

Callose detection and quantification at plasmodesmata in bryophytes

Arthur Muller¹, Tomomichi Fujita² and Yoan Coudert^{1,3}

¹Laboratoire Reproduction et Développement des Plantes, Université de Lyon, ENS de Lyon, UCB Lyon 1, CNRS, INRA, INRIA, Lyon 69007, France

²Department of Biological Sciences, Faculty of Science, Hokkaido University, Kita 10 Nishi 8, Kita-ku, Sapporo, Hokkaido, 060-0810 Japan

³Lead contact: yoan.coudert@ens-lyon.fr

Abstract

In bryophytes (i.e. mosses, liverworts and hornworts), extant representatives of early land plants, plasmodesmata have been described in a wide range of tissues.

Although their contribution to bryophyte morphogenesis remains largely unexplored, several recent studies have suggested that the deposition of callose around plasmodesmata might regulate developmental and physiological responses in mosses. In this chapter, we provide a protocol to image and quantify callose levels in the filamentous body of the model moss *Physcomitrium* (*Physcomitrella*) *patens* and discuss possible alternatives and pitfalls. More generally, this protocol establishes a framework to explore the distribution of callose in other bryophytes.

Key words

aniline blue, bryophytes, callose, moss, *Physcomitrella patens*, *Physcomitrium patens*, plasmodesmata

1. Introduction

Bryophytes, which comprise mosses, liverworts and hornworts, originated between 460 and 506 million years (My) ago [1] and constitute extant representatives of early land plants. Most recent phylogenomic studies support bryophytes as a monophyletic sister lineage to tracheophytes (vascular plants) [2–5]. Comparative ultrastructural studies have shown that extant bryophytes and tracheophytes possess similar plasmodesmata with desmotubules and spoke-like extensions that bridge the endoplasmic reticulum and the plasma membrane lining the intercellular cytoplasmic strand. These similarities suggest that the plasmodesmata of bryophytes and tracheophytes may be homologous, i.e. inherited from a shared ancestor [5–10]. In bryophytes, the structure of plasmodesmata has been characterized using transmission electron microscopy in various cell types and tissues, including apical meristematic cells, leptoids (food-conducting cells), phyllids (leaves), rhizoids and protonemata (filaments) in mosses, and placental cells at the gametophyte-sporophyte junction in hornworts and liverworts [11–15]. Whilst plasmodesmata are a conserved structural feature of land plants, their contribution to the development and physiology of bryophytes remains poorly understood.

The reversible deposition of callose (β -1,3-glucan) at plasmodesmata provides a mechanism for dynamically controlling symplasmic trafficking in response to developmental or environmental cues in flowering plants [16–19]. Callose deposits can be visualized with fluorescence microscopy following aniline blue staining [20]. Using this method, callose has been observed around plasmodesmata connecting protonemal cells in *Funaria hygrometrica* and

Physcomitrium (Physcomitrella) patens, and in various tissues of the gametophore and the sporophyte in other moss species [21–24]. Exogenous treatment of *P. patens* with the phytohormone abscisic acid (ABA) triggers callose accumulation at cell-cell junctions within 72 hours, when the intercellular movement of macromolecules such as the photoconvertible fluorescent protein Dendra2 is reduced [22, 23]. Inhibition of callose biosynthesis with the glucose analog, 2-deoxy-*D*-glucose, strongly reduces branching in *P. patens* gametophores, and this effect might be caused by an increase of auxin diffusion capacity through plasmodesmata in the stem [25]. Beyond these studies, the mechanisms and function of callose accumulation and turn-over in bryophytes remain largely unexplored and deserve more attention.

Hereafter, we describe a method that uses aniline blue to stain callose deposits in protonemata of the model moss *P. patens* [26]. We provide a procedure to image and quantify aniline blue fluorescence levels using confocal imaging and the ImageJ software. In the **Notes** section, we highlight some of the pitfalls that we encountered when setting up this protocol. It must be noted that the main protocol described below has been established by Arthur Muller and Yoan Coudert to measure callose levels between adjacent protonemal cells. Alternative steps to this protocol (referred to as “Alternative steps” in the Materials and Methods sections) have been established independently by Tomomichi Fujita to detect changes in global callose levels and punctate signals in protonemal cell walls.

2. Materials

2.1 Plant tissue growth

1. Sterile 90 mm Petri dishes.
2. 250 ml autoclavable glass bottle.
3. Deionized water.
4. BCDAT growth medium stock solutions:
 - a. Stock solution B (100x): 25 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$.
 - b. Stock solution C (100x): 25 g/L KH_2PO_4 , adjust pH to 6.5 with 4 M KOH.
 - c. Stock solution D (100x): 101 g/L KNO_3 , 1.25 g/L $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$.
 - d. Stock solution AT (100x): 92 g/L $\text{C}_4\text{H}_{12}\text{N}_2\text{O}_6$.
 - e. Stock solution TE (1000x): 614 mg/L H_3BO_3 , 55 mg/L $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 389 mg/L $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 55 mg/L $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 55 mg/L $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 28 mg/L KI, 25 mg/L $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$.
 - f. Stock solution CaCl_2 (500 x): 73.5 g/L CaCl_2 .
5. Agar.
6. Micropore surgical tape (3M).
7. Laminar flow hood for plant tissue culture.
8. Tweezers for plant tissue culture.
9. Glass bead sterilizer.
10. Growth cabinet.
11. *Physcomitrium (Physcomitrella) patens*.

2.2 Aniline blue solution preparation

1. 0.1 mg/mL aniline blue fluorochrome solution (Biosupplies) in sterile deionized water. Solution must be stored at 4°C in the dark.
2. 0.1 M phosphate buffer, pH 8.5: 97 mM K_2HPO_4 , 3 mM KH_2PO_4 .

2.3 Alternative steps to 2.2 for aniline blue preparation

1. Dissolve aniline blue (Wako) in 100 mM phosphate buffer (pH 8.7) to a concentration of 5% (w/v) and sterilize by filtration (pore size = 0.22 μm). This is the stock solution (blue color).
2. Dilute the stock solution in 100 mM phosphate buffer to a concentration of 1.6% (w/v). This is the working solution to be used within a year.
3. Incubate the working solution for 2 or more days at room temperature with exposure to air (see **Note 1**).

2.4 Biological sample preparation

1. Aluminum foil.
2. Microscope slides.
3. Microscope cover slips.
4. Thin forceps.
5. Sterile deionized water.
6. 200 μL automatic pipette.
7. 200 μL tips for automatic pipette.
8. Binocular microscope.
9. 24-well cell culture plates.

2.5 Biological sample observation and data analysis

1. Confocal microscope equipped with 10x dry and 40x water objectives, a 405 nm UV laser diode, a PhotoMultiplier Tube (PMT) spectral detector and a UV light source were used to image aniline blue fluorescence.
2. The Leica Application Suite X software is used for image acquisition.
3. ImageJ (<https://imagej.nih.gov/ij/download.html>), an open source image processing software, is used for quantification of fluorescence intensity.

4. Microsoft Excel or a free equivalent (e.g. Apache OpenOffice), or R (<https://www.r-project.org>), is used to make plots.

3. Methods

3.1 Growth of plant material

1. Make up 100 ml of 1x BCDAT agar growth medium: Pour about 80 ml of sterile deionized water into a clean autoclavable bottle, add 1 ml of stock solution B, 1 ml of stock solution C, 1 ml of stock solution D, 1 ml of stock solution AT, 100 μ l of stock solution TE and 0.7 g of agar, top up with sterile deionized water to 100 ml, autoclave the bottle and cool it down in a laminar flow hood, add 200 μ l of sterile CaCl_2 stock solution while the medium is still warm.
2. Distribute warm autoclaved BCDAT agar growth medium into five sterile 90 mm petri dishes (i.e., 20 ml per petri dish).
3. Inoculate solid BCDAT agar medium with 1-week-old protonemata or sterile spores of the moss *P. patens* under sterile conditions.
4. Seal petri dishes with Micropore tape.
5. Incubate petri dishes for 2 weeks at 23°C under a 16 h light/8 h dark cycle, at 50-150 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$.

3.2 Alternative steps to 3.1 for growth of plant material

1. Prepare 100 ml of BCDAT medium containing 5 g/L glucose and 5 g/L gellan gum (BCDATG (gellan gum) medium) in a bottle. Autoclave and cool it down (see **Note 2**).
2. Melt the BCDATG (gellan gum) medium in a microwave (see **Note 3**).

3. Pour 500 μL of cooled BCDATG medium into each well of a four-well chamber slide and let it solidify.
4. Inoculate a small number of 3- to 5-day-old protonemata into each well.
5. Cover protonemata with 250 μL of cooled BCDATG.
6. Put on the lid and seal the chamber slide by wrapping parafilm around the chamber slide one and a half times (see **Note 4**).
7. Cultivate the protonemata for 10-15 days under continuous red light with the glass surface of the chamber slide up (see **Note 5**).

3.3 Sample preparation

The protocol below has been optimized for moss filaments (see **Notes 6-11**).

1. Prepare a fresh 1:3 dilution of the aniline blue stock solution (i.e., 33 $\mu\text{g}/\text{mL}$ final concentration) in 0.1 M phosphate buffer, pH 8.5.
2. Dispense 100 μL of diluted aniline blue solution into each well of a 24-well cell culture plate. Prepare one well per sample.
3. Harvest a few filaments from the edge of a well-grown moss colony. Make sure not to take any agar with the filaments.
4. Place the filaments in aniline blue solution.
5. Wrap 24-well cell culture plate in aluminum foil to keep the tissue in the dark.
6. Incubate for 30 minutes at room temperature.
7. Drop 30 μL of distilled water onto a microscope slide.
8. Transfer a few filaments into the water droplet.
9. Gently tease apart the filaments with thin forceps.
10. Place a cover slip onto the sample.
11. Check with a binocular microscope that filaments are individualized.

3.4 Alternative steps to 3.3 for sample preparation

1. Add 150 μL of the aniline blue solution to the samples to a final concentration of 0.1% (w/v).
2. Keep the slide with the glass surface down for 12-24 hours to let the aniline blue solution diffuse through the medium (see **Note 12**).
3. Incubate the sample for 12-24 hours with the glass surface up (see **Note 13**).

3.5 Confocal microscope settings and data collection

After the filaments have been stained and mounted between slides and coverslips, they can be imaged directly with the confocal microscope. The following steps describe the image acquisition procedure with a Leica SP8 confocal microscope, but any equivalent imaging system can be used.

1. Set 405 nm UV laser diode power at 10%.
2. Set 440-520 nm (blue/cyan) band-pass emission filter for aniline blue.
3. Set scanning parameters as follows: frame scanning mode; frame size 1,024 x 1,024; pixel depth 12-bit; numerical zoom 1.5 x; pinhole 1 A.U.; frame average 8; scan speed 8,000 Hz.
4. Use the UV light (not the laser diode) and 10x dry lens to locate the aniline blue signal at the cell wall junction between the apical and sub-apical cell.
5. Select the 40x water immersion lens and focus on the cell wall.
6. Set Z-stack parameters. Select the beginning and end point of the volume to image, set number of steps to 30 and Z-step size to 0.7 μm .
7. Perform image acquisition.

3.6 Data visualization

1. In the microscope software, perform a maximum intensity projection for each Z-stack and save resulting image as a TIFF file. Alternatively, maximum intensity projections can be performed in ImageJ, as described below.
2. Open .lif project (for Leica confocal microscopes) with ImageJ
3. Tick *Open files individually*, click *OK* and select the Z-series to analyze
4. Perform maximum intensity projection: go to *Image > Stack > Z-project*, chose “Max Intensity” in “Projection type”
5. Select the aniline blue channel.
6. Normalize the image contrast (**Figure 1A**): go to *Image > Adjust > Contrast > Set*. Set the minimum displayed value to 0 and the maximum displayed value to 4,095 for 12-bit pictures, and to 63,353 if working with 16-bit pictures.
7. Apply false colors to the aniline blue fluorescence channel, use the “Fire” look up table (LUT): go to *Image > Lookup tables > Fire*. This will help visualize intensity gradients (**Figure 1B-C**).

3.7 Alternative steps to 3.5 for confocal microscope settings and data collection

1. Detection of changes in global callose levels.

Take images of five z-stack images (1,024 × 1,024 pixels) with a 20× objective (zoom 4 ×) and a 405-nm diode laser using a confocal laser-scanning microscope. The pinhole size is set to 14.05µm, which yields a 1.77-µm optical slice (see **Note 14**).

2. Detection of punctate signals in cell walls

To detect punctate callose signals, acquire images with a 100× objective (zoom 4 ×) and a 405-nm diode laser. The pinhole size is set to 40.87 μm, which yields a 0.31-μm optical slice. (see **Notes 15-17**).

3.8 Signal intensity quantification

1. Open .lif project (for Leica confocal microscopes) with ImageJ.
2. Tick *Open files individually*, click *OK* and select the Z-series to analyze.
3. Sum grey values for each pixel: go to *Image> Stack> Z-project*, chose “Sum slices” in “Projection type”.
4. Select the aniline blue channel.
5. Open the ROI (Region Of Interest) manager: go to *Analyze> Tools> ROI manager*. Draw a rectangle around the area to analyze, such as the wall between the apical and sub-apical cell. The size of the ROI should be large enough to fit all the images analyzed. Name the ROI (click *Rename*) and save it (click *More > Save*) in the ROI manager menu. It is important to keep an ROI of constant size for all the images analyzed.
6. Set measurements to retrieve « area » and « integrated density » values from the ROI: in the main toolbar go to *Analyze> Set measurements*, tick “area” and “integrated density”.
7. Place the ROI in the background and measure pixel intensity in the ROI: go to *Analyze> Measure (Figure 1B)*. The measured “RawIntDens” value is considered as *background noise*.
8. Place the ROI around the cell wall between the apical and sub-apical cell and measure pixel intensity (**Figure 1C**). The measured “RawIntDens” value is considered as the *raw signal*.

9. Save both measurements and repeat steps 7-8 for all the images of the experiment.
10. Open an Excel spreadsheet (or equivalent) and transfer the data to three columns: (A) *image number*, (B) *background noise* and (C) *raw signal*.
11. In a fourth column (D), calculate the net fluorescence intensity value for each image as follows: *net signal (D) = raw signal (C) - background noise (B)*.
12. Represent data as boxplots or similar graphical representations (**Figure 1D-F**) (see **Note 18**).

4. Notes

1. The color of the solution should change from blue to brownish yellow.
Subsequent staining is carried out after this color change. This color change is observed with the pH 8.7 phosphate buffer, although it has previously been reported with K_3PO_4 (pH 12) [20]. Aniline blue dissolved in K_3PO_4 (pH 12) is unsuitable for time-lapse imaging due to the deleterious effect of pH on cells. pH 8.7 phosphate buffer alleviates damage permitting time-lapse imaging [23].
2. Application of glucose facilitates growth of *P. patens*. The high translucence of gellan gum improves imaging. BCD medium (BCDAT without ammonium tartrate) may alternatively be used to grow and observe gametophore tissues.
3. This medium can be used several times but should be discarded when it no longer solidifies.
4. Parafilm prevents drying of the medium but may delay protonemal growth as it limits gas exchange.

5. Red light suppresses initiation of side branching and makes observation of protonemata easier.
6. As aniline blue is expensive, we tried to dilute it further (e.g. 1:30) but found that it did not yield sufficient staining.
7. Tissues treated in the same conditions but without aniline blue fluorochrome were used as a negative control.
8. Previous reports suggested that tissue fixation may help visualize callose deposition [20]. We tested the effect of tissue fixation on aniline blue staining intensity in moss and found that a 30-minute treatment with an FAA-based fixative (5% acetic acid, 50% ethanol, 13.5% FAA (37% stock solution), 31.5% H₂O), prior to aniline blue staining, altered tissue color and significantly reduced fluorescence levels (**Figure 1D**).
9. We tested the effect of washing tissues after aniline blue treatment and found that it significantly reduced fluorescence levels (**Figure 1E**).
10. We tested different staining times and found that treating tissues with aniline blue solution more than 30 minutes (e.g. overnight) did not increase fluorescence levels (**Figure 1F**).
11. It took about 5 min to prepare one slide and locate the sample to image under the microscope, and about 5 min to perform a Z-stack. This must be considered when performing large-scale experiments.
12. Since the light is coming from above the sample, the protonemata tend to grow away from the glass surface during this period.

13. This step is important for the observation of the most apical cell walls because it will encourage the tips of the protonemata to grow on the glass surface, making them easier to observe.
14. The signal from callose stained with aniline blue was stable during time-lapse imaging over 180 min at 20-min intervals.
15. Manual adjustment of brightness and contrast are required to calculate density of punctate signals.
16. This method is unsuitable for time-lapse imaging to detect changes of global callose levels at lower magnification, because the higher laser power causes photobleaching.
17. Septa of *P. patens* in proximal regions of protonemata (i.e. closer to the colony center) are rich in callose, which makes it difficult to distinguish individual punctate signals
18. Aniline blue fluorescence signal intensity was very variable between and within experiments, which could be attributable to biological stochasticity (age of sampled tissues, location of the filaments within the colony). A minimum of 30 different cell walls per treatment ($n > 30$) was imaged to take variability into account in the measurements (**Figure 1D-F**).

Acknowledgements

YC acknowledges the CNRS (ATIP-Avenir fellowship) and the University of Lyon (Impulsion grant) for funding, the PLATIM team for technical assistance, Teva Vernoux for his continual support and Gwyneth Ingram for rereading this article. The work in TF's laboratory was supported by JSPS KAKENHI; Grant-in-Aid for

Challenging Exploratory Research (25650089) and Grant-in-Aid for Scientific Research on Innovative Areas (18H04829).

Conflict of interest

The authors declare that they have no conflict of interest.

5. References

1. Morris JL, Puttick MN, Clark JW, et al (2018) The timescale of early land plant evolution. *Proceedings of the National Academy of Sciences* 115:E2274–E2283. <https://doi.org/10.1073/pnas.1719588115>
2. Puttick MN, Morris JL, Williams TA, et al (2018) The Interrelationships of Land Plants and the Nature of the Ancestral Embryophyte. *Current Biology* 1–16. <https://doi.org/10.1016/j.cub.2018.01.063>
3. Harris BJ, Harrison CJ, Hetherington AM, Williams TA (2020) Phylogenomic Evidence for the Monophyly of Bryophytes and the Reductive Evolution of Stomata. *Current Biology* 30:2001–2012.e2. <https://doi.org/10.1016/j.cub.2020.03.048>
4. Leebens-Mack JH, Barker MS, Carpenter EJ, et al (2019) One thousand plant transcriptomes and the phylogenomics of green plants. *Nature* 574:679–685. <https://doi.org/10.1038/s41586-019-1693-2>
5. Delaux P-M, Hetherington AJ, Coudert Y, et al (2019) Reconstructing trait evolution in plant evo-devo studies. *Curr Biol* 29:R1110–R1118. <https://doi.org/10.1016/j.cub.2019.09.044>

6. Raven JA (1997) Miniview: Multiple origins of plasmodesmata. *European Journal of Phycology* 32:95–101
7. Nishiyama T, Sakayama H, de Vries J, et al (2018) The Chara Genome: Secondary Complexity and Implications for Plant Terrestrialization. *Cell* 174:448-464.e24. <https://doi.org/10.1016/j.cell.2018.06.033>
8. Cook M, Graham L, Botha C, Lavin C (1997) Comparative ultrastructure of plasmodesmata of Chara and selected bryophytes: toward an elucidation of the evolutionary origin of plant plasmodesmata. *Am J Bot* 84:1169–1169
9. Cook ME, Graham LE (1999) Evolution of Plasmodesmata. In: van Bel AJE, Van Kesteren WJP (eds) *Plasmodesmata: Structure, Function, Role in Cell Communication*. Springer, Berlin, Heidelberg, pp 101–117
10. Lucas W, van der Schoot C (1994) Plasmodesmata and the supracellular nature of plants. *New Phytol* 126:719–719. <https://doi.org/10.1111/j.1469-8137.1994.tb02967.x>
11. Brunkard JO, Zambryski PC (2017) Plasmodesmata enable multicellularity: new insights into their evolution, biogenesis, and functions in development and immunity. *Curr Opin Plant Biol* 35:76–83. <https://doi.org/10.1016/j.pbi.2016.11.007>
12. Pressel S, Ligrone R, Duckett JG (2006) Effects of De- and Rehydration on Food-conducting Cells in the Moss *Polytrichum formosum*: A Cytological Study. *Ann Bot* 98:67–76. <https://doi.org/10.1093/aob/mcl092>

13. Pressel S, Ligrone R, Duckett JG (2008) Cellular differentiation in moss protonemata: a morphological and experimental study. *Ann Bot* 102:227–245.
<https://doi.org/10.1093/aob/mcn080>
14. Kitagawa M, Fujita T (2015) A model system for analyzing intercellular communication through plasmodesmata using moss protonemata and leaves. *J Plant Res* 128:63–72. <https://doi.org/10.1007/s10265-014-0690-7>
15. Gambardella R, Ligrone R (1987) The development of the placenta in the anthocerote *Phaeoceros laevis* (L.) Prosk. *Planta* 172:439–447.
<https://doi.org/10.1007/BF00393859>
16. Carafa A, Duckett JG, Ligrone R (2003) The Placenta in *Monoclea forsteri* Hook. and *Treubia lacunosa* (Col.) Prosk: Insights into Placental Evolution in Liverworts. *Ann Bot* 92:299–307. <https://doi.org/10.1093/aob/mcg140>
17. Benitez-Alfonso Y, Faulkner C, Pendle A, et al (2013) Symplastic intercellular connectivity regulates lateral root patterning. *Dev Cell* 26:136–147.
<https://doi.org/10.1016/j.devcel.2013.06.010>
18. Chen X-Y, Liu L, Lee E, et al (2009) The Arabidopsis callose synthase gene *GSL8* is required for cytokinesis and cell patterning. *Plant Physiol* 150:105–113
19. Vatén A, Dettmer J, Wu S, et al (2011) Callose biosynthesis regulates symplastic trafficking during root development. *Dev Cell* 21:1144–1155.
<https://doi.org/10.1016/j.devcel.2011.10.006>

20. Caillaud M-C, Wirthmueller L, Sklenar J, et al (2014) The Plasmodesmal Protein PDL1 Localises to Haustoria-Associated Membranes during Downy Mildew Infection and Regulates Callose Deposition. *PLoS Pathogens* 10:e1004496.
<https://doi.org/10.1371/journal.ppat.1004496>
21. Zavaliev R, Epel BL (2015) Imaging callose at plasmodesmata using aniline blue: quantitative confocal microscopy. *Methods Mol Biol* 1217:105–119.
https://doi.org/10.1007/978-1-4939-1523-1_7
22. Hébant C (1968) Observations en microscopie à fluorescence sur la répartition de la callose chez les mousses (Bryopsida). *Naturalia monspeliensia* 1–3
23. Kitagawa M, Tomoi T, Fukushima T, et al (2018) Abscisic Acid Acts as a Regulator of Molecular Trafficking through Plasmodesmata in the Moss *Physcomitrella patens*. *Plant Cell Physiol* 11:36–14.
<https://doi.org/10.1093/pcp/pcy249>
24. Tomoi T, Kawade K, Kitagawa M, et al (2020) Quantitative Imaging Reveals Distinct Contributions of SnRK2 and ABI3 in Plasmodesmatal Permeability in *Physcomitrella patens*. *Plant Cell Physiol* 61:942–956.
<https://doi.org/10.1093/pcp/pcaa021>
25. Schuette S, Wood A, Geisler M, et al (2009) Novel localization of callose in the spores of *Physcomitrella patens* and phylogenomics of the callose synthase gene family. *Ann Bot*

26. Coudert Y, Palubicki W, Ljung K, et al (2015) Three ancient hormonal cues coordinate shoot branching in a moss. *eLife* 4:e06808.
<https://doi.org/10.7554/eLife.06808>

27. Rensing SA, Goffinet B, Meyberg R, et al (2020) The Moss *Physcomitrium* (*Physcomitrella*) *patens*: A Model Organism for Non-Seed Plants. *The Plant Cell*. <https://doi.org/10.1105/tpc.19.00828>

Figure captions

Figure 1. Aniline blue staining of callose in *P. patens* protonemata.

(A) Confocal image of a filament tip stained with aniline blue and shown in grey levels. Arrowhead indicates the junction between the sub-apical cell (sac) and the apical cell (ac). (B-C) Same images as A shown in “Fire” LUT of ImageJ. ROI 1 and ROI 2 indicate ROIs for quantification of background noise and raw signal, respectively. (D-F) Boxplots showing the effect of tissue fixation (D), tissue wash (E) and staining duration (F) on aniline blue fluorescence intensity (Wilcoxon signed-rank test different from control, $*p < 0.05$). Net signal values are indicated in millions. “fix.” means FAA-based fixative.