bio-101 www.bio-protocol.org/e3612
Bio-101: e3612.
DOI:10.21769/BioProtoc.3612

Toluidine Blue O Staining of Paraffin-Sectioned Maize Tissue

Josh Strable*, Jeffrey R. Yen, Michael J. Scanlon and Anne W. Sylvester

School of Integrative Plant Science, Plant Biology Section, Cornell University, Ithaca, NY 14853, USA
*For correspondence: jjs369@cornell.edu

[Abstract] Histology incorporates microtechnique, the preparation of living material for observation and microscopy to study the anatomy of cells, tissues and organs. Interpreting histological patterns in a specimen may help to correlate form and function at the cellular, tissue and organ levels. Many stain options and staining methods ("schedules") for plant tissues have been described previously (Johansen, 1940; Jensen, 1962; O'Brien and McCully, 1981; Sylvester and Ruzin, 1994; Ruzin, 1999). Here, we present a straightforward, inexpensive protocol that uses Toluidine Blue O (TBO) to stain maize tissue. TBO is a general metachromatic stain that can be used on paraffin-embedded histological sections, as well as fixed, hand-sectioned material. The value of TBO as a stain is the inherent metachromasia, whereby the dye is altered by pH resulting in violet blue color variants. This property is particularly useful even in sectioned material for identifying acidic components of plant cells. We demonstrate the utility of TBO staining for cellular-, tissue- and organ-level studies using maize seedling root, shoot apex and mature leaf (Figure 2).

Keywords: Maize, Histology, Toluidine Blue O, Paraffin-section, Deparaffinized-staining

Materials and Reagents

A. Consumables
   1. Paper towels
   2. Kimwipes
   3. Razor blades (single edge, steel)
   4. Pencil
   5. Parafilm
   6. Adhesive Glass Microscope Slides (VWR, VistaVision™ HistoBond®, catalog number: 16004-406)
   7. Cover slips (25 x 60 mm; VWR, catalog number: 89082-272)
   8. Glass Pasteur pipettes
   9. Glass vials with caps (VWR, catalog number: 66012-022)
   10. Paraplast Plus® (McCormick Scientific, catalog number: 15159-464)
   11. Cups with lids (VWR, catalog number: 89508-714)
   12. Biopsy cassettes (VWR, catalog number: 25608-756)
   13. Disposable base molds (VWR, catalog number: 100501-996)
   14. Embedding ring (VWR, catalog number: 87002-374)

Bio-101: e3612.
DOI:10.21769/BioProtoc.3612

B. Biological material

Maize tissue (*e.g.*, shoot apex, stem, fully expanded leaf blade or root)

C. Reagents

1. Distilled water
2. Ethanol (VWR, catalog number: EM-EX0276-3S)
3. Histo-Clear II (VWR, catalog number: 101412-882)
4. Acetic acid glacial (VWR, catalog number: BDH3094-2.5LG)
5. 37% Formaldehyde solution (VWR, catalog number: 97064-604)
6. Sodium borate (VWR, catalog number: 470302-454)
7. Toluidine Blue O (VWR, catalog number: 97062-168)
8. Permount (Fisher, catalog number: SP15-100)
9. Ethanol series: dilutions to 95%, 85%, 70%, 50% in distilled water
10. Eosin Y (VWR, catalog number: 97061-034)
11. FAA fixative (100 ml) (see Recipes)

Equipment

1. Forceps (VWR, catalog number: 82027-386)
2. Slide warmer (set to 37-42 °C)
3. Oven set at 42 °C
4. Vacuum pump
5. Microtome
6. Fine paintbrushes (*e.g.*, Virtuous Arts Fine Paintbrushes, amazon.com)
7. Stainless steel slide rack with handle (VWR, catalog number: 25461-014)
8. Tupperware (*e.g.*, medium rectangular 15.5 x 8.1 x 7.3 cm of volume 412 ml)
9. Glass staining dish (VWR, catalog number: 25461-016) with cover (VWR, catalog number: 25461-018)
10. Orbital shaker
11. Microscope equipped with camera

Procedure

A. Fixation of maize material

1. Prepare fresh FAA fixative (Recipe 1) and place on ice. Formaldehyde is toxic; therefore, FAA solution should be prepared and used in a fume hood and disposed of properly.
2. Harvest maize tissue samples and place immediately in ~20 ml fresh FAA fixative in glass vials on ice.
3. Apply a vacuum (~500 mm Hg) to the glass vials with samples in FAA on ice. Maintain vacuum

Bio-101: e3612.
DOI:10.21769/BioProtoc.3612

for 15-20 min; effervescence released from the tissue should be obvious, but fixative should not bubble.

4. Release vacuum gently (over a minute) and renew the FAA fixative.
5. Keep the glass vials with samples in FAA overnight at 4 °C shaking at 50-70 rpm.

B. Embedding fixed maize material

1. Pre-cool the ethanol series (Dilutions to 95%, 85%, 70%, 50% in distilled water) on ice or at 4 °C in plastic cups.
2. Transfer samples from glass vials to biopsy cassettes to facilitate moving samples through the following ethanol and Histo-Clear series.
   Note: If biopsy cassettes are not used, decant and replace the liquid through each series while leaving the specimens in the glass vial.
3. Dehydrate samples by moving the biopsy cassettes with specimens through a graded ethanol series in lidded cups on ice, shaking on an orbital shaker at 90 rpm:
   50% EtOH for 1 h.
   70% EtOH for 1 h.
   85% EtOH for 1 h.
   95% EtOH for 1 h.
   100% EtOH #1 for 1 h.
   100% EtOH #2 for 1 h.
   100% EtOH #3 for 1 h.
   100% EtOH with 0.01% (w/v) Eosin overnight at overnight at 4 °C.
   Note: Any volume is sufficient as long as it covers the specimens. The plastic cups hold 100 ml. EtOH solutions can be reused and stored at room temp.
4. Transfer samples to fresh 100% EtOH at room temperature for 1 h.
5. Replace ethanol with Histo-Clear II through the following graded series in lidded cups at room temperature, shaking on an orbital shaker at 90 rpm:
   EtOH to Histo-Clear (3:1 v/v) for 1 h.
   EtOH to Histo-Clear (1:1 v/v) for 1 h.
   EtOH to Histo-Clear (1:3 v/v) for 1 h.
   100% Histo-Clear #1 for 1 h.
   100% Histo-Clear #2 for 1 h.
   100% Histo-Clear #3 for 1 h.
   100% Histo-Clear #4 for 1 h.
6. Add 0.25 vol of Paraplast Plus® chips to the samples in 100% Histo-Clear #4 and place at 42 °C oven overnight.
7. Add Paraplast Plus® chips to several cups and place in a 60 °C to ensure fresh molten wax is available for subsequent wax changes.
8. Replace molten Paraplast Plus®, kept at 60 °C, at least 6 times by decanting molten Paraplast

Bio-101: e3612.
DOI:10.21769/BioProtoc.3612

Plus® from samples and replacing with fresh molten Paraplast Plus®. This can be accomplished 3 times every 4 h over 2 days, or once a day for 6 days.

9. Final embedding of tissue for sectioning will vary depending on the tissue type and desired plane of section. Disposable plastic molds and rings are useful to maintain correct orientation of tissue. Mounted, embedded samples are stable for a year or more at 4 °C.

C. Microtome sectioning of embedded maize material

1. Warm embedded blocks to room temperature.
2. Trim the block into a trapezoidal shape (see Figure 1) using a razor blade, leaving a ~1 mm perimeter of wax around the tissue.

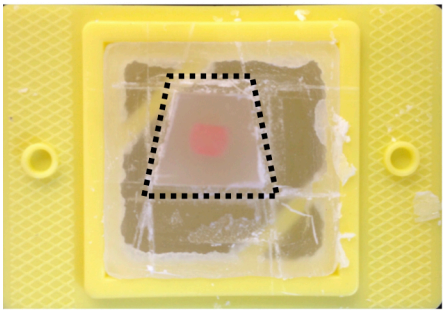

Figure 1. Trapeziod shape in paraffin block. Image of trapezoid shape trimmed into paraffin block around embedded tissue stained pink with Eosin.

3. Mount tissue block onto the block holder of the microtome by positioning the longer of the parallel edges of the trapezoid at the bottom (closet to the microtome blade).
4. Section to a thickness of 8-10 µm.
5. Use fine paintbrushes to move wax ribbons to a bench lined with parafilm, which will allow selecting tissue sections of interest by directly cutting with a razor blade.
6. Pipet 1 ml of MilliQ water at room temperature onto the surface of HistoBond glass microscope slide and carefully move selected wax ribbon onto the water as to float the sections of interest on the slide.
7. Transfer the slide slowly to the slide warmer to heat the slide-water-wax ribbon combination. This method of heating helps to spread the ribbon on the surface of the water to achieve even adhering of the tissue to the surface of the slide.
8. Use a kimwipe to wick away water carefully to lower the ribbon evenly on the slide.
9. Allow slides to dry and tissue to adhere for a minimum of six hours to overnight at 37-42 °C on the slide warmer.
10. Transfer slides to metal slide rack. Slides with adhered sectioned tissue should proceed to TBO staining; however, they can be stored in an air-tight Tupperware with packets of silica desiccant for several days to a few weeks at 4 °C.

Bio-101: e3612.
DOI:10.21769/BioProtoc.3612

D. Deparaffinization and rehydration of sectioned tissue

Deparaffinize and rehydrate samples by bathing slides in slide rack in 350 ml of the following reagents at room temperature in Tupperware containers unless otherwise stated:

100% Histo-Clear for 10 min in glass staining dish, do not discard as it will be used again.

100% Histo-Clear for 10 min in glass staining dish.

100% EtOH #1 for 1 min.

100% EtOH #2 for 1 min.

95% EtOH for 1 min.

85% EtOH for 1 min.

70% EtOH for 1 min.

50% EtOH for 1 min.

Distilled water for 1 min.

Note: EtOH series can be made fresh or stored in glass bottles at room temperature.

E. TBO staining of tissue sections

Stain samples in Tupperware by bathing slides in 350 ml of the following reagents at room temperature.

1% Sodium borate solution (w/v) for 1 min.

0.5% TBO solution for 1-5 min (determine concentration and staining time empirically).

Distilled water

Using an orbital shaker set at 90 rpm, wash slides 1-2 min with several changes of distilled water until water remains mostly colorless.

F. Dehydration of samples and non-aqueous mounting slides

1. Dehydrate samples by bathing slides briefly in 350 ml of the following reagents at room temperature:

   50% EtOH (from previous use) for 20 s.

   70% EtOH (from previous use) for 20 s.

   85% EtOH (from previous use) for 20 s.

   95% EtOH (from previous use) for 20 s.

   100% EtOH #2 (from previous use) for 20 s.

   100% EtOH #3 (fresh) for 20 s.

   100% EtOH #4 (fresh) for 20 s.

   100% Histo-Clear #1 (from previous use) for 2 min in glass staining dish with slight movement of slide rack.

   100% Histo-Clear #2 (from previous use) for 2 min in glass staining dish with slight movement of slide rack.

   100% Histo-Clear #3 (fresh) for 2 min in glass staining dish.

Bio-101: e3612.
DOI:10.21769/BioProtoc.3612

2. With slide rack immersed in 100% Histo-Clear #3, process earch slide individually for Steps F2-F4. Remove slides individually and allow them to drip mostly dry.
3. With a glass Pasteur pipette, add 2-4 drops of Permount mounting medium to the slide surface. This should be performed in the fume hood.
4. Using forceps, gently place cover slip on slide, lowering the cover slip slowly to avoid air bubbles forming in the Permount.
5. Dry slides in the hood.

G. Observe and image with a light microscope equipped with camera (Figure 2)

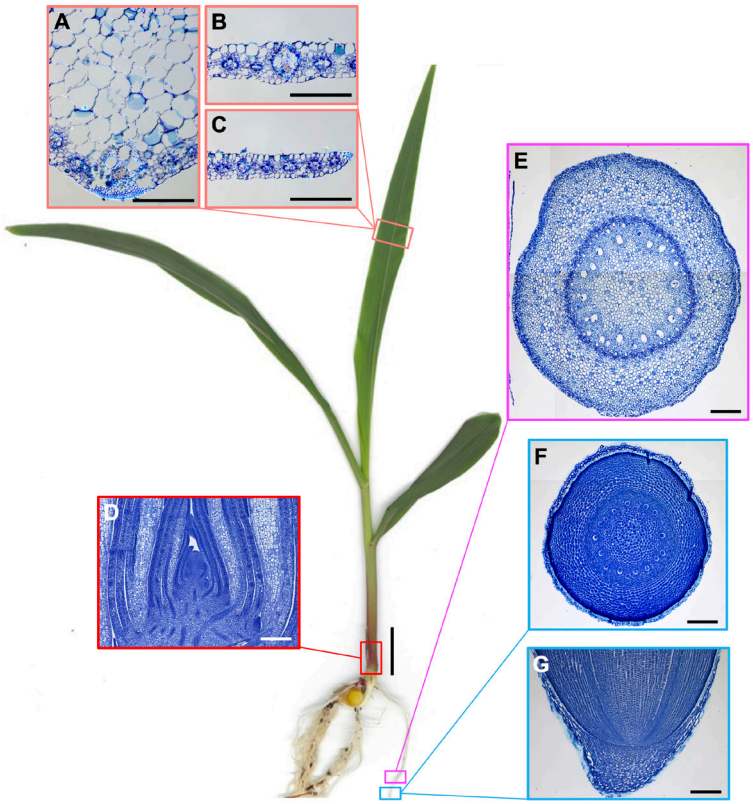

Figure 2. TBO staining of paraffin-sectioned maize tissue. Two-week old maize seedling with representative tissues that were paraffin-sectioned, deparaffinized, rehydrated and stained with TBO. A-C. Transverse sections of leaf 3: leaf midrib (A), lateral and intermediate veins (B) and leaf margin (C). D. Median longitudinal section of the shoot apex showing the shoot apical meristem (center) surrounded by leaf primordia. E-F. Transverse section of crown root. G. Median longitudinal section of crown root apex showing root cap and root apical meristem. All sections of 10 µm thickness. Vertical scale bar = 2 mm; Horizontal scale bars = 200 µm.

Notes

1. Fixed samples can be stored in 70% EtOH at 4 °C for several months (Step B3).

2. Embedding schedule is optimized for sample blocks ~27 mm^3 or smaller; larger samples may require adjustment of longer incubation times.
3. Eosin (~0.01%) will stain cell walls to facilitate visualizing samples during embedding and sectioning and washes out of sectioned samples at 70-85% EtOH during sample rehydration (Step D1. 70-85% EtOH) and will not impact TBO staining.
4. Degree of staining should be determined empirically (*e.g.*, concentration of TBO; duration of staining). Overstaining will result in poor cellular resolution in stained tissue.
5. 1% Sodium borate solution (w/v) is stable at room temperature for a year or more but can also be prepared fresh at each staining.
6. It is recommended that a 1% TBO stock (w/v) be prepared and used to make the working 0.5% TBO solution (v/v).
7. Label slides with pencil as markers and pens will be removed by ethanol.

Recipes

1. FAA fixative (100 ml)
   50 ml EtOH (200 proof)
   5 ml acetic acid glacial
   10 ml 37% formaldehyde solution
   35 ml MilliQ water
2. 1% Sodium Borate (400 ml)
   400 ml MilliQ water
   4 g sodium borate
3. 0.5% TBO solution (400 ml)
   400 ml MiiliQ water
   0.5 g toluidine blue O

Acknowledgments

A version of this protocol was described previously by Strable and coworkers (2017). J.S. was supported by a National Science Foundation Postdoctoral Research Fellowship in Biology (IOS-1710973); J.R.L, M.J.S. and A.W.S were supported by a National Science Foundation grant (DEB-1457070).

We declare that we have no conflicting or competing interests with respect to the implementation of the protocol outlined herein.

References

1. Jensen, W. A. (1962). *Botanical Histochemistry*. W.H. Freeman, San Francisco, CA.

2. Johansen, D. A. (1940). *Plant Microtechnique.* McGraw-Hill, New York, NY.
3. O'Brien, T. O. and McCully, M. E. (1981). *The study of plant structure: principles and selected methods.* Termarcarphi, Melbourne, Australia. ISBN: 0959417400, 9780959417401.
4. Ruzin, S. E. (1999). *Plant microtechnique and microscopy.* Oxford University Press, New York, NY.
5. Strable, J., Wallace, J. G., Unger-Wallace, E., Briggs, S., Bradbury, P. J., Buckler, E. S. and Vollbrecht, E. (2017). Maize *YABBY* genes *drooping leaf1* and *drooping leaf2* regulate plant architecture. *Plant Cell* 29: 1622-1641.
6. Sylvester, A.W. and Ruzin, S.E. (1994). Light microscopy I: Dissection and microtechnique. In: Freeling, M. and Walbot, V. (Eds.). In: *The Maize Handbook*. Springer Lab Manuals, Springer, New York, NY. pp 83-95.

