Protocol

Imaging Callose utilizing Aniline Blue Protocol

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Introduction

Aniline Blue is a widely used dye to help stain, image, and quantify various structures in human cells and plants. In plants, aniline blue can be used to identify callose, a polysaccharide associated with plasmodesmata, pollen tubes, and microsporocytes. In plasmodesmata, the change in callose accumulation determines the diffusion of molecules from one cell to another. This change in callose structure can also be used as an indicator of infection by *Agrobacterium Tumefaciens*. Thus, accurate assessment of changes in the level of plasmodesmata-associated callose using aniline blue has become a preferred experimental approach in the study of intercellular communication in plants. Here, we describe a method that can be used identify callose structures using aniline blue at plasmodesmata in leaf tissues of various plants including *Arabidopsis thaliana*, *Nicotiana benthamiana,* and *N. tabacum*, as well as in other tissues and plant species.

Storage and Handling

- Aniline blue powder should be stored at room temperature and protected from light.
- When stored under the recommended conditions and handled correctly, this product should be stable for at least 2 years from date of purchase.

Materials

- Leaf tissue from plants at similar developmental stage
- 0.01% Aniline blue (Methyl Blue GoldBio Catalog # A-298) in 0.01M K_3PO_4, pH 12

> **Note: Run the solution through a 0.45 µm Millipore filter and store at room temperature in a container wrapped with aluminum foil. Before use, incubate mixture at room temperature for at least 48 hours for complete decolorization until the solution reaches a bright yellow color.**

- 96% ethanol
- Double-distilled water (DDW) with 0.01% Tween-20
- Large-size (24 x 60 mm) microscope cover glasses
- Microscope wire loop
- Aluminum foil
- Parafilm
- Razor blades
- Plastic containers

- Glass tubes (25 ml)
- Thin forceps (GoldBio Catalog # F3001)
- Vacuum desiccator
- Confocal microscope equipped with a 405 nm laser for aniline blue excitation and with 475-525 or 420-480 nm band-pass emission filters for aniline blue fluorescence collection.

Method

Staining and sample preparation

1. Cut entire leaf by holding at the petiole with forceps and submerge in 96% ethanol.

   Note: Avoid touching the leaf because it can be easily damaged.

2. Cover and seal in 96% ethanol. Incubate at RT on a shaker at 30-40 rpm for 2-5 hours or until bleaching is complete.

   Note: Speed up the bleaching by changing the ethanol solution after a 1-hour incubation.

3. Remove the bleached leaf from the ethanol by holding it from the petiole.

   Note: The leaf is easily breakable at this stage. Handle gently.

4. Place the leaf on a flat surface and cut into 5 mm wide strips with a razor blade.

5. Rehydrate the tissue by placing in DDW with 0.01% Tween-20. Incubate at RT on a slow shaker at 30-40 rpm for 1 hour.

   Note: Ensure the strips sink into the water. Proper rehydration aids in dye penetration.

6. Remove strips from DDW and place in a 25 ml glass tube filled with 1/3 aniline blue solution.

7. Place tubes in a vacuum desiccator and apply vacuum for ~10 minutes followed by slow release of pressure.

   Note: Good dye penetration is indicated by yellowing of the strips.

8. Cover the tubes with aluminum foil by wrapping them, and incubate at RT for 2 hours on a shaker at 100 rpm.

9. The samples are now ready for imaging using a confocal microscope. De-staining is not needed.

Image Acquisition

1. Place a stained leaf sample between 2 large cover glasses.

   Note: Do not use a microscope slide for observation of both sides of the sample.

2. Use a 40x water immersion objective for observation.

3. Use transmitted light to determine which side of the leaf to analyze (adaxial or abaxial).

   Note: The adaxial side is characterized by tightly packed round palisade mesophyll cells under the epidermal layer. Analyze the abaxial side by flipping the sample.

4. Use transmitted light to focus on the sample.

5. Position the objective at the upper-left corner of the specimen, 2-3 cells away from the cut cells of the edge.

   Note: DO NOT use UV light to focus because it will bleach the area.

6. Use single-track mode when building the configuration of the microscope. Use the 405 nm laser for excitation and 475-525 nm filter for aniline blue fluorescence. Use white pseudo-color for aniline blue emission. Turn off the transmitted light channel.

7. Use the following scanning parameters:
   a. Frame scanning mode
   b. Frame size 1,024 x 1,024
   c. Scan speed 8
   d. Pixel depth 8 bit
   e. Line scanning
   f. Scan average 4

g. Zoom 1x

8. Start with pinhole aperture set to open to observe as many plasmodesmata as possible.

9. Adjust laser intensity, detector gain, amplifier offset, and amplifier gain to optimize the plasmodesmata fluorescence against the background signal.

Note: Adjust the values to minimize oversaturation of callose sites.

Note: Ensure the brightest plasmodesmata sites do not reach saturation (255) and remain in the upper 5th section of the histogram (200-220).

Note: Ignore saturation of stomata.

10. Begin acquiring images once the microscope settings have been optimized.

Note: If plasmodesmata oversaturation occurs, reduce the laser intensity or amplifier gain.

Note: If plasmodesmata show weak fluorescence intensity (below 200), increase the laser intensity or amplifier gain.

11. Take 10-15 images and always follow the same systematic way of image acquisition with each sample.

Associated Products

- Aniline blue (Methyl Blue GoldBio Catalog # A-298)
- Forceps (GoldBio Catalog # F3001)

References

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