**Steedman wax protocol for Plant samples for Light Microscipy:**

I) FIXATION

1. Fixative: 3-4% Paraformaldehyde (EMS, from liquid stock) in 50mM PIPES, pH 6.9
2. Cut out the sample after submerging in the fixative with a surgical blade
3. Put them in the 10 ml tube with 5 ml fixative
4. Vacuum for 0.5-1 hr in a vacuum chamber
5. Wash with PIPES buffer for 10 min x 3 times
6. Wash with PBS for 10 min x 3 times

II) DEHYDRATION

1. 10 (ice), 25(-20C →), 35, 50, 70, 95, 100 % cold ethanol for 30 min each (dilutions in PBS when needed).
2. Another 100 % ethanol (RT) 30 min.
3. Another 100% ethanol (37C) 15 min or until solution reaches 37C in a 37C oven

III) INFILTRATION (everything is done in 37C)

1. 2 parts of 100% ethanol + 1 part melt Steedman's wax, 2 hr
2. 1 part of 100 % ethanol + 1 part melt Steedman's wax, 2 hr
3. 2 parts of Steedman's wax + 1 part 100 % ethanol, 2 hr
4. 100 % Steedman's wax, 2 hr
5. 100 % Steedman's wax, overnight

<If tissue was cut additionally after infiltration, leave the cut tissues in fresh wax for 15 min before embedding>

IV) EMBEDDING (procedure operated in the oven but power was off and door was left open for cooling down)

1. Pour fresh in the embedding mold and place the tissues with a forceps.
   *Tissue should not be disturbed during embedding
2. After wax was completely hardened, mold was moved to the hood

V) TRIMMING

1. (optional for big mold) A long cutting blade was slightly heated over the alcohol lamp and gently cut into pieces of small individual blocks to separate the pistil.
2. Extra wax was trimmed away with the heated blade to make smaller perpendicular block around the tissue.
3. With a lightly heated butter knife, melt-wax was applied on the wooden block, and also the bottom of the wax block was touched with heated knife to melt the wax slightly.
4. Place the wax block on the wooden block
5. With heated cutting blade, melt the joint of wax block and wood block to ensure the attachment.

VI) ADDITIONAL TRIMMING

* Top surface of the block where the tissue lays in were not touched. However, additional trimming was done to make the flat pyramid shape on top part of the block

VII) CUTTING on the microtome (Spencer 820, American Optical Company)

1. By gently pulling the section out from the blade while cutting, a series of section (ribbon) were cut.
2. Apply some drops of DI water on the adhesive glass slide.
3. Gently pick up the ribbon with a forceps and put on the water
   * cut side of the sections are to be contacted with water
4. Gently move around the water to spread the sections
5. Remove water by tilting the slide or by use of kimwipes
6. Air dry by having the slide standing in an angle
7. Remove residual water from the slide with kimwipes
8. VERY IMPORTANT Store slides in the storage box and leave the lid open for at least 18 hr in the hood to completely dry the section

VIII) DEWAXING

1. Dip slides in to 100% Eth for 10 min X 3 (fresh water free Eth each time)
2. Transfer slides in to 75, 50, 25 and 0% Eth in PBS each 15 min
3. 45 min in PIPES buffer

4. Incubate in an enzymatic mixture (5 mL): 2% Pectolyase (from Sigma) in 0.5 M EGTA, 0.4 M mannitol, 1% Triton X-100 and 0.3 mM PMSF all dissolved in PIPES buffer for 15 min.
5. Transfer slides to PIPES for 10 min.
6. Transfer slides to PIPES containing 1% Triton X-100 for 10 min
7. Block with 100% GBB (if you use Goat secondary antibodies) for 2-3 h (goat serum, BSA (both Igg free), Micro-O-Protect)
8. Rinse in PBS (brief)
9. Incubate with primary Anti-bodies containing 10% GBB overnight
10. Rinse 3X 10 min in PBS
11. Incubate with secondary anti-bodies 3-4 h
12. Rinse 3X 10 min in PBS
13. Mount in Mowiol/prolong gold (Invitrogen) mounting medium
14. Remove excess and seal with 'Top coat' 'instant dry' type nail polish and store the slides in 4 degree C until analysis.

Please cite the following reference if you are satisfied with this method and used in your experiments. Also if you have made any improvement to this, please let us know that will help us update the protocol.

Thanks

Shiv

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**Impacts of Aluminum on the Cytoskeleton of the Maize Root Apex. Short-Term Effects on the Distal Part of the Transition Zone**

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