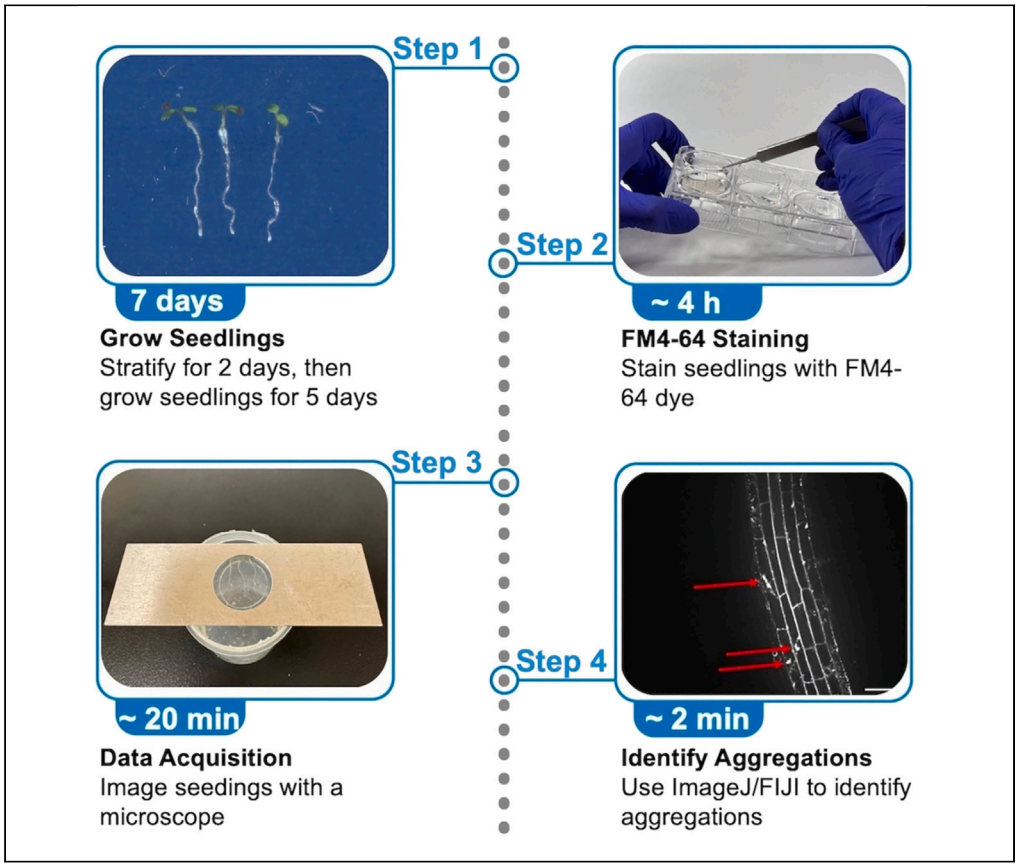
STAR Protocols

CellPress
OPEN ACCESS

Protocol

Protocol for detecting intracellular aggregations in *Arabidopsis thaliana* cell wall mutants using FM4-64 staining



Here, we present a step-by step protocol to visualize intracellular aggregations in *Arabidopsis* mutants with cell wall secretion defects using FM4-64, a lipophilic styryl dye. We describe steps for growing seedlings, staining them with FM4-64, and identifying intracellular aggregates in cell wall synthesis and/or secretion mutants in root and hypocotyl epidermal cells via confocal microscopy. Additionally, we provide troubleshooting suggestions for common pitfalls.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Fabrizio Chow, Eskandar Mohammad, Heather E. McFarlane

h.mcfarlane@utoronto.ca

Highlights

Protocol to detect intracellular aggregates in *Arabidopsis* cell wall synthesis/secretion mutants

Instructions for staining and imaging aggregations in live root or hypocotyl cells

Guidance and troubleshooting on distinguishing aggregations from common artifacts

Chow et al., STAR Protocols 6, 103665
March 21, 2025
https://doi.org/10.1016/j.xpro.2025.103665

STAR Protocols

CellPress
OPEN ACCESS

Protocol

Protocol for detecting intracellular aggregations in *Arabidopsis thaliana* cell wall mutants using FM4-64 staining

Fabrizio Chow,[1,2] Eskandar Mohammad,[1] and Heather E. McFarlane[1,3,*]

[1]Department of Cell and Systems Biology, University of Toronto, 25 Harbord Street, Toronto, ON M5S 3GS, Canada

[2]Technical contact

[3]Lead contact

*Correspondence: h.mcfarlane@utoronto.ca
https://doi.org/10.1016/j.xpro.2025.103665

SUMMARY

Here, we present a step-by step protocol to visualize intracellular aggregations in *Arabidopsis* mutants with cell wall secretion defects using FM4-64, a lipophilic styryl dye. We describe steps for growing seedlings, staining them with FM4-64, and identifying intracellular aggregates in cell wall synthesis and/or secretion mutants in root and hypocotyl epidermal cells via confocal microscopy. Additionally, we provide troubleshooting suggestions for common pitfalls.

For complete details on the use and execution of this protocol, please refer to Hoffmann and McFarlane.[1]

BEFORE YOU BEGIN

The cell wall exists in a constant state of remodeling to respond to biotic and abiotic signals.[2] These responses require precise coordination of the endomembrane system.[3] In the model plant *Arabidopsis thaliana*, mutants lacking key proteins required for synthesis and/or trafficking of matrix polysaccharides to the cell wall can display various phenotypic abnormalities including dwarfism and intracellular aggregations of cell wall components.[1,4–7] Here, we describe a protocol using the lipophilic styryl dye, FM4-64, to visualize intracellular aggregations in Arabidopsis mutants defective in cell wall synthesis and/or secretion. This protocol outlines sample preparation, visualization, and analysis of intracellular aggregations in root and hypocotyl epidermal cells via confocal microscopy, using Arabidopsis Columbia-0 wild type, plus *mur3*,[8] *ech*,[9] and *yip4a yip4b*[5] mutants as examples. We have also used this protocol to visualize intracellular aggregations in other cell wall mutants and using several different microscopy setups.[1] We provide comprehensive troubleshooting information for effective analysis. Methods used here are as described and executed in Hoffmann and McFarlane, 2024.[1]

Preparation of $^1/_2$ MS media

Timing: 2 h

The following steps are to prepare solid growth media for *Arabidopsis* seedlings and liquid media for staining seedlings.

1. Dissolve 881.0 mg of Murashige & Skoog Medium (including vitamins) (final concentration: half-strength) and 195.3 mg of MES monohydrate (final concentration: 2.5 mM) in 350 mL of ddH_2O.
2. Adjust pH to 5.8 with 1 M KOH solution.
3. Add 2.8 g of agar (final concentration: 0.7% w/v).

4. Adjust volume to 400 mL with ddH_2O.
5. Autoclave on liquid cycle for 20 min at 121°C.
6. In a sterile environment, add 20 mL of sterile 20% sucrose solution (final concentration: 1% w/v) and stir well.
7. Allow the media to cool slightly, and pour into 10 square petri dishes.
8. Let the plates cool until solidified and store at 4°C for up to 4 weeks.

Note: To prepare $^1/_2$ MS liquid media omit step 3.

$^1/_2$ MS media with 1% Sucrose

Reagent	Final concentration	Amount
Murashige & Skoog Medium (including vitamins)[a]	N/A	881.0 mg
MES [2(n-morpholine) ethane sulfonic acid] Monohydrate	2.5 mM	195.3 mg
Sucrose	1%	20 mL of 20% (w/v) solution
Agar (omit for liquid media)	0.7%	2.8 g
ddH_2O	N/A	Adjust to 400 mL
Total	N/A	**400 mL**

Autoclaved media without sucrose can be stored at room temperature for up to 8 weeks; poured plates (including sucrose) can be stored at 4°C for up to 4 weeks.
[a]Please refer to the instructions outlined by the manufacturer to achieve $^1/_2$ strength MS media.

Growing *Arabidopsis* seedlings: Light-grown roots or etiolated hypocotyls

Timing: 7 days

The following steps are to grow *Arabidopsis* seedlings (roots or hypocotyls) on solid media.

9. Add 250 mL of bleach (6% sodium hypochlorite, final concentration: 3% v/v) and Triton X-100 (0.5 g, final concentration: 0.1% w/v) to 250 mL of ddH_2O to make 2x Seed Sterilization Solution.
10. Add 400 μL of ddH_2O and 400 μL of 2x Seed Sterilization Solution (1:1 ratio) to a microcentrifuge tube containing ~30 *Arabidopsis* seeds, mix continuously by inversion for 10 min.
11. In a sterile environment, wash seeds with 800 μL of sterile ddH_2O 5 times.
12. Use a sterile 1000 μL pipette tip to individually transfer seeds onto the $^1/_2$ MS plate.

Note: To ensure that there is sufficient space between seeds to allow for root or hypocotyl growth, as required (e.g., 3 rows of 30 seeds per row on 12 cm square plates), consider using a template (Figure S1).

13. Seal plates using micropore tape.
14. Wrap plates in aluminum foil to protect from light and stratify for 2 days at 4°C.
15. After 2 days of stratification.
    a. For Roots:
        i. Remove aluminum foil.
        ii. Place plates vertically in a growth chamber for 5 days.

Note: Growth chamber conditions: long day photoperiod (18 h light, 6 h dark), ~120 mmol $m^{-2}s^{-1}$ photosynthetically active radiation (PAR), 21°C.

    b. For Hypocotyls:
        i. Remove aluminum foil.
        ii. Place plates vertically in the growth chamber for 3 h of light exposure.

Note: Growth chamber conditions: ~120 mmol $m^{-2}s^{-1}$ PAR, 21°C.

iii. After 3 h of light exposure, re-wrap plates in aluminum foil.
iv. Incubate plates vertically in the growth chamber for 5 days, protected from light.

Note: Growth chamber conditions: dark, 21°C.

2x Seed Sterilization Stock Solution		
Reagent	Final concentration	Amount
Bleach (6% sodium hypochlorite)	3% (v/v)	250 mL
ddH_2O	N/A	Adjust to 500 mL
Triton X-100	0.1% (w/v)	0.5 g
Total	N/A	**500 mL**
Store away from any light at 4°C for up to 8 weeks.		

Seed Sterilization Working Solution		
Reagent	Final concentration	Amount
2x Seed Sterilization Stock Solution	1x (1.5% sodium hypochlorite, 0.05% Triton X-100)	400 µL
ddH_2O	N/A	400 µL
Total	N/A	**800 µL per Microcentrifuge tube**

Preparation of agarose pads

Timing: 10 min

The following steps are to prepare agarose pads required to mount samples for imaging on an inverted microscope.

Note: Preparation of agarose pads does not require sterile conditions; any number of agarose pads can be prepared at one time, but they should be prepared fresh each day.

16. Prepare a 0.8% agarose solution by adding 0.4 g of agarose to 50 mL of ddH_2O. Microwave until the solution begins to boil and the agarose is fully dissolved. Allow it to cool slightly (~5 min).
17. Prepare a hydration chamber with lid (e.g., a petri dish or similar): line the bottom of the container with parafilm, and attach a folded and wet Kimwipe to the underside of the lid.
18. Place round coverslips (one per sample that will be imaged) onto the parafilm-lined petri dish.
19. Pipette 100 µL of 0.8% agarose solution onto a round coverslip, then place another round coverslip on top to create a sandwich-like structure with the agarose in between.

Note: In open air, agarose pads may dry out within ~2 h. By storing them in a closed, humidified environment, they can remain usable for one day.

Preparation of FM4-64

Timing: 5 min

The following steps are to prepare a solution of FM4-64 for staining intracellular aggregates in *Arabidopsis* seedlings.

20. Add 10.97 µL of dimethyl sulfoxide (DMSO) directly to a 100 µg aliquot of FM4-64 (molecular weight: 607.5136 g/mol) to prepare a 15 mM stock solution of FM4-64.

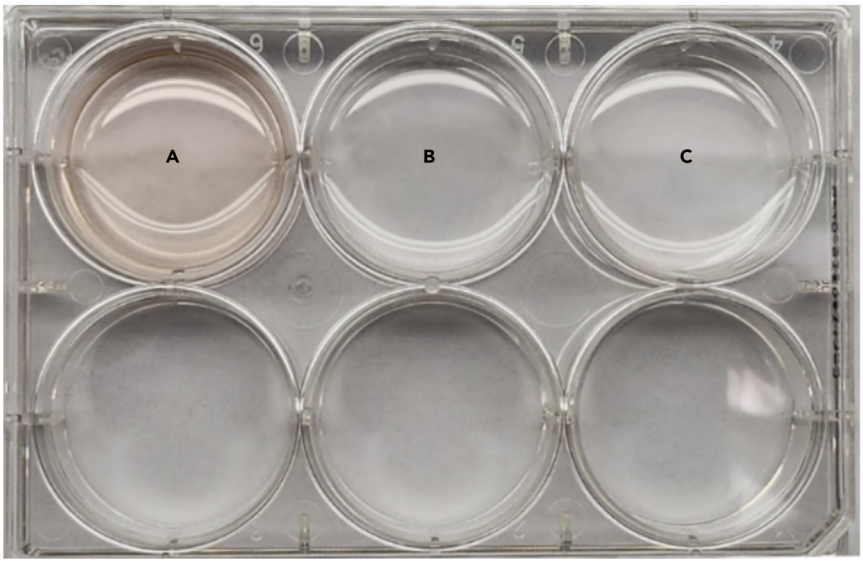


Figure 1. Preparation of a 6-well plate for FM4-64 staining

(A) Staining solution: 5700 μL of $^{1}/_{2}$ MS liquid + 300 μL of 20% sucrose (final concentration: 1%) + 0.6 μL of 15 mM FM4-64 (final concentration: 1.5 μM).

(B and C) Wash 1 and Wash 2/mounting solution: 5700 μL of $^{1}/_{2}$ MS + 300 μL of 20% sucrose (final concentration: 1%) for each.

21. Store the solution at −20°C, protected from light, until use and avoid multiple freeze-thaw cycles.
22. Add 5700 μL of $^{1}/_{2}$ MS liquid to 3 wells of a 6-well plate followed by 300 μL of 20% sucrose solution (final concentration: 1% w/v) (Figure 1).
23. Add 0.6 μL of 15 mM FM4-64 dye (final concentration: 1.5 μM) to the first well only (Figure 1A). This well will be used for staining the samples, while the other wells will be used for washing and mounting.

15 mM FM4-64 Stock Solution

Reagent	Final concentration	Amount
FM4-64 Dye (*N*-(3-Triethylammoniumpropyl)-4-(6-(4-(Diethylamino) Phenyl) Hexatrienyl) Pyridinium Dibromide) (molecular weight: 607.5136 g/mol)	15 mM	100 μg
Dimethyl Sulfoxide	N/A	10.97 μL
Total	N/A	**10.97 μL**

Store away from any light at −20°C for up to 2 months.

1.5 μM FM4-64 Staining Solution

Reagent	Final concentration	Amount
15 mM FM4-64 Stock Solution	1.5 μM	0.6 μL
$^{1}/_{2}$ MS liquid media	N/A	5700 μL
20% Sucrose solution	1%	300 μL
Total	N/A	**6 mL**

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Murashige & Skoog medium (including vitamins)	PhytoTechnology Laboratories	Catalog No. M519-50L
MES [2(n-morpholine) ethane sulfonic acid] monohydrate	Fisher Scientific	Catalog No. BP300-100
Potassium hydroxide pellets	Fisher Scientific	Catalog No. P251-500
BD Bacto dehydrated agar	BD	Catalog No. 214010
UltraPure agarose	Thermo Scientific	Catalog No. 16500500
Sucrose	Fisher Scientific	Catalog No. BP-220-212
Household bleach (6% sodium hypochlorite solution)	Lavo Pro	e.g., SKU: 1952D
Triton X-100	Fisher Scientific	Catalog No. BP151.500
Dimethyl sulfoxide (DMSO)	Fisher Scientific	Catalog No. D128-1
FM4-64 dye (*N*-(3-triethylammoniumpropyl)-4-(6-(4-(diethylamino) phenyl) hexatrienyl) pyridinium dibromide)	Invitrogen	Catalog No. T3166
Arabidopsis thaliana seeds	Arabidopsis Biological Resource Center (ABRC)	e.g., Col-0: CS70000 e.g., *mur3-3*: SALK_141953
Software and algorithms		
Fiji	Schindelin et al., 2012[10]	https://imagej.net/software/fiji/
Nikon NIS Elements Software	Nikon	https://www.microscope.healthcare.nikon.com/products/software/nis-elements (Version 6.02.03)
Other		
Growth chamber	Conviron	e.g., Model No. PGC20
Refrigerator	Whirlpool	e.g., Model WRR56X 18FW02
P20 Pipette	Rainin	e.g., 17014412
P200 Pipette	Rainin	e.g., 17014411
P1000 Pipette	Rainin	e.g., 17014407
Inverted spinning disk confocal microscope	Nikon	Eclipse Ti2-E equipped with a 60 mW 488 nM laser (LUN-F XL), and a Photometrics Prime 95B-22 mm Back-Illuminated sCMOS Camera.
40x N.A. 1.3 (Plan Fluor corrected) oil-immersion objective	Nikon	Material No. MRH01401
Immersion oil	Nikon	e.g., SKU: MXA22168
Metal slide (with round opening)	Custom made (refer to Verbančič et al.[11])	N/A
Silicon vacuum grease	Beckman Coulter	Catalog No. 335148
Rectangular cover glasses	Fisherbrand	Catalog No. 12541019CA
Round micro cover glasses	Matsunami Glass	Catalog No. C015001
Square Petri dishes 120 W x 120 L x 17H mm	Greiner Bio-One	Catalog No. 688161
Microcentrifuge tubes	Fisher Scientific	Catalog No. 05-541-38
Aluminum foil	U-LINE	e.g., Catalog No. S-22909
Pipet tips	VWR	e.g., Catalog No. 76323-456
3M Micropore surgical paper tape	Fisher Scientific	Catalog No. 19-027761
Forceps	Fisherbrand	e.g., Catalog No.12-000-131
Bemis Parafilm M laboratory wrapping film	Bemis	Catalog No. PM999
Kimberly-Clark Science Kimwipes	Fisher Scientific	e.g., Catalog No. 06-666-2
6-well plate	VWR	e.g., Catalog No. 10861-554

STEP-BY-STEP METHOD DETAILS

Staining procedure: Roots

Timing: 30 min

The following steps describe how to stain *Arabidopsis* roots for visualization of intracellular aggregates.

1. Using fine forceps, pick up a seedling by the cotyledons without closing the forceps and carefully transfer seedlings from solid media plates into the 6-well-plate containing liquid media and FM4-64 (Figure 1A), ensuring that the seedling is fully submerged.

   Note: Do not close forceps on the tissue; rather use forceps to gently lift the seedling into the media.

2. Incubate for 30 min. Cover the plate in aluminum foil as FM4-64 is light sensitive.
3. Immediately before imaging, gently dip the seedling into each of the 2 wells of liquid media without FM4-64 for ~5 s (Figures 1B and 1C).

Staining procedure: Hypocotyls

Timing: 4 h

The following steps describe how to stain *Arabidopsis* etiolated hypocotyls for visualization of intracellular aggregates.

4. Using fine forceps, pick up a seedling at the root without closing the forceps and carefully transfer seedlings from solid media plates into the 6-well-plate containing liquid media and FM4-64 (Figure 1A), ensuring that the seedling is fully submerged.

   Note: Do not close forceps on the tissue; rather use forceps to gently lift the seedling into the media.

5. Incubate for 4 h. Cover the plate in aluminum foil as FM4-64 is light sensitive.
6. Immediately before imaging, gently dip the seedling into each of the 2 wells of liquid media without FM4-64 for ~5 s (Figures 1B and 1C).

Sample mounting for confocal microscopy: Roots or hypocotyls

Timing: ~2 min per sample; proceed directly to step 11 after mounting each sample

The following steps describe how to mount either roots or hypocotyls for visualization of intracellular aggregates.

7. Prepare a metal slide for imaging:
   a. Add a small amount (~toothpick tip sized amount, ~5 μL) of silicone vacuum grease on each side of the metal slide opening.
   b. Add a 22 × 32 mm coverslip and firmly press to secure the cover slip onto the metal slide. Avoid touching the center of the coverslip (i.e., the area through which light will travel during imaging).
   c. Flip the slide over and place it on a stand to keep the coverslip from touching other surfaces.
8. Transfer seedlings to the slide:
   a. Using a P20 pipette, pipette 3 droplets, ~4 μL each, of sterile $^1/_2$ MS liquid solution to the center of the coverslip. Ensure there is enough space between each droplet so that the droplets do not aggregate.
   b. For Roots:
      i. Using fine forceps, pick up a seedling by the cotyledons without closing the forceps and transfer the seedling to one of the drops of liquid on the prepared cover slip, placing the tip of the root onto one of the droplets.
      ii. Place a total of three seedlings onto the coverslip.

c. For Hypocotyls:
   i. Using fine forceps pick up a seedling at the root without closing the forceps and transfer the seedling to one of the drops of liquid on the prepared cover slip, placing the etiolated seedling onto of one of the droplets.
   ii. Place a total of three seedlings onto the coverslip.

9. Mount the samples:
   a. Grab an agarose pad and separate the two round coverslips. The agarose will remain on one of the round coverslips; discard the coverslip without the agarose pad.
   b. For Roots: Place the round coverslip, agarose pad face down, below the cotyledons so that it completely covers the root from the tip, but the cotyledons are not covered.
   c. For Hypocotyls: Place the round coverslip, agarose pad face down, over the etiolated hypocotyls so that the hypocotyls are completely covered; the roots may not be completely covered.
10. Steps 7–9 above describe sample mounting for imaging on an inverted microscope. Alternatively, for imaging on an upright microscope, create an imaging space using double-sided tape:
   a. Do not prepare any agarose pads.
   b. Place two small pieces of double-sided tape to each side of the center of a glass slide, leaving ~20 mm between.
   c. Transfer samples to the center of the glass slide according to step 8, then place a 22 × 32 mm coverslip on top of the seedlings. The double-sided tape will create a small space on the slide to ensure that the seedlings are not damaged by the coverslip.

⚠ CRITICAL: Handle the seedlings with care to avoid applying pressure, as this can damage the cells and make it difficult to identify aggregations (see troubleshooting steps below).

⚠ CRITICAL: Complete the mounting process quickly to minimize light exposure of the FM4-64 dye and to ensure that the seedling does not dry during this process. Proceed immediately to imaging (Step 11) after each sample (3 seedlings) is mounted.

⚠ CRITICAL: Ensure that the agarose pad does not touch the rim of the metal slide, otherwise this can wick water away between the rectangular cover slip and the metal slide, which can cause sample movement during imaging.

⚠ CRITICAL: Ensure no air bubbles are trapped under the coverslip. If bubbles form, add more solution between the coverslip and the slide, and use a tissue to absorb any excess liquid and to stabilize the sample.

Visualization of intracellular cell wall aggregates via confocal microscopy

Timing: Depending on the sample numbers, each slide should take ~20 min

The following steps outline visualization of root and hypocotyl epidermal cell aggregations in cell wall mutants.

Note: Here, we use an inverted spinning disk confocal microscope (e.g., Nikon Eclipse Ti2-E with a CSU-W1 spinning disk head), an sCMOS camera (Photometrics Prime 95B), and a 40x N.A. 1.3 oil immersion objective, and the Nikon NIS Elements Software. A 561 nm excitation laser (70 mW at 24% power) is used to excite FM4-64. Fluorescence emission is collected using a band emission filter of 605/52 nm for FM4-64. Because of the large size of the aggregations (~5 μm), a high N.A. oil immersion objective should not be required, and 40x water or glycerol immersion or air objectives should be suitable. This protocol has also been successfully applied using an upright Leica SP5 laser scanning confocal microscope with either a 63x N.A. 1.4 Plan Apo or a 40x N.A. 1.25 Plan Apo objective utilizing an Argon 514 nm laser for

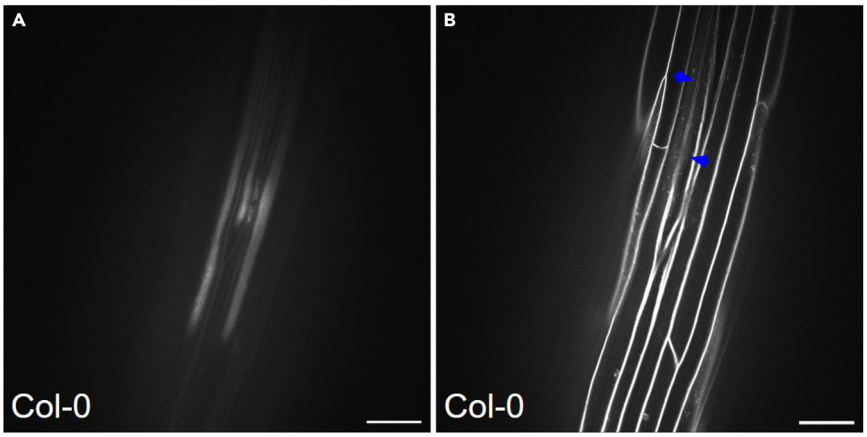


Figure 2. Images of 4 h FM4-64-stained hypocotyls illustrating depth for image collection

(A) Outer surface of wild-type Col-0 etiolated hypocotyl epidermal cells.

(B) Inner surface of wild-type Col-0 etiolated hypocotyl epidermal cells. Blue arrows indicate areas where deeper cell layers begin to come into focus. Scale bars represent 50 μm.

excitation, with emission detected from 600–650 nm via a photomultiplier tube. Given the distinct differences between these spinning disk and laser scanning confocal microscope setups, we anticipate that any confocal microscope with appropriate excitation and emission parameters for FM4-64 would be suitable for executing this protocol. Many microscopes should be capable of imaging this fluorophore. Since FM4-64 has wide excitation and emission curves, it can also be excited with a 488 nm laser (i.e., "GFP") or a 514 nm laser (i.e., "YFP") and emission captured with an RFP filter. For microscopes with filters that can only image GFP emission, the blue-shifted FM1-43 dye should also be suitable for this protocol.

11. Set up imaging parameters:
    a. Initiate microscope and log into Nikon NIS Elements Software.
    b. Switch to the 40x objective lens.
    c. Add a drop of immersion oil onto the 40x objective lens and transfer the slide from the stand to the microscope.
    d. Use an excitation wavelength between 445–565 nm (e.g., 561 nm laser) and emission wavelength ranging from 580–840 nm (e.g., 605/52 nm filter).
12. Adjust the focus to locate the root or hypocotyl cells. Use an exposure time of 100–200 ms to avoid photobleaching the sample.
    a. For Roots: Locate root epidermal cells in late elongation zone (starting from the cells closest to the root tip and stopping up to the point just before the first root hairs appear) or maturation zone (after root hairs develop).
    b. For Hypocotyls: Locate mature hypocotyl epidermal cells 1–2 mm above the collet hairs at the hypocotyl-root junction.

Note: Aggregations are more visible in mature cells that have already undergone significant cell wall synthesis and cell elongation, as younger cells may not have synthesized enough material for large aggregations to be visible.[1]

13. Capture z-stack images with an exposure time of 500 ms, beginning from the outer surface of the epidermal of the cell, just before the plasma membrane comes into focus, and progressing to the inner surface of the epidermal cell, where the next cell layer of the subsequent cell in the root or hypocotyl becomes visible (Figure 2).

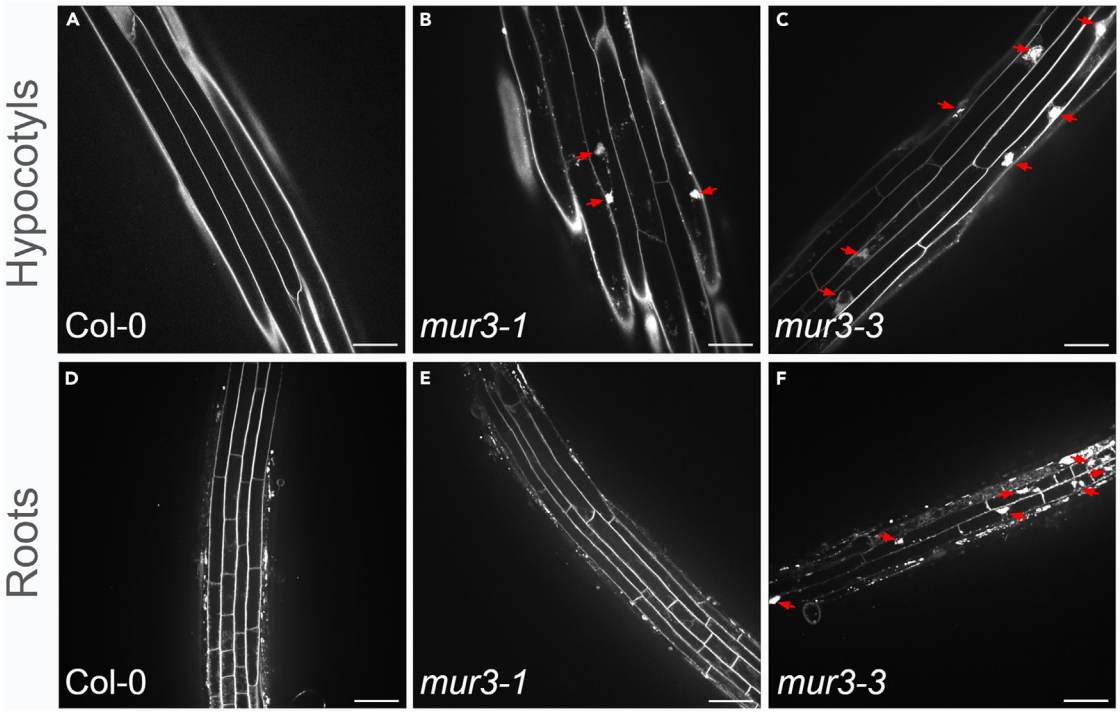


Figure 3. FM4-64 staining can be used to detect intracellular aggregations in cell wall synthesis mutants, but the severity of aggregations can differ depending on the nature of the mutation or the tissue examined

(A–C) Representative images of etiolated hypocotyl epidermal cells from (A) wild-type Col-0, (B) *mur3-1* (weak allele), and (C) *mur3-3* (strong allele). (D–F) Representative images of light-grown root late elongation zone epidermal cells from (D) wild-type Col-0, (E) *mur3-1*, and (F) *mur3-3*. Red arrows indicate locations of intracellular aggregations. Scale bars represent 50 μm.

Note: This process typically covers a 10–20 μm range, depending on the cell type and developmental stage (Figure 2). Use spacing between the images of the z-stack that is appropriate for the objective lens (0.3 μm for our 40x objective) to avoid over- or under-sampling.

14. Save images as uncompressed .tiff or .nd2 files.

⚠ CRITICAL: Cellular damage can be mistaken for cell wall aggregations. Care must be taken to avoid imaging damaged or dead cells (see troubleshooting steps below).

Analysis of intracellular cell wall aggregates via Fiji

Timing: ~2 min per sample

The following steps describe how to analyze intracellular aggregates.

15. Open the image in FIJI and click the "play" button in the bottom left to display all slices; alternatively, drag the slider at the bottom to manually scroll through each slice.
16. Scan through the focal planes to identify intracellular aggregations within the cell. Look for structures that differ from those typically observed in wild-type cells, since aggregations will appear as unusual accumulations within the cell (Figures 3, 4, and 5).

⚠ CRITICAL: Be cautious of "aggregations" observed in dead cells, as these may stain intensely and mimic true aggregations (see troubleshooting, Problem 5 and Figure 6). Moreover, when looking at root cells in the early elongation zone, be vigilant not to

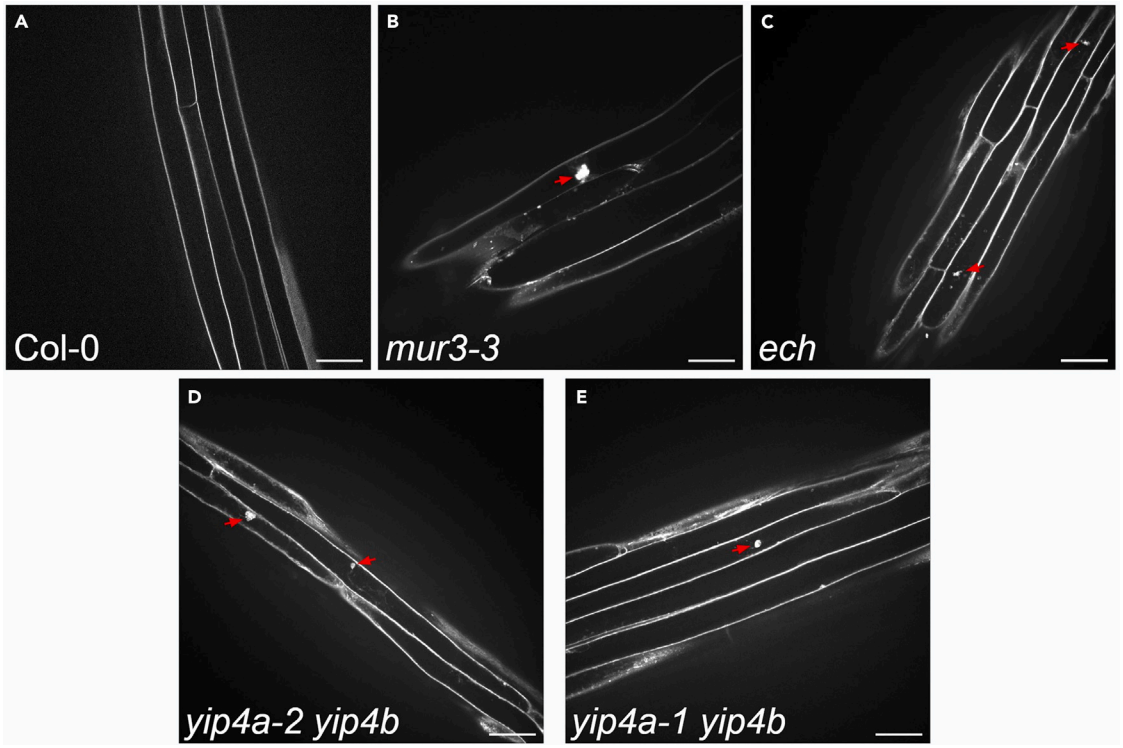


Figure 4. FM4-64 staining can be used to detect intracellular aggregations in cell wall secretion mutants

Representative images of etiolated hypocotyl epidermal cells of (A) Col-0, (B) *mur3-3*, (C) *ech*, (D) *yip4a-2 yip4b* (strong allele), and (E) *yip4a-1 yip4b* (weak allele). Red arrows highlight locations of intracellular aggregations. Scale bars represent 50 μm.

mistake root cap debris or endosomal staining for intracellular aggregations (see troubleshooting, problem 6, Figure 7; Figures 3D–3F). Similarly, when analyzing projections of z-stacks (e.g., Figure 5D), be mindful that debris or out-of-focus material near the cell surface can resemble intracellular aggregations. Carefully scan through all layers of the z-stack to differentiate true intracellular aggregations from artifacts.

EXPECTED OUTCOMES

This protocol is suitable for imaging FM4-64-stained aggregation phenotypes in a variety of mutants affecting cell wall polysaccharide synthesis and secretion. For example, *mur3* mutants affect a galactosyltransferase required for adding a galactose side chain onto the hemicellulose xyloglucan[8] and these mutants frequently display intracellular aggregations of cell wall material[1,4,7] (Figures 3, 4, and 5). Similarly, other mutants that affect xyloglucan synthesis and create xyloglucan lacking this galactose side chain also display FM4-64 aggregation phenotypes.[1] Polysaccharides also accumulate in mutants affecting the *trans*-Golgi Network-localized ECH/YIP4 complex.[5,6] Here we demonstrate that FM4-64 aggregation phenotypes are also detected in *ech* single mutants and *yip4a yip4b* double mutants (Figures 4C–4E).

This protocol is also appropriate for imaging FM4-64 aggregation phenotypes in either roots or hypocotyls, since it is sufficient to detect aggregations in either cell type of *mur3-3* mutants, but not in Col-0 cells of the same tissue (Figure 3). However, there can be phenotypic variation depending on the tissue type examined (Figure 3) or the mutant allele (Figures 3 and 4). Differences in the severity of the FM4-64 phenotypes across different alleles is correlated with the type of mutation (i.e., *mur3-3* and *yip4a-2 yip4b* are strong alleles while *mur3-1* and *yip4a-1 yip4b* are weaker alleles in which some functional protein is still produced[4,5]); however it remains unclear why some cells develop dramatic

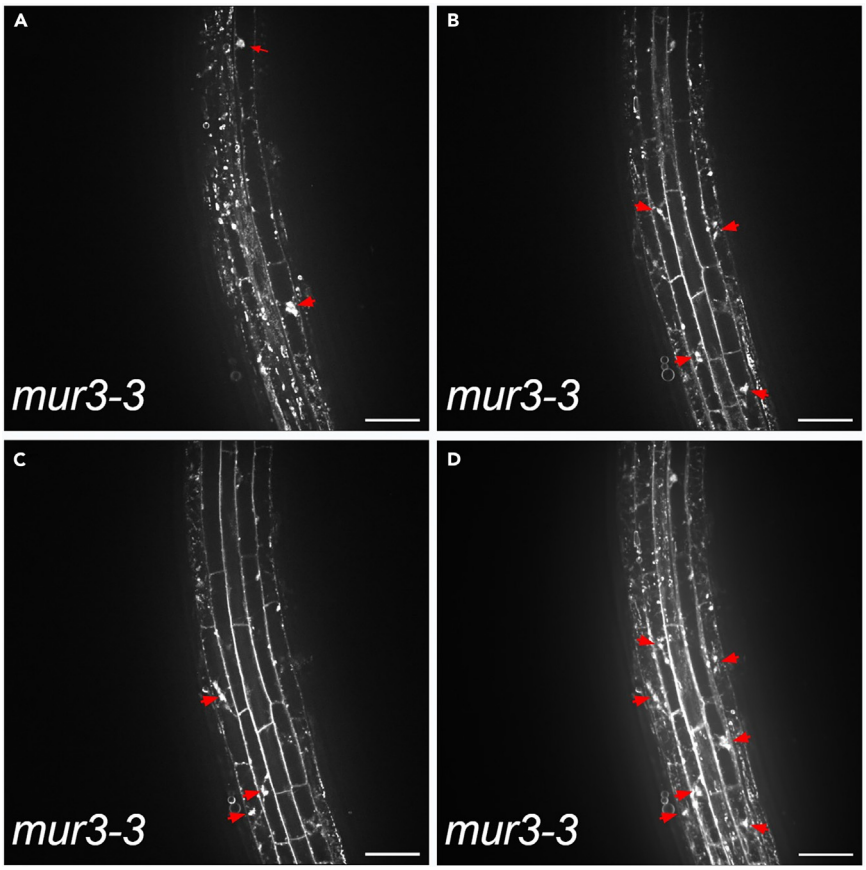


Figure 5. Intracellular aggregations can be detected though the thickness of cells

Z-stack imaging of *mur3-3* mutant light-grown root epidermal cells stained with FM4-64, illustrating deeper focal planes in the z-direction.

(A) Initial focal plane that includes parts of the surface and underlying epidermal tissue.

(B) Image captured 4 μm deeper than (A).

(C) Image captured 6 μm deeper relative to (A) and 2 μm deeper than (B).

(D) Average projection image of the entire z-stack. Red arrows indicate locations of intracellular aggregations. Scale bars represent 50 μm.

FM4-64 labeled aggregations in the weak alleles while other cells appear wild-type[1] (Figure 3B; Figures 4B–4E).

LIMITATIONS

In our initial study,[1] we used FM4-64 as a qualitative tool to screen for intracellular aggregates, based on its ability to strongly identify *mur3-3* aggregations.[7] Our screen found significant variation in aggregation size, abundance, and intensity across mutants with characterized or hypothesized defects in xyloglucan synthesis.[1] This variability may pose a challenge when attempting to identify aggregations in novel cell wall synthesis or trafficking mutants, particularly those with mild phenotypes and fewer aggregates. Additionally, uneven penetration of FM4-64 dye in epidermal cells could lead to false negatives. Dye penetration may be affected by different cell wall properties in cell wall synthesis/secretion mutants being studied. This penetration variability, along with aggregation variability among mutants, prevents quantification and direct comparison of the number of aggregations among different alleles, though aggregation size can be compared. Aggregation phenotypes may also be sensitive to abiotic conditions, including temperature,[4] or the inclusion of sucrose in the media.[1] To quantify and compare aggregation frequency among genotypes, we advise crossing a stable and constitutive marker line for the endomembrane system

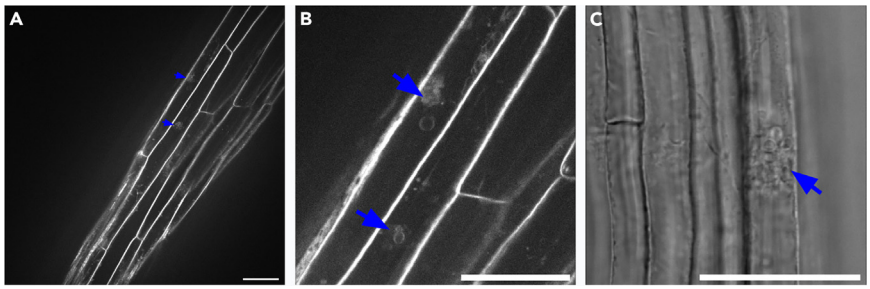


Figure 6. Intracellular debris from damaged or dead cells can be mistaken for cell wall aggregations

(A) Imaging of *yip4a-1 yip4b* etiolated hypocotyl epidermal cells stained with FM4-64, displaying apparent intracellular aggregations (blue arrows).

(B) Zoomed-in image at a deeper focal plane within the same root, revealing damaged intracellular structures such as vacuole shrinkage or fragmentation in dead cells.

(C) Bright-field microscopy of *ech* epidermal root cells showing comparable damaged intracellular structures visible without fluorescent staining. Scale bars represent 50 μm. See also Video S1.

(e.g., GFP-sSC[7] or GFP-NAG[12]), or using a double marker for the Golgi apparatus and cell outlines (e.g., RFP-NAG + GFP-LTI6b[1]), since these will consistently identify aggregations in most or all cells. It is important to note that FM4-64 has a low penetration capability and will primarily stain epidermal cells, and potentially stain cortical cells, so this protocol may not be applicable to studying secondary cell wall synthesis/secretion mutants without significant adaptation (e.g., using the inducible VND system to trigger secondary cell wall development in epidermal cells[13]). Despite these limitations, we have successfully used this protocol to screen a large number of cell wall synthesis mutants and identify trends in the FM4-64 aggregation phenotypes amongst different mutants that led to novel biological insights.[1]

TROUBLESHOOTING

Problem 1: Quantification of aggregates

In Steps 15 & 16, quantification and comparison of the frequency of intracellular aggregates in cell wall synthesis/trafficking mutants is unreliable due to unequal penetrance of the stain.

Potential solution

The size of intracellular aggregations can be measured and compared among genotypes. However, to confirm FM4-64 identified aggregates and to quantify the frequency of aggregations, we crossed a dual Golgi and plasma membrane fluorescent marker N-ACETYLGLUCOSAMINIE TRANSFERASE-RFP/ LOW-TEMPERATURE-INDUCED-PROTEIN6B-GFP (NAG-RFP/ LTI6B-GFP)[12,14] into lines that were positive for aggregations in our initial FM4-64 screen.[1] Clustering of Golgi stacks were used as a proxy for intracellular aggregations due to Golgi stacks being found within these aggregates.[1]

Problem 2: Uneven staining

In Steps 12 & 13, FM4-64 staining may appear uneven, particularly in root epidermal cells.

Potential solution

Uneven staining can occur because the seedlings tend to float on top of the staining solution in Steps 1 & 2 (for roots) or Steps 4 & 5 (for hypocotyls). Without complete submersion, some regions of the seedling may not be exposed to the dye, resulting in uneven or incomplete staining. To address this issue, ensure the seedlings are fully submerged in the staining solution in Steps 1 & 2 (for roots) or Steps 4 & 5 (for hypocotyls) by gently breaking the surface tension of the staining solution without damaging the tissue. Tilting the plate when submerging the seedlings into the incubation solution helps. Adding a small amount of detergent (e.g., <0.01% (v/v) of Triton X-100) when preparing the staining solution can also help to break the surface tension.

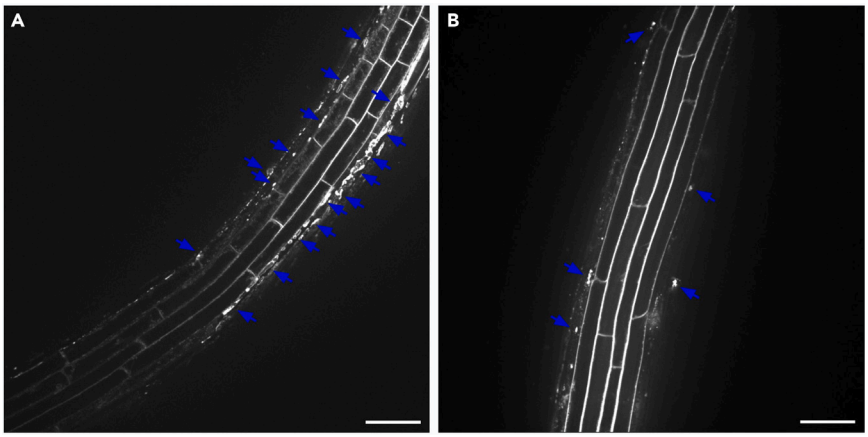


Figure 7. Cellular debris is less likely to complicate imaging of mature cells

Image of Col-0 light-grown root epidermal cells stained with FM4-64, demonstrating differences in debris levels and their impact on identifying intracellular aggregations.

(A) Younger root cells in the early elongation zone with increased cellular and extracellular debris, highlighted by blue arrows.

(B) Col-0 root in the upper elongation zone with reduced debris. Scale bars represent 50 μm.

Problem 3: Low signal

In Steps 12 & 13, low signal can result from insufficient dye concentration, old/expired dye or inadequate incubation time. In some cases, the signal may appear too weak, making it difficult to visualize intracellular features. Photobleaching can also reduce signal.

Potential solution

When preparing the FM4-64 staining solution ("before you begin" Step 23), increase the concentration of FM4-64 to a maximum of 50 μM[15] or extend the incubation period in Step 2 (for roots) or Step 5 (for hypocotyls) to strengthen the signal. Additionally, optimize the imaging conditions in Steps 12 & 13 by increasing the exposure time or using higher laser power during microscopy. However, if staining intensity fades during imaging, this is an indication that photobleaching is occurring. In this case, decrease laser power or exposure time/collection time to reduce photobleaching. If low signal persists, consider purchasing new dye and protecting the new aliquot from light and repeated freeze-thaw cycles.

Problem 4: Aggregations not detected, despite confirmation that they should be present

Intracellular aggregations may not be detected in Steps 12 & 13, even though they are expected in mutants or under certain conditions. This can happen when the cells being examined are too young, as early-stage cells may not yet display the aggregations.[1] Another reason could be insufficient staining time, resulting in a weak signal that remains limited to the plasma membrane and does not reveal internal structures.

Potential solution

First, ensure that the cells being imaged are from a mature part of the seedling, such as the late elongation zone of roots, or closer to the base of the hypocotyl, where aggregation formation is more likely to be visible. Aggregations may not be visible if the cells being examined are too young, as early-stage cells may not yet display the aggregations.[1] If FM4-64 signal appears too faint or only localized to the plasma membrane, extend the staining time in Step 2 (for roots) or Step 5 (for hypocotyls) to allow the dye to penetrate deeper into the cells and enhance the internal signal. Alternatively, increase the concentration of FM4-64 (to a maximum of 50 μM)[15] in "before you begin" Step 23.

Problem 5: Damaged or dead cells mistaken for aggregates

Dead or damaged cells can be mistaken for aggregations in Steps 12–16. Cells that have been mishandled during mounting or left exposed to air for too long can become damaged or die. Trichoblasts (root hair forming cells) are especially prone to damage since each root hair forms as a projection from a root epidermal cell and these delicate root hairs are prone to breakage during staining and mounting, which will damage the entire epidermal cell. Damaged or dead cells can contain structures that stain intensely with FM4-64 and therefore resemble aggregations (Figures 6A and 6B).

Potential solution

Handle seedlings with extreme care, being gentle during the staining (Steps 1–6) and mounting (Steps 8–10) to avoid physical damage. Ensure that the mounting steps (Steps 8–10) are completed quickly to minimize air exposure, which can cause the cells to dry out and die. Ensure that imaging steps (Steps 11–14) are completed quickly to minimize time that the live seedling spends on a slide, since aging samples can display signs of stress. Damage from broken root hairs can be avoided by imaging late elongation zone cells, just before the maturation zone where root hairs begin to form. Damaged cells can often be identified by irregular Brownian motion of intracellular structures and decreased cytoplasmic streaming (Video S1), a lack of normal intracellular structures, vacuole shrinkage or fragmentation (“bubble-like” structures; Figure 6, blue arrows). Damaged cells can also be identified using bright-field microscopy (Figure 6C), or with a vital stain like propidium iodide, which stains the nucleus when cell integrity is compromised. If damaged cells are detected in Steps 12 & 13, discard the sample and use a fresh seedling for imaging.

Problem 6: Difficulty distinguishing between true aggregations and false positives

In Steps 12–16, it can sometimes be difficult to differentiate between true intracellular aggregations and debris or artifacts. This is especially challenging close to the root tip, where extracellular debris from root cap cells that have been sloughed-off is more prevalent (Figures 3D–3F). In other cases, surface-level structures or irregularities can resemble intracellular aggregates, making it hard to distinguish between them (e.g., Figure 5A).

Potential solution

To reduce the likelihood of mistaking debris for aggregations, focus on cells further from the root tip in Steps 12 & 13, as these areas generally contain less debris (Figure 7). Additionally, collect a complete z-stack that goes fully through each cell in Step 13 (Figure 2). Aggregations deeper within the cell are often easier to confidently identify, as they are less likely to be confused with surface-level debris or structures located at the plasma membrane.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Heather E. McFarlane (h.mcfarlane@utoronto.ca).

Technical contact

Technical questions on executing this protocol should be directed to and will be answered by the technical contact, Fabrizio Chow (fabrizio.chow@mail.utoronto.ca).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate unique datasets or code.

ACKNOWLEDGMENTS

H.E.M. is the Canada Research Chair in Plant Cell Biology. Imaging was conducted using instruments supported by Canada Foundation for Innovation and Ontario Research Fund grants (38721) to H.E.M. This work was supported by the Natural Sciences and Engineering Research Council of Canada NSERC Discovery Grant (2020-05959) and an Ontario Early Researcher Award (ER21-16-256) to H.E.M. and by an Ontario Graduate Scholarship (OGS) awarded to E.M.

AUTHOR CONTRIBUTIONS

Conceptualization, F.C., E.M., and H.E.M.; methodology, F.C. and E.M.; investigation, F.C. and E.M.; visualization, F.C. and E.M.; resources, H.E.M.; writing – original draft, F.C.; writing – review and editing, F.C., E.M., and H.E.M.; supervision, H.E.M.; project administration, H.E.M.; funding acquisition, H.E.M.

DECLARATION OF INTERESTS

The authors declare no competing interests.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2025.103665.

REFERENCES

1. Hoffmann, N., and McFarlane, H.E. (2024). Xyloglucan side chains enable polysaccharide secretion to the plant cell wall. Dev. Cell *59*, 2609–2625.e8. https://doi.org/10.1016/j.devcel.2024.06.006.

2. Wolf, S. (2022). Cell Wall Signaling in Plant Development and Defense. Annu. Rev. Plant Biol. *73*, 323–353. https://doi.org/10.1146/annurev-arplant-102820-095312.

3. Hoffmann, N., King, S., Samuels, A.L., and McFarlane, H.E. (2021). Subcellular coordination of plant cell wall synthesis. Dev. Cell *56*, 933–948. https://doi.org/10.1016/j.devcel.2021.03.004.

4. Kong, Y., Peña, M.J., Renna, L., Avci, U., Pattathil, S., Tuomivaara, S.T., Li, X., Reiter, W.-D., Brandizzi, F., Hahn, M.G., et al. (2015). Galactose-Depleted Xyloglucan Is Dysfunctional and Leads to Dwarfism in Arabidopsis. Plant Physiol. *167*, 1296–1306. https://doi.org/10.1104/pp.114.255943.

5. Gendre, D., McFarlane, H.E., Johnson, E., Mouille, G., Sjödin, A., Oh, J., Levesque-Tremblay, G., Watanabe, Y., Samuels, L., and Bhalerao, R.P. (2013). *Trans* -Golgi Network Localized ECHIDNA/Ypt Interacting Protein Complex Is Required for the Secretion of Cell Wall Polysaccharides in *Arabidopsis*. Plant Cell *25*, 2633–2646. https://doi.org/10.1105/tpc.113.112482.

6. McFarlane, H.E., Watanabe, Y., Gendre, D., Carruthers, K., Levesque-Tremblay, G., Haughn, G.W., Bhalerao, R.P., and Samuels, L. (2013). Cell Wall Polysaccharides are Mislocalized to the Vacuole in echidna Mutants. Plant Cell Physiol. *54*, 1867–1880. https://doi.org/10.1093/pcp/pct129.

7. Tamura, K., Shimada, T., Kondo, M., Nishimura, M., and Hara-Nishimura, I. (2005). KATAMARI1/MURUS3 Is a Novel Golgi Membrane Protein That Is Required for Endomembrane Organization in Arabidopsis. Plant Cell *17*, 1764–1776. https://doi.org/10.1105/tpc.105.031930.

8. Madson, M., Dunand, C., Li, X., Verma, R., Vanzin, G.F., Caplan, J., Shoue, D.A., Carpita, N.C., and Reiter, W.-D. (2003). The *MUR3* Gene of Arabidopsis Encodes a Xyloglucan Galactosyltransferase That Is Evolutionarily Related to Animal Exostosins. Plant Cell *15*, 1662–1670. https://doi.org/10.1105/tpc.009837.

9. Gendre, D., Oh, J., Boutté, Y., Best, J.G., Samuels, L., Nilsson, R., Uemura, T., Marchant, A., Bennett, M.J., Grebe, M., et al. (2011). Conserved *Arabidopsis* ECHIDNA protein mediates *trans* –Golgi-network trafficking and cell elongation. Proc. Natl. Acad. Sci. USA *108*, 8048–8053. https://doi.org/10.1073/pnas.1018371108.

10. Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., et al. (2012). Fiji: an open-source platform for biological-image analysis. Nat. Methods *9*, 676–682. https://doi.org/10.1038/nmeth.2019.

11. Verbančič, J., Huang, J.J., and McFarlane, H.E. (2021). Analysis of cellulose synthase activity in Arabidopsis using spinning disk microscopy. STAR Protoc. *2*, 100863. https://doi.org/10.1016/j.xpro.2021.100863.

12. Grebe, M., Xu, J., Möbius, W., Ueda, T., Nakano, A., Geuze, H.J., Rook, M.B., and Scheres, B. (2003). Arabidopsis Sterol Endocytosis Involves Actin-Mediated Trafficking via ARA6-Positive Early Endosomes. Curr. Biol. *13*, 1378–1387. https://doi.org/10.1016/S0960-9822(03)00538-4.

13. Yamaguchi, M., Goué, N., Igarashi, H., Ohtani, M., Nakano, Y., Mortimer, J.C., Nishikubo, N., Kubo, M., Katayama, Y., Kakegawa, K., et al. (2010). VASCULAR-RELATED NAC-DOMAIN6 and VASCULAR-RELATED NAC-DOMAIN7 Effectively Induce Transdifferentiation into Xylem Vessel Elements under Control of an Induction System. Plant Physiol. *153*, 906–914. https://doi.org/10.1104/pp.110.154013.

14. Cutler, S.R., Ehrhardt, D.W., Griffitts, J.S., and Somerville, C.R. (2000). Random GFP-cDNA fusions enable visualization of subcellular structures in cells of *Arabidopsis* at a high frequency. Proc. Natl. Acad. Sci. USA *97*, 3718–3723. https://doi.org/10.1073/pnas.97.7.3718.

15. Bolte, S., Talbot, C., Boutte, Y., Catrice, O., Read, N.D., and Satiat-Jeunemaitre, B. (2004). FM-dyes as experimental probes for dissecting vesicle trafficking in living plant cells. J. Microsc. *214*, 159–173. https://doi.org/10.1111/j.0022-2720.2004.01348.x.