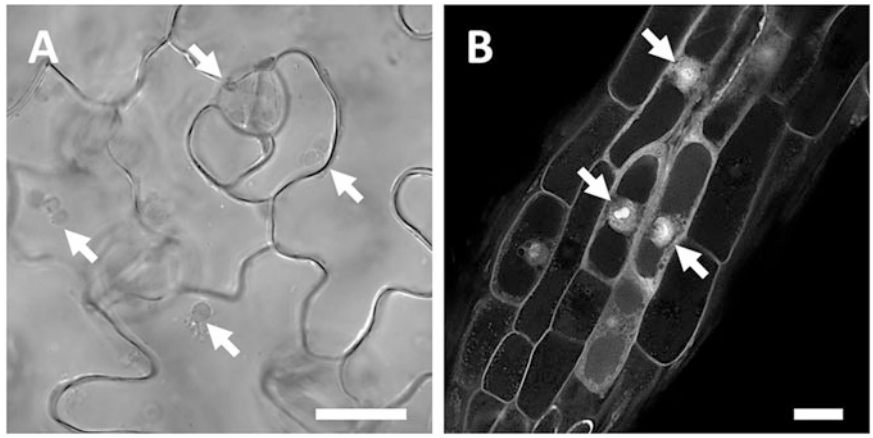

Fig. 3 Samples showing stress symptoms during live imaging. Bright-field image of an *Arabidopsis thaliana* leaf (**a**). The formation of spherical vesicle-shaped structures (arrows) within the cells is an indicator that the cells are stressed by the imaging process. The stress could be induced by the mechanical pressure created by the slide and the coverslip on the sample and/or by the phototoxic effect of the laser. Fluorescence micrograph (confocal, single optical section) of *Arabidopsis* root epidermal cells stained with propidium iodide (**b**). In healthy plant samples, propidium iodide only stains the cell wall and does not traverse the plasma membrane. In stressed or dead cells, the plasma membrane becomes permeable to propidium iodide which then predominantly stains the nuclei as it binds to DNA (arrows). Scale bars = 20 μm

Table 1
Image acquisition parameters for each dye

Dye	Excitation (nm)	Emission (nm)	Laser on the system	Laser power (%)	Detector gain	Dwell time (μs/pixel)
Propidium iodide	543	566–618	543 nm (HeNe, 1 mW)	2	480	1.44
COS^{488}	488	510–610	488 nm, (Argon, 25 mW)	2	445	1.00
S4B	514	520–620	514 nm (Argon, 25 mW)	3.5	755	1.4
Calcofluor White	405	410–523	405 nm (Diode, 30 mW)	2	427	0.90

For each acquisition, the pinhole was adjusted to 1 AU, and the scanning was performed bidirectionally. The acquisition was performed with a Zeiss LSM710 laser scanning confocal microscope equipped with the following objectives: Plan-Apochromat 20×/0.8 and Plan-Apochromat 40×/1.4 oil

2. Use the objective that suits your experimental needs. Preference must be given to objectives with longer working distance and high numerical aperture, to maximize the spatial resolution while preventing the application of a physical pressure on the sample (*see* **Note 19**).

3. When imaging, use the minimum required laser power to minimize phototoxicity and local heating of the sample (*see* **Note 20**) (Table 1).
4. Adjust the pinhole to 1 AU (airy unit) (*see* **Note 21**).
5. To reduce the total exposure time of the sample to the laser light, perform bidirectional scanning instead of unidirectional scanning (*see* **Note 22**), and use the "crop" function to only image the region of interest. Do not image the part of the sample that is not of interest or out of focus.
6. Adjust the detector gain sensitivity to obtain a clear image. Make sure there is no saturated pixel in the image (*see* **Note 23**) (Table 1).
7. If the emission spectrum of the dye overlaps with the autofluorescence of the chlorophyll in the sample, adjust the emission bandwidth of the spectral detector to eliminate the autofluorescence (*see* **Note 24**).
8. Image the samples as fast as possible to minimize phototoxicity. As a rule of thumb, a 1024 × 1024 pixel image should be imaged in less than 30 s (Table 1).
9. Imaging software offers the possibility of scanning the sample multiple times to obtain an averaged image that has an improved signal to noise ratio. Use this feature cautiously so as not to damage the sample through excessive irradiation. Do not reimage the same area twice.
10. Always take a bright-field image to assess the health/status of the sample (Fig. 3a).
11. Do not spend more than 15 min imaging the same sample. It is advisable to prepare multiple samples and image them sequentially rather than to image one sample repeatedly. Prepare the samples as you go.
12. If time-lapse imaging over a period of a few hours is required, gently remove the coverslip after 10 min of imaging, and place the sample back in water or half-MS liquid media to allow recovery between scans (*see* **Note 25**). Depending on the time elapsed between one acquisition and the next, re-staining of the sample might be necessary.
13. Image the negative control sample using the exact same microscope settings as the treated sample. There should be no signal in the fluorescence image other than autofluorescence if present. If there is unexplained signal, troubleshooting should be done to check for possible contamination with dyes or other substances.

4 Notes

1. For the purpose of this chapter, we used yellow onion bulb leaves. White onions are also suitable.
2. For more information on how to grow *Arabidopsis* seedlings on half-MS medium, please refer to [10].
3. We tested the propidium iodide from Fisher Scientific and Sigma-Aldrich, and both worked perfectly at the indicated concentrations.
4. Go as deep as you can with the blade to get a clear and clean cut of the leaf. The smaller the area, the easier it will be to handle in the next steps. Do not cut more than 1 cm^2.
5. Each onion leaf is made of two epidermal layers sandwiching mesophyll and vascular tissues. The outer epidermal layer (the one facing the outside of the bulb) is the abaxial epidermis, and the inner epidermal layer (facing the center of the onion bulb) is the adaxial epidermis.
6. While removing the cut section of the third leaf, the adaxial epidermis can remain stuck to the fourth leaf. If this happens, gently pick up the epidermal layer with the forceps, and place it on the slide.
7. When peeling the epidermis from the tissue block, a few mesophyll cells may detach and adhere to the epidermis. This generally is not a problem. Make sure you place the side of the epidermis that was in contact with the mesophyll on the slide. This facilitates imaging, as the smooth outer periclinal wall faces up (i.e., facing the coverslip).
8. Glass coverslips should have a thickness of 0.17 mm (#1.5). To avoid the formation of air bubbles, place one end of the coverslip on the slide, and slowly lower the other end using a forceps.
9. The staining can be done in the fridge at 4 °C or on the bench on a layer of ice. If the experiment is performed on the bench, cover the slides with aluminum foil or any other opaque material during incubation.
10. This step might take a little practice to master. Once the absorbent paper starts absorbing the dye, make sure that the water is released at the opposite end of the coverslip. The trick is to never interrupt the flow of water. Change the absorbent paper frequently.
11. Image the sample within 30 min. Waiting longer can result in artifacts induced by imaging-related stress. If you need to image more than one sample, prepare them as you go.
12. Place the extremities of a fine forceps under the cotyledons of the seedling. Gently close the forceps without squeezing the

hypocotyl and slowly retrieve the seedling without breaking the root.

13. Deposit the seedling at the bottom of the tube. Make sure it is well immersed in the staining solution.
14. Place the tubes on ice until ready to image.
15. The area that will be imaged should be facing the coverslip.
16. If you need to perform a time-lapse series or subsequent set of experiments with the same seedling, carefully mount the whole seedling on the slide with fine forceps. Take care not to damage any organ. If you do not need to reuse the same seedling, it is more practical for imaging to dissect the organ of interest using a razor blade or scalpel before mounting between a slide and a coverslip.
17. Make sure the coverslip sticks to the slide by applying a light pressure at the corners of the coverslip. Do *not* squeeze the sample between the slide and the coverslip. The surface tension created at the interface of the water and glass is sufficient to hold the coverslip on the slide. If needed, add a few microliters of water to hold the slide on the coverslip.
18. Turn on the system, turn on and warm up the lasers, and set up the system for the experiment.
19. Objectives with both long working distance and high numerical aperture (NA) might not be available on all microscopes. If a high spatial resolution is not required for the experiment, use a lower NA objective with a long working distance. If high resolution is required, use a high NA objective with the appropriate immersion medium and acquire the images as fast as possible.
20. On most imaging software, laser power is displayed as percentage. For the purpose of this chapter, the laser powers used are summarized in Table 1.
21. Choose a pinhole opening between 0.6 and 1.2 AU. A smaller pinhole will result in a higher lateral and axial resolution but will reduce the amount of signal detected. For the figures shown in this chapter, the pinhole was adjusted to 1 AU.
22. On many confocal laser scanning systems, a quick calibration of the directional scanning should be performed before acquiring the image. Refer to the user manual of the microscope.
23. Most imaging system software are equipped with a function allowing the user to verify if fluorescence intensity values are within the dynamic range of the detector and highlights pixels that are saturated. Adjust the detector gain sensitivity to have not more than one or two saturated pixels in the entire image. If you intend to acquire a Z-stack, the gain should be adjusted

identically throughout the stack by changing the focal plane manually and checking that all images obtained at the different planes are within the dynamic range of the detectors.

24. Chlorophyll (present in the mesophyll and guard cells) exhibits very strong autofluorescence in red, with a peak emission at approximately 680 nm. To eliminate the autofluorescence from the images, set the spectral detector's upper limit to 620 nm. Make sure that no autofluorescence is captured when imaging the negative control sample. If no spectral detector is available on your system, choose a bandwidth emission filter with an upper limit of 620 nm.
25. Slide a fine forceps between the slide and the coverslip. While holding the slide with one hand, gently lift the coverslip, and flip it on its back. Retrieve the sample.

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