Histology Protocol, Supplementary Data

Supplementary data to

Buzgo M, AS Chanderbali, S Kim, Z Zheng, D Oppenheimer, PS Soltis, DE Soltis 2007 Floral developmental morphology of *Persea americana* (avocado, Lauraceae): the oddities of male organ identity. *International Journal of Plant Sciences* (in press)

Table of Content

Fixation and dehydration

Material

- Ethanol (EtOH)
- Distilled H_2O, or DEPC-treated H_2O (H_2O_{depc}, for *in situ* hybridizations)
- Tissue samples: fixed (e.g., FAA, PFA), transferred and washed in 70 % EtOH several times

Fixation

FAA (Formalin, Acetic acid, Alcohol)

Good for histology and *in situ* hybridization.

Mix:

(Ruzin, S.E. 1999 *Plant Microtechnique and Microscopy*. Oxford: University Press. pp. 322. ISBN: 0-19-508956-1)

EtOH 95%	50 ml
Acetic acid	5 ml
Formalin (37% Formaldehyde)	10 ml
H_2O	35 ml

Can be stored several days in dark and cool.

- Drop samples into FAA
- Apply vaccum for few seconds (20-60) several times in fast succession (3-6x) with careful release in between.
- Incubate for 4-24 hours (depending on size, postfixation possible).
- Transfer into EtOH 50 %
- Transfer into EtOH 70 % for storage

PFA (Paraformaldehyde 4%)

Particularly for *in situ* hybridization.

Mix in beaker & stirrer

H_2O	90 ml
PFA	4 g
NaOH 10M	4-5 drops

- Microwave; heat to 65˚C, with occasional stirring, until PFA is dissolved.
- Cool solution to room temperature.

H_2SO_4 0.8M	adjust pH 7.0
PBS 10X	10 ml

- Cool the fixation solution on ice
- Drop samples into PFA
- Apply vaccum for few seconds (20-60) several times in fast succession (3-6x) with careful release in between.
- Incubate for 4-24 hours (depending on size, postfixation possible).
- Transfer into EtOH 50 %
- Transfer into EtOH 70 % for storage

Dehydration series

Incubate samples for 1 hour up to 1 day, depending on toughness and size, in:

- EtOH 70 %
- EtOH 85 %
- EtOH 95 %
- EtOH 100 %

Samples can be stored

Paraplast embedding

Material

- Paraplast™ or *Paraplast Plus*™ Tissue Embedding Medium [#23-021399 and #23-021400, respectively, *Fisher Scientific Com. L.L.C.*; Houston, TX 77038, USA]. *Paraplast Plus*™ contains DMSO for rapid infiltration).
  Melt *Paraplast* in advance (56°C, takes pretty long)
- Incubator or oven with steady temperature (56°C)
- *Histoclear*® (simple histology) [#HS3200/EA, *National Diagnostics*, Atlanta, GA 30336, USA], Xylenes (for *in situ* hybridizations)
- Embedding molds: commercial device. Or form molds: deep enough but ≤1cm, wide enough from cardboard, aluminum foil, plastic lid, small petri-dish, etc.
- Optional: warm bench and glycerol
- Flame (gas or spiritus), dissection needles and forceps
- Bowl with ice water or cool water
- Firm razor blades
- Wood blocks or manufactured microtome sockets.
- Fixed samples, dehydrated to 100% EtOH according to Dehydration above.

Infiltration

- Cover samples with Xylene or *Histoclear* 100 %
- Fill up with molten *Paraplast*, let melt again at 56°C
- Replace with molten *Paraplast*, let melt again at 56°C
- Replace with molten *Paraplast*, let melt at 56°C and let all Xylene evaporate (no more scent is detectable).

Incubate samples for 1 hour up to 1 day, depending on toughness and size, in:

- EtOH 100 % +0.01% Eosine (dye that increases sample visibility when embedded in *Paraplast*)
- EtOH:Xylene 2:1
- EtOH:Xylene 1:1
- EtOH:Xylene 1:2
- Xylene 100 % (samples can be stored)
- Xylene 100 % (samples can be stored)

Embedding

Some prefer to work on pre-warmed bench (slower solidifying)

- Lubricate inner surface of mold with Glycerol: may help removal of wax block from after hardening
- Pour *Paraplast* and sample swiftly into embedding mold, fill up with molten *Paraplast* if necessary
- Using the flame-warmed needles & forceps: place samples in appropriate position while *Paraplast* hardens. If placement goes awry, if too many air bubble occur, if added *Paraplast* forms separate layer, re-melt tub in stove (on a petridish to avoid spilling and leaking).
- Let *Paraplast* go somewhat firm ("skin").
- Drop mold into ice water till cold
- Store until use

Block

- Remove samples from mold
- Cut and trim with firm razor blade to appropriate shape
- Weld sample onto wood block: flame-warm blade and streak between wood block and sample bottom.
- Let cool completely
- Trim sample to trapezoid shape and remove excessive *Paraplast*

Continue with Microtome sectioning using rotation microtome (10-5 µm).

Metacrylate resin embedding

Replacing *Kulzer Technovit H8100* for about half the price.

Material

Metacrylate Mix

Work in hood, use gloves and wipe traces from bottle (odor)

- Butyl methacrylate [*Sigma-Aldrich #23,586-5*] 80 ml
- Methyl methacrylate [*Sigma-Aldrich #64200*] 20 ml
- Benzoin ethyl ether [*Sigma-Aldrich #17,200-6*] 0.5 g
- DTT (reductant) 10 mM (=0.154 g)
- **Gas removal:**
  Gently bubble N_2 through mixture for ~30min (get rid of O_2).
  Use an 250 ml Erlenmeyer with double-hole plug, one hole with plastic pipette tip as regulation walve, one hole for plastic tube with pasteur pipette reaching into mix, put Erlenmeyer into a styrofoam box, put liquid N_2 into Erlenmeyer, plug it, regulate by opening of styrofoam box and plastic pipette tip.
- Store in darkness at -20°C

Other material

- Ethanol (EtOH)
- Distilled H_2O, or DEPC-treated H_2O (H_2O_{depc}, for *in situ* hybridizations)
- Optional: DTT dissolved in EtOH (DTT 1mM for up to EtOH 95%, 10mM for 100%)
- Embedding molds: commercial device, or form molds: deep enough but ≤1cm, wide enough from aluminum foil, plastic lid, small petri-dish etc.
- Optional: long wave UV source (~420nm)
- Firm razor blades
- Improvised or manufactured microtome sockets (preferably plastic or aluminum).
- Bi-component epoxy-resin or similar glue (*Araldite, Loctite Quick Set™ Epoxy*, etc.)
- Fixed samples, dehydrated to 100% EtOH according to Dehydration above (optional with DTT, 1mM for up to 95%, 10mM for 100%).

Embedding: infiltration and polymerization

Then incubate in

- 2:1 EtOH:Metacrylate mix 30min, -20°C
- 1:1 EtOH:Metacrylate mix 30min, -20°C
- 1:2 EtOH:Metacrylate mix 30min, -20°C
- 100% Metacrylate mix overnight -20°C (dark), one day 4°C
- 100% Metacrylate mix 1h, -20°C
- 100% Metacrylate mix 2h, -20°C

- 100% Metacrylate mix, evacuate on 10min at 4°C, leave in hood overnight (dark)
- 100% Metacrylate mix exchange, seal and keep air completely out (O_2 inhibits polymerization)
- Expose to long wave UV (~420nm) for up to 6h at 4°C, or on ice, to fluorescent ceiling light or to sunlight behind a window.
- Samples can be stored

Block

- Remove hardened block with included sample from mold
- Trim to appropriate size and easy positioning with firm blade
- Glue to microtome socket using bi-component epoxy-resin glue
- Let harden

Continue with Microtome sectioning using rotation microtome (≤5 µm) and microscope glass slides (clean, untreated)

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