**Chemistry protocols for CEFE/CRNS – *Arabidopsis* herbivory experiment**

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**Clearing protocol – strong leaves**

1. Prepare solution ‘A’ of 5% sodium hydroxide dissolved in water.
2. Place individual leaves in tissue cassettes, labeling each with pencil (not pen). If leaves are very curved (non-planar) consider making incisions to allow them to flatten along desired lines, rather than forcing leaves to rip and tear later during slide mounting.
3. Place samples in solution ‘A’ for 12-96 hours. Samples should stay in this solution until the solution turns yellow-brown and the leaf samples are transparent white. This solution will have to be changed regularly to maintain its effectiveness (dispose of as basic [high pH] chemical waste). Samples must be removed in a timely manner from this solution to prevent full digestion.
4. Transfer samples to 100% water and agitate gently to wash.
5. Repeat previous step.
6. Transfer samples to 50% ethanol, 50% water.
7. Continue with Step 4 for normal leaves protocol.

**Clearing protocol – normal leaves**

1. Prepare stock solution ‘B’ of 95% ethanol, 5% glacial acetic acid.
2. Transfer the samples to 33% Solution ‘B’, then 67% water, 67% Solution ‘B’, 33% water. Keep the sample in each solution for at least 10-15 minutes (longer is OK).
3. Transfer samples to 100% Solution ‘B’. Samples should stay in this solution for at least 12 hours and probably 72-96 hours until the solution turns green and the leaf samples are transparent white. Cassettes can be carefully opened during this process to check progress. This solution will have to be changed regularly to maintain its effectiveness. Samples can stay in this solution indefinitely.
4. When each sample is ready (and not before), transfer it in tissue cassette to solution of 1% safranin dissolved in ethanol. Keep the sample in this solution for 30-60 minutes.
5. Transfer the sample to clean 100% ethanol, being careful to transfer as little extra stain as possible. Destain in this solution for at least 1 hour. Gentle agitation will increase the rate of destaining. Samples can stay in this solution for several days.

*If samples become too brittle and cannot be mounted: instead perform dehydrations at 25% ethanol : 75% water and 50% ethanol : 50% water and do staining in 0.1% safranin in 50% ethanol : 50% water, and destaining only at 50% ethanol: 50% water. Image contrast will be lower but samples will remain more pliable.*

**Mounting method - temporary**

1. Transfer the sample to 67% ethanol, 33% glycerol for 10-15 minutes.
2. Transfer the sample to 33% ethanol, 67% glycerol for 10-15 minutes.
3. Transfer the sample to 100% glycerol for 5-15 minutes.
4. Prepare a glass slide with approximately 200 µL (a few drops) glycerol spread in a circular region on the surface.
5. Using tweezers or a wooden stick (coffee stirrer), carefully transfer the leaf onto the slide, avoiding air bubbles as possible.
6. If necessary add an additional 100 – 200 µL (a drop or two) glycerol above the leaf.
7. Place a coverslip on the slide, lowering it onto the sample from the side. Press to remove air bubbles and wipe edges clean with paper tissue.
8. If air bubbles persist, place slide under vacuum for 1 minute or heat on hotplate to 60-80°C for 1-2 minutes until bubbles expand and move to sides of slide.
9. To make a semi-permanent mount, use clear nail polish to seal the edges of the coverslip.
10. Allow slide to rest for 1-2 days before imaging.

**Mounting method - permanent (requires chemical hood)**

1. Transfer the sample to 50% ethanol, 50% toluene or xylene for 1-2 minutes.
2. Transfer the sample to 100% toluene or xylene.
3. Prepare a slide mount with 200 µL Permount resin or other similar mounting medium placed in a circular region.
4. Using tweezers or a wooden stick (coffee stirrer), carefully transfer the leaf onto the slide, avoiding air bubbles as possible.
5. If necessary add an additional 100 – 200 µL (a drop or two) Permount above the leaf.
6. Place a coverslip on the slide, lowering it onto the sample from the side. Press to remove air bubbles and wipe edges clean with paper tissue.
7. If air bubbles persist, place slide under vacuum for 1 minute.
8. Allow slide to rest for 1-2 days before imaging.

**Imaging method**

1. Place leaf parallel to plane of camera sensor.
2. Set up trans-illumination (light underneath sample) on a clear glass plate
3. Set camera to f/7.1, ISO 100, manual focus, with 2-second timer delay, auto-exposure. If possible shoot in highest-quality JPEG file storage
4. Set camera frame to capture the maximum amount of un-damaged leaf underneath the cover glass.
5. Focus the lens manually.
6. Take photograph of sample. After pressing camera button, do not touch anything to allow vibrations to stabilize for the 2-second delay.
7. At the same camera and distance settings take a calibration image of an object with known distance scale.