Harvest and 3:1 Ethanol: Acetic Acid Fixation of Arabidopsis Seeds for LCM 030707 RK

-Always harvest seed in the afternoon (after 2:00 PM) in order to avoid excessive fixation overnight prior to processing the sample through alcohol and dehydration series

-Retrieve plants to be used in the days harvest from growth chambers. Age of plants will depend on growth conditions and desired seed stage. Check for siliques with characteristics exhibiting desired age (see: Developmental Series)

-ALWAYS collect seed out of a few test siliques and clear seed to confirm that your silique criteria match the stage of seeds contained within.

Clearing Solution:
2.5g Chloral Hydrate
0.7 ml ddH20
0.3 ml glycerol

Prepare in hood. Chloral Hydrate is toxic, handle with gloves and avoid breathing fumes.

Clearing Protocol:
Under a dissecting scope, cut open siliques with a needle and transfer seeds onto a microscope slide with a droplet of distilled water. You should be able to fit two water droplets on a slide and collect seed from different siliques into each. Be sure to leave enough room between the droplets for each to get a cover slip. Do not let your water droplet with your seeds dry out, it is okay to add additional water if needed.

Once you have your slides with your seeds, wick away the water using the edge of a kimwipe. Before the seeds start to dry, quickly add a few drops of Chloral Hydrate clearing solution to cover your seeds. Add cover slip atop the seeds; remove any excess clearing solution and let sit to clear. Clearing time will vary depending on stage of seed, with older seeds taking longer. Glob stage seeds should clear almost instantaneously.

Examine seeds under Nomarsky Optics to determine the stages of seed present in siliques. If it appears that your silique selection criteria are off for a particular set of plants, make appropriate adjustment to silique collection and again test clear seeds to confirm that you are going to be harvesting the correct stage. Capture representative images of the stages of embryo observed.

Tissue Harvest:
-Prepare fresh 3:1 Ethanol: Acetic acid fixative before each harvest. Use RNase free precautions. Transfer ~5ml into 2-dram glass vial. Keep vial capped and on ice until harvest.

You will need:

Dissecting scope and illumination source
Forceps
1ml syringe
Small hypodermic needle (30.5 gauge)
Microscope slide
Double-sided tape (optional)
Bucket with ice to keep fixative cold at bench.
RNase ZAP

1) Wipe down all work surfaces and instruments with RNase ZAP. Wipe down dissecting scope stage, focus knob, light source switch etc. with RNase ZAP. You want to ensure that anything that you are likely to touch during several hours of harvesting has been treated.

2) Using a new or well cleaned and RNase ZAP treated slide as a work surface on the microscope stage, pluck an appropriately staged silique with forceps and lay it down. Depending on personal preference, you can place a strip of double sided tape on the slide and use it to fix the silique in place.

3) Keep fixative on ice during harvest process. Vial may be left capped or uncapped during harvest.

4) Affix needle to syringe. Using beveled edge of needle, slice open silique along septum while being careful to avoid damaging seeds. If using double-sided tape you can push the silique walls down so they stick. Doing this will expose the seeds for easy collection.

5) Gather seeds into clumps using forceps and scoop into vial of fixative. Minimize the time between cutting open of silique and getting seeds into fixative. Avoid collecting damaged seeds.

6) Be sure to keep track of the number of siliques you collect seed from.

7) Use a new region of slide or tape for different siliques to avoid contamination with dried or damaged seeds. Clean slide and/or replace tape as needed.

8) Once harvest is complete, transfer vial to 4º C and let sit overnight. **Be sure to process samples into at least 70% EtOH the next morning** to avoid over fixation.

DAY TWO

Dehydration on rotator at room temperature:

-70% EtOH, 1 hour minimum. Samples can be left at this stage for upwards of a week if there are multiple harvests from different days that you want to process together.
-85% EtOH, 1 hour.
-95% EtOH, 1 hour.

-100% EtOH, 1 hour.
-100% EtOH, 1 hour.
-100% EtOH, overnight.
* All EtOH solution made with 2X autoclaved Depc'd water*

DAY THREE
Going into Xylenes on rotator at room temperature
-3:1, 100% EtOH: Xylenes, 2 hours.
-1:1, 100% EtOH: Xylenes, 2 hours.
-1:3, 100% EtOH: Xylenes, 2 hours.
-100% Xylenes, 2 hours.
-100% Xylenes, overnight.

DAY FOUR
-100% Xylenes, 2 hours.
-100% Xylenes (half full) + 6 paraffin chips, rotate.
-add 10 paraffin chips at the end of the day and rotate overnight.

DAY FIVE
-Incubate at 42 degrees until paraffin is dissolved, ~1.5 hours.
-Remove paraffin: xylenes solution and add melted paraffin. Incubate at 60 degrees.
-Paraffin change at end of day.

DAY SIX
-Paraffin change in morning.
-Paraffin change in afternoon.
-Paraffin change at night.

DAY SEVEN
-Paraffin change in morning.
-Paraffin change in late afternoon.
-Incubate at 60 degrees for ~1 hour and place in 4 degree until ready for embedding.