Date:

TISSUE PREPARATION FOR LASER MICRODISSECTION (LCM) WITH ETHANOL:ACETIC ACID FIXATION

Reference: This protocol is used for preparing plant material for laser micro-dissection specific cells/tissues, which are then used for isolation RNA. General fixation and embedding protocol is used by Tim Nelson group (Kerk et al. (2003). Plant Physiol . 132:27-35) and Goldberg lab. Written by Anhthu Bui (July 17, 2004 and Revised Oct. 6, 2008).

A. FIXING SAMPLES

1. Prepare a fresh ~500 mL of 0.1% DEPC water in a 500-mL glass bottle as following:
    a. Fill the glass bottle with ~500 mL of double-distilled or Milli-Q water.
    b. Add 0.5 mL of DEPC aliquot (stored in a plastic box in a refrigerator in room 2826) to the water. *Note: if DEPC aliquot is not available, get the stock bottle stored in the cold room and pipet 0.5 mL of DEPC solution*. Close the cap of the stock bottle tight and put the bottle back in the cold room, immediately. *Caution: DEPC is suspected as carcinogen*. Tighten the cap of the 500-mL bottle and shake it vigorously to dissolve DEPC micells.
2. Use the freshly prepared DEPC water to clean rinse the glass scintillation vials for fixing tissues and decontaminate the gloves. Then, rinse the vials with fixative solution to remove the excess DEPC water.
3. Prepare a fresh ethanol:acetic acid (3:1, v/v) solution in a **glass graduated cylinder** if > **50 mL** of fixative solution is prepared or in a 20-mL **glass** scintillation vial (**preferable** for 20 mL) or in a 50-mL **polypropylene** centrifuge tube as example below

Final Concentration	**20 mL**
3 volumes Absolute Ethanol	15 mL
1 volume Glacial Acetic Acid	5 mL

Mix the solution well. Keep the vial containing fixative solution on ice.

4. Excise/collect the tissue/organ and keep it in the fixative solution.
5. Fix the plant material in the Ethanol:acetic acid solution on ice or at **4°C for 4-16 hours.** Anhthu fixed tobacco anther sections (stage +6) for 4 hours.

B. DEHYDRATION AND INFILTRATION

1. Dehydrate plant material in the following ethanol solutions at room temperature (**3 hours/ solution**):

	75%	**85%**	**95%**	**100%**	**100%**	**100%**
Absolute Ethanol	15 mL	17 mL	19 mL	20 mL	20 mL	20 mL
DEPC treated water (autoclaved)	5 mL	3 mL	1 mL	0 mL	0 mL	0 mL
Time Changing Solution						

2. Infiltrate the plant material in the xylenes -solutionseries below for at least 3 hours in each solution at room temperature in a fume hood

Ratio of Ethanol (E) and Xylenes (X)	**E : X (75:25)**	**E : X (50:50)**	**E : X (25:75)**	**E : X (0:100)**	**E : X (0:100)**	**E : X (0:100)**
Volume of 100% Ethanol (E)	15 mL	10 mL	5 mL	0 mL	0 mL	0 mL
Volume of Xylenes (X)	5 mL	10 mL	15 mL	20 mL	20 mL	20 mL
Total Volume	20 mL	20 mL	20 mL	20 mL	20 mL	20 mL
Time Changing Solution						

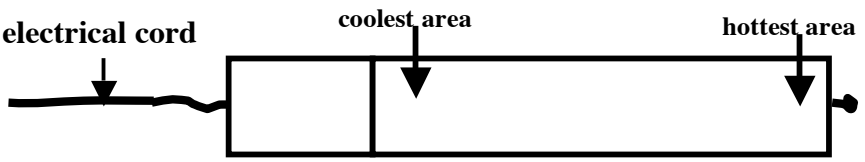
Dispose xylenes solutions in a glass "organic waste" bottle.

3. Add ~10 chips of Paraplast to the vial containing plant material in the last xylenes solution. Leave the vial in the fume hood for >4 hours or overnight (for convenience).
4. At the meantime, prepare a molten paraplast solution as follows:
   a. Fill a 1-liter glass beaker (RNase-free) to the rim with paraplast chips.
   b. Cover the beaker with a piece of brand new folded (2 layers) aluminum foil.
   c. Place the beaker full of paraplast chips in a 58-60ºC oven (dedicated for histology work) overnight (it would take >5-6 hours to melt the paraplast chips, 1-Liter chips will give ~400 mL of molten paraplast solution).
5. Next day, put the vial in a 42ºC air-incubator to melt the remaining of Paraplast chips for 3-4 hours.
6. **Infiltrate** tissues with paraplast by replacing xylenes:paraffin mixture with molten paraplast solution at 3- to 6-hour intervals until the xylenes odor is gone. Normally, Anhthu carries out 6 to 8 changes before embedding the samples in the paraffin.

Date	Time	Time

C. EMBEDDING AND STORAGE

a. Treat aluminum, fluted, low-form weighing dishes (Fisher, Cat # 08-732-101) with fresh DEPC water (**prepared as in step 1**) to remove any RNase.
b. Pour a freshly prepared DEPC water in a 500-mL glass beaker.
c. Submerge each weighing dish in the DEPC water for a few seconds.
d. Air-dry the DEPC-treated weighing dished on a new piece of aluminum foil in a fumehood with the dishes upside down for >1 hour or until no more water on the dishes.
e. Stack all DEPC-treated dishes and wrap them with a new piece of aluminum.
f. Put a piece of white tape with label as "DEPC treated and Date".
g. Embedding the plant material in the paraffin.
h. Warm up a slide warmer for at least 1 hour.
i. At the meantime, write on a piece of white tape "sample name, Date, your initial".
j. Place a DEPC-treated aluminum weighing dish on a hottest end of a slide heater (the end without an electric cord attachment, see figure below)



k. Pour the **infiltrated** tissues into the aluminum dish
l. Top off the dish with molten paraplast solution

m. Arrange the tissues in the dish so that pieces are at least 5 mm away from each other using either a warm wired needle or a warm flat-end spatula. *Note:*

- *A warm needle or spatula is briefly heated 2-3 seconds with a burner or a . Heat the needle or spatula occasionally to prevent cooled paraplast sticks to it.*
- *THINK in advance how you want to position your samples in the paraplast, so that when you cut it in blocks and trim it for the microtome sectioning, you will have a desirable angle.*

n. Once all the samples are arranged, slowly slide the dish toward the coolest area to allow paraplast to harden. Leave the dish there for about 10-15 minutes (after 10 minutes, the paraplast at the bottom of the dish harderns enough, carefully move the cooled dish from the slide heater to the lab bench so the "coolest area" of the slide heater is available for the next dish with samples)

o. Put the piece of label white tape prepared in step 13b above to the dish's handle

p. Repeat steps 13c-13h for other samples.

q. Once finish with all samples, unplug the slide heater.

r. Clean the surface of the slide heater by swiping with several layers of Kimwipes to remove spilled paraplast solution while the slide heater is still warm.

s. Allow paraplast in all dishes to harden at room temperature for >1-2 hours

t. Wrap all dishes with a piece of aluminum foil.

u. Store at 4ºC for at least 4 hours or until ready for sectioning.