Stain Dicot and Monocot Stem Sections

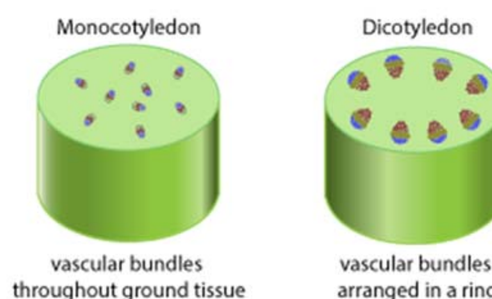
GTAC
GENE TECHNOLOGY ACCESS CENTRE

Introduction

Cellulose is the main component of all plant cell walls, and is the most abundant organic compound in most plants. Lignin, another polysaccharide, is the next most abundant. Lignin forms a major support system by providing rigidity to xylem, the water transport structure of plants. The vascular tissues of plants can be distinguished by their structure and molecular composition using stains that react differentially with cellulose and lignin.

In the microscopic analysis of animal and plant tissues, thin sections of tissue are required to achieve a slice with only one or a few layers of cells. If the section is too thick, the light of the microscope cannot penetrate the specimen. To achieve this, an instrument called a **microtome** is used. Tissue is usually fixed (preserved), embedded in wax or a similar material, then passed across a sharp blade to slice off very thin sections.

In this practical you will use a 'hand microtome', which replicates the principle on which a standard laboratory microtome works. You will prepare thin sections of plant stem of dicotyledons or monocotyledons. The sections are then stained with differential stains to help distinguish between cell walls made of cellulose and lignin, and thus identify phloem and xylem.



Aims

- To learn the principles of using a microtome to prepare thin tissue sections
- To understand the principles and applications of differential staining
- To section and stain plant stems, for microscopic observation of vascular tissue

Materials

- ❒ Hand microtomes (nut and bolt)
- ❒ Plant stem e.g. thyme, geranium
- ❒ Melted paraffin wax
- ❒ Forceps/tweezers – fine point
- ❒ Razor – single edge
- ❒ Multiwell dish or Small Petri dish
- ❒ 4 transfer pipettes (ethanol and wash)
- ❒ Microscope slides and Coverslips
- ❒ Mountant (glycerol:water 1:1)
- ❒ 10 mL 70% ethanol
- ❒ 10 mL 95% ethanol
- ❒ 10 mL distilled water
- ❒ filter paper (cut into squares/arcs)
- ❒ Waste beaker & paper towel

Stains in dropper bottles

- ❒ 0.5% safranin-O in 50% ethanol
- ❒ 0.5% methyl green
- ❒ 0.025% toluidine blue

Method – thin sections with a hand microtome ***SAFETY SHARPS handling***

1. Choose a stem 2-5mm diameter. The stem tissue should be firm but not woody. Cut a piece about 1cm long (make sure its longer than the depth of the nut of your hand microtome. The tissue can be used freshly cut from the plant, or cut and placed in 70% ethanol up to a day before sectioning.

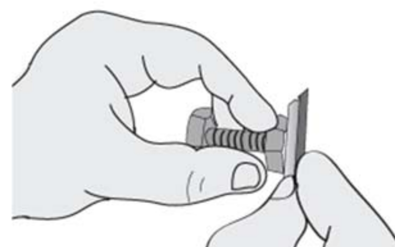
2. Loosen the nut so it is barely secured to the end of the bolt. This creates a “well” in the nut.

3. Stand the bolt up so its head is on the table.

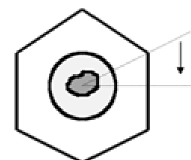
4. ***Perform this step quickly*** *so that the tissue is embedded uniformly; the wax starts to set rapidly in small volumes at room temperature*:
   Place some molten wax in the well formed in the nut. Immediately stand the stem in the molten wax. When the stem can stand up by itself, add more melted wax around the tissue to form a mound around the protruding stem. Allow the wax to completely harden (about 10 minutes).

5. *SAFETY: Blade motion away from you*
   Hold the head of the bolt flat on the table with one hand. Hold the blade with the other hand. With the razor blade, carefully shave off the mound of wax and stem from above the top of the nut. Run the blade over the nut’s surface to make the wax completely smooth. This portion is discarded.

   Alternatively, place the nut-bolt horizontally on a cutting board, hold the nut firmly (keeping fingers out of the way) then **slice downwards** against the edge of the nut.

6. Turn the bolt clockwise about 1/12th of a turn to make the wax cylinder protrude slightly. Again holding the microtome and blade slice the blade over the nut’s surface to make a shaving of the wax surface through the stem. **A smooth quick slicing motion is the best.** Continue the procedure to get several thin sections of the stem.

7. For staining, choose the 2-4 thinnest sections (the most transparent sections). Stain 2 sections with each of the staining protocols below.

Staining method 1- double stain – safranin-O and methyl green

Cell walls composed mostly of cellulose will appear thin and blue or blue-green (stained with methyl green) while those containing lignin will appear thicker and red-orange or red (stained with safranin-O).

Double stain – Safranin-O & Methyl green				
Stain	**Colour of stained structure**	**Molecule stained**	**Structure stained**	**Other features**
Safranin-O	Red-orange, red	lignin	Xylem	thick
Methyl green	Blue, blue-green	cellulose	Phloem, pith, epidermis	thin

1. Using forceps, gently transfer the sections into a small petri dish or well (separated them from the wax as you do this) and immediately add a drop of **safranin** stain to cover the sample. Let the section sit in the stain for 3 minutes.

2. Remove safranin by blotting it with filter paper and immediately add 70 % ethanol. Let the section sit in 70% ethanol for 1 minutes. Remove the ethanol wash, add more 70% ethanol for another 1-2 minutes to completely remove excess safranin from the section.

3. Remove the remaining 70% ethanol by blotting and immediately add methyl green and let it sit for 1 minute. Remove methyl green by blotting.

4. Immediately add 95% ethanol and allow it to sit with gentle agitation for 1 minute. Remove the ethanol wash. Add more 95% ethanol for another 1-2 minutes to remove excess of both stains. Blot out the ethanol.
   - Do not leave the section for too long in ethanol as it may remove too much of the stain. The section should appear translucent to blue. If it appears red, then the excess stain has not been effectively removed (this happens when the sections are too thick).

5. Gently transfer the section to a microscopic slide and with filter paper blot out excess ethanol.

6. Immediately add 1-2 drops of mountant (glycerol: water, 1:1) and add a coverslip over the section. Water can be used for mounting.

7. Label the slide and examine under low power (4x objective) and higher power (10x objective) of your microscope.

Staining Method 2 - Toluidine blue

Toluidine blue is a polychromatic stain, meaning that different components of the cell wall stain different colours. Cellulose stains pink/purple, lignin stains blue/blue-green.

Toluidine blue Polychromatic – different components of cell wall stain different colour			
Tissue	**Colour seen under microscope**	**Molecules stained**	**Other features**
Phloem	Pink-purple	Cellulose (and other pectic substances, but not lignin)	Thin cell wall
Xylem	Blue, blue-green	Lignin	Thick cell wall

1. Gently transfer the sections into the Petri dish (if the tissue was fixed in ethanol before sectioning, sit in dH2O for 3 minutes, then remove the water before staining)
2. Add a drop of **Toluidine blue** stain. Let the section sit in the stain for 5 minutes.
3. Remove stain by blotting with filter paper. Add distilled water to the dish, swirl and remove water. Repeat until excess stain washes out.
4. Gently transfer the section to a microscopic slide and with filter paper blot out excess water.

8. Immediately add 1-2 drops of mountant (glycerol: water, 1:1) and add a coverslip over the section. Water can be used for mounting.

5. Label the slide and examine under low (4x objective) and high (10x objective) powers of your microscope.

Results

Make annotated diagrams of the stained stem.

Preparation and procedural notes

Suppliers of stains

Stain	Supplier and product	Cost in 2015
Toluidine Blue, 0.05% solution (dilute to 0.02-0.025% in dH_2O for stem staining procedure)	Southern Biological SI18 - Toluidine blue, 0.05% available in 100mL & 500mL	$21.90, $34.25
	Science Supply Australia 3268/10G Toluidine Blue (C.I.52040)	$74.95 (ex GST)
Safranin O, 1% solution	Southern Biological SI15 - Safranin O, 1% available in 100mL & 500mL	$12.90, $34.25
	Science Supply Australia SL110/25G Safranin O (microscopy stain) Unit: 25g	$117 (ex GST)
Methyl green , powder	Science Supply Australia VE0120/5G Methyl green (C.I. 42590) for microscopy Unit: 5g	$89.00 (ex GST)
	Sigma-Aldrich **M8884-5G**	**$45**

- This procedure can be done with fresh stem tissue. It can also be done with tissue placed in 70% ethanol for fixation prior to the practical.
- Stains: easiest if you can purchase the solution ready-made. Otherwise follow the instructions with the powder for dissolution. Safranin-O and toluidine blue in solution are classed non-hazardous. Methyl green powder has hazard associated with handling. Dispense stains into dropper bottles and keep in the dark.
- Safranin-O is in 50% ethanol. Apart from dissolving the stain, the ethanol also also fixes the tissue sections.
- Toluidine blue – purchase 0.05% solution. Dilute 1:1 in dH_2O for stem staining procedure; e.g. 10mL of 0.05% Toluidine blue + 10mL dH_2O.
- Only small volumes of stain (1-2 drops) are needed when using small stems (3-5mm diameter. Best to have stains in dropper bottles.
- To save some class time, stems may be mounted in wax in the 'microtome' before class (Steps 1-3 of procedure above). Mount the stem in wax not too long before class to prevent too much drying out of the stem. Make sure there is at least 5mm excess stem above the top of the nut, so this will be trimmed off to reach good fresh tissue
- The sections can be removed from the surrounding wax (handle them carefully if doing this before staining), or they can remain surrounded by wax until mounting on the slide, then remove the wax.
- Apply the stain directly to the tissue section. Use **multiwell dishes/trays** or small (e.g. 35mm) petri dishes. Small wells need less ethanol volume
- 10mL of 70% and 95% ethanol (in capped tubes or bottles, dispensed with transfer pipettes or Pasteur pipettes & bulbs) should be sufficient for the destaining/washing steps as long as excess stain is not used and the procedure times are adhered to. Longer staining makes it harder to remove the excess stain. The result is lack of colour differentiation of the tissues.

- Mountant: many protocols suggest 50% glycerol in water (1 part glycerol: 1 part dH_2O). To prepare 50mL of 50% glycerol, pour 25mL glycerol into a capped tube or other vessel with a well sealed cap or stopper. Add 25 mL of dH_2O. Cap or stopper the tube and mix by inversion. Alternatively, mix equal volumes into a beaker with a magnetic stir bar, stir gently until uniformly mixed. Dispense into small volumes, 1-2 mL per group. Glycerol doesn't dry out as occurs with water mounts, so stains can be done one day and viewed in a subsequent class. It also prevents microbial growth. If students prepare really good sections and stains, they can be kept for a long period in glycerol mountant. The edges of the coverslip can be sealed with nail polish to keep it in place.

- Bubbles may appear from the stem tissue after mounting - it seems air gets trapped in the tissue sections. The bubbles could be misleading for students, who may misinterpret them as cells.

- Plant tissue may be fixed in 70 % ethanol before sectioning. Otherwise, if you do not want the students to do the sectioning, fresh tissue sections can be fixed in 50-70% ethanol immediately upon sectioning, then distributed to students in small dishes.

- These plant staining procedures produce variations in colour depending on the plant and tissue stained. It can become confusing and may vary depending on the thickness of the tissue section and whether the tissue was fixed in ethanol prior to staining. Focus on the main elements for this level of students, the vascular tissue, xylem and phloem

- Further detail of possible colours from toluidine blue: from www.saps.org.uk citing Parker, A. J., Haskins, E. F. and Deyrup-Olsen, I. (1982) Toluidine Blue: A simple, Effective Stain for Plant Tissues. The American Biology Teacher., Vol. 44, No. 8, pp. 487-489

  The table below show the colours that you should expect to see in your preparation. Generally non-lignified tissue should be pink/purple and lignified tissue should be green/blue. Both colours tend towards dark blue when over stained.

Tissue Element or Structure	Colour
Xylem	Green or Blue-green
Phloem	Red
Sclerenchyma	Blue-green, sometimes Green
Collenchyma	Red-Purple
Parenchyma	Red-Purple