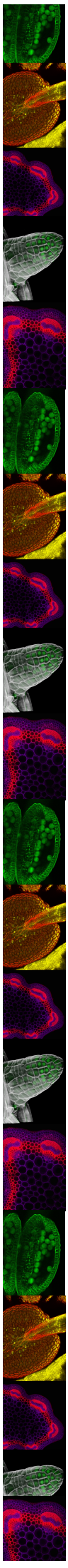


Protocol for combined observation of fluorescent proteins with classical histological stains

(A modified protocol by Robertas Ursache, based on the Kurihara et al. CLEARSEE METHOD, Development 2015; http://www.ncbi.nlm.nih.gov/pubmed/26493404)

Chemicals:

The following chemicals were used to develop the protocol: PFA (Paraformaldehyde) (Merck, CAS-No: 30525-89-4), Xylitol (Sigma, CAS-No: 87-99-0), Sodium Deoxycholate (Sigma, CAS-No: 302-95-4), Urea (Sigma, CAS-No: 57-13-6), Calcofluor White M2R (Fluorescent brightener 28) (Polysciences, CAT#4359), Direct Yellow 96 (Solophenyl Flavine 7GFE 500, Sigma, Product Number: S472409-1G, CAS-No: 61725-08-4), Direct Red 23 (Pontamine Fast Scarlet 4B, Sigma, CAS-No: 3441-14-3), Basic Fuchsin (Fluka, Analytical, CAS-No: 58969-01-0), Nile Red (Sigma, CAS-No: 7385-67-3) and Auramine O (Sigma, CAS-No: 2465-27-2). The most optimal results were achieved using the chemicals purchased from the above mentioned suppliers.

ClearSee solution:

XYLITOL [final 10% (w/v)] (Sigma),

SODIUM DEOXYCHOLATE [final 15% (w/v)] (Sigma)

UREA [final 25% (w/v)] (Sigma)

WATER to the final volume

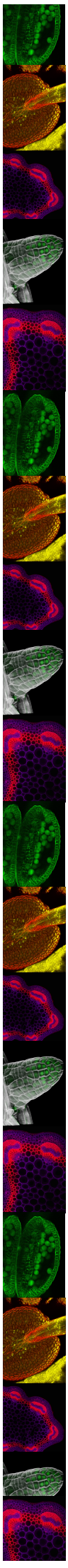
Mix the solution well for at least 30min to 1 hour on the magnetic stirrer until you get everything completely dissolved!

FIXATION AND CLEARING:

- Fix your samples with 4% **PFA (paraformaldehyde)** in 1 x PBS for 1 hour at room temperature with gentle agitation. For young roots (5-6 days old) it works well without vacuum. For older roots, cotyledons, leaves, stems, flowers vacuum treatment is highly recommended. For such samples apply at least 1 hour of vacuum treatment;

  Tip1: to prevent tissue damage, the samples can be dipped into fixation solution by using a soft brush;

  Tip2: to decrease the surface tension when transferring the sample into fixative, a small drop of Brij® L23 solution can be applied;
- Wash twice the fixed tissues for **1 min in 1 x PBS.** The washing step is very important!
- Transfer the seedlings to **ClearSee** solution and clear them at room temperature with gentle agitation. For young roots (4-6 days) overnight/1 day is sufficient. Otherwise clear longer for older roots, stems, shoots and leaves. If the clearing takes several days or weeks, the solution has to be changed at least every second day. After clearing proceed to the staining procedure.



EASY PROTOCOL FOR 4% PFA (PARAFORMALDEHYDE) PREPARATION

1. Take 4g of paraformaldehyde powder and add 1 X PBS to 100ml to 4% final concentration;
2. Transfer to the stirrer. Heat while stirring to approximately 60 °C. **Take care that the solution does not boil! Don't go over 70**°C!
3. The powder will not immediately dissolve. Slowly raise the pH by adding NaOH or KOH dropwise from a pipette until the solution clears;
4. Once the paraformaldehyde is dissolved, recheck the pH, and adjust it with small amounts of HCl to approximately 6.9 pH;
5. Cool down the solution before use.

NOTE: the solution can be aliquoted, kept at +4ºC or frozen. Try to use always fresh PFA and store it in +4ºC no longer than for a week and in -20Cº no longer than for two weeks!

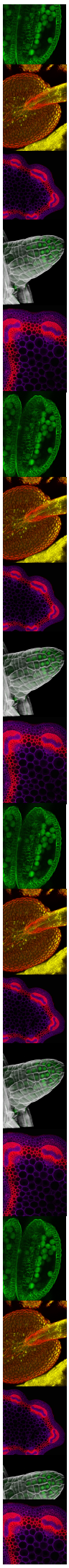
PREPARATION OF THE STAINING SOLUTIONS AND THEIR STORAGE

- All of the dyes used in this study were purchased as a powder and dissolved in ClearSee solution as described in the "Staining Procedure" section.
- To prepare the staining solutions, weight the indicated amount of the corresponding dye powder, dissolve it in ClearSee by mixing the solution on the magnetic stirrer for at least 30 min to 1 hour.
- To store the solutions, wrap them in aluminium foil to avoid exposing them to light.
- Calcofluor White, Direct Red 23, Direct Yellow 96 can be stored for long time (several months) protected from light at room temperature. Basic Fuchsin, Auramine O and Nile Red can stay longer at +4ºC (at least for 2-3 weeks). For the best results, it is advisable to prepare fresh solutions of Basic Fuchsin, Auramine O and Nile Red after one week of storage at +4ºC.

STAINING PROCEDURE

1. Basic Fuchsin staining for lignin

- Prepare 0.2% **Basic Fuchsin** directly in ClearSee and stain the fixed seedlings overnight;
- Remove all **Fuchsin** solution and rinse once in ClearSee;
- Wash for 30 min in ClearSee with gentle agitation;
- Wash once more in ClearSee for at least 1 h or overnight (samples can always be kept for prolonged periods in ClearSee, prolonged storage in ClearSee will only remove background florescence and not de-stain lignin);
- Mount the seedlings on slides with ClearSee solution for imaging;
- Image **Basic Fuchsin** with 561 nm excitation and detect at 600-650 nm.



2. Auramine O staining for lignin and suberin

- Prepare 0.5% solution of **Auramine O** directly in ClearSee. Stain for 12-16 hours;
- Remove all **Auramine O** solution and rinse in ClearSee once;
- Wash in ClearSee for 30 min, then again for at least 1 h;
- Mount the seedlings on slides with ClearSee solution for imaging;
- Image **Auramine O** with 488 nm excitation and detect at 505-530 nm;

Note: Auramine O stains lignin and is visible in Casparian strips and xylem. Later on, when suberin starts to appear, it is also stained with this dye.

3. Calcofluor White staining for cell walls

- Prepare a 0.1% solution of **Calcofluor White** directly in ClearSee. Stain for 30-60 min;
- Remove all **Calcofluor White** solution and rinse in ClearSee once;
- Wash for at least 30min in ClearSee;
- Mount the seedlings on slides with ClearSee solution for imaging;
- Image **Calcofluor White** with 405 nm excitation and detect at 425-475 nm;

4. Nile Red staining for suberin and lipids

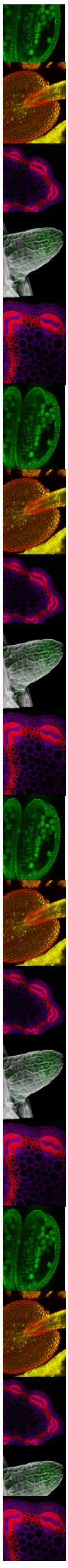
- Prepare 0,05 % solution of **Nile Red** directly in Clearsee. Stain for 12-16 hours;
- Remove all **Nile Red** solution and rinse once;
- Wash for 30 min in ClearSee, then again for at least 1 h, replace the solution several times;
- Mount the seedlings on slides with ClearSee solution for imaging;
- Image **Nile Red** with 561 nm excitation and detect at 600-620 nm;

5. Direct Yellow 96 staining for cell walls

- Prepare a 0.1% solution of **Direct Yellow 96** directly in ClearSee. Stain for 1-2 hours;
- Remove all **Direct Yellow 96** solution and rinse in ClearSee once;
- Wash for at least 30 min in ClearSee;
- Mount the seedlings on slides with ClearSee solution for imaging;
- Image **Direct Yellow 96** with 488 nm excitation and detect at 519 nm;

6. Direct Red 23 staining for cell walls

- Prepare a 0.1% solution of **Direct Red 23** directly in ClearSee. Stain for at least 2 hours;
- Remove all **Direct Red 23** solution and rinse in ClearSee once;
- Wash for at least for 30 min in ClearSee;
- Mount the seedlings on slides with ClearSee solution for imaging;
- Image **Direct Red 23** with 561 nm excitation and detect at 580-615 nm;



NOTE: In order to combine **Basic Fuchsin** or **Nile Red** stains with **Calcofluor White**:

1. Clear and stain the seedlings with **Basic Fuchsin** or **Nile Red** as described above;
2. Wash for 30 min in ClearSee with gentle agitation;
3. Wash once more in ClearSee for at least 1 h;
4. Move the seedlings to 0.1% **Calcofluor White** (in ClearSee) and stain for 30min;
5. Wash the seedlings for at least 30 min, mount them directly in ClearSee. For visualization use the settings for **Basic Fuchsin** and **Calcofluor White** as described above.

NOTE: **Calcofluor White** can be combined together with **Basic Fuchsin**, **Nile Red** or **Auramine O** into one solution at the concentrations described above. The overnight staining is sufficient to visualise the double-staining using the confocal settings indicated above.

To combine the **Basic Fuchsin** or **Nile Red** staining with **Direct Yellow 96**:

1. Clear and stain the seedlings with **Basic Fuchsin** or **Nile Red** as described above;
2. Wash for 30 min in ClearSee with gentle agitation;
3. Wash once more in ClearSee for at least 1 h;
4. Move the seedlings to 0.1% **Direct Yellow 96** (in ClearSee) and stain for 1 hour;
5. Wash the seedlings for at least 30 min, mount them directly in ClearSee. For visualization use the settings for **Fuchsin**, **Nile Red** and **Direct Yellow 96** as described above.

To combine the **Auramine 0** staining with **Direct Red 23**:

1. Clear and stain the seedlings with **Auramine 0** as described above;
2. Wash for 30 min in ClearSee with gentle agitation;
3. Wash once more in ClearSee for at least 1 h;
4. Move the seedlings to 0.1% **Direct Red 23** (in ClearSee) and stain for 2 hours;
5. Wash the seedlings for at least 30 min, mount them directly in ClearSee. For visualization use the settings for **Auramine 0** and **Direct Red 23** as described above.

NOTE:

- **Calcofluor White** **and** **Basic Fuchsin** stained roots are compatible with all **GFP** or **YFP** lines. To separate signals, use 405 nm excitation and 425-475 nm emission for **Calcofluor White**, 561 nm excitation and 600-650 nm emission for **Basic Fuchsin**, for **YFP** - 514 nm and its emission peak is 527 nm, for **GFP** – 488 nm and 509 nm;

- **Calcofluor White** and **Nile Red** stained roots are compatible with **GFP** or **YFP** lines. For **Nile Red** use 561 nm excitation and 600-620 nm emission, for **Calcofluor White** use 405 nm excitation and 425-475 nm emission, for **GFP** and **YFP** use 488 nm excitation/509 nm emission and 514 nm excitation/527 nm emission respectively.

- **Direct Yellow 96** is compatible not only with **Fuchsin** or **Nile Red**, but also with **RFP** and **mCherry** lines. Use the settings for **Direct Yellow 96**, **RFP** or **mCherry** as described above.

- **Direct Red 23** is compatible not only with **Auramine 0**, but also with **YFP** and **GFP** lines. Use the settings for **Direct Red 23**, **YFP** and **GFP** as described above.