

MICROBOTANICAL LABORATORY PROCEDURES 2013
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1. Preparation. In this step, you will generate your initial laboratory sample, referred to as your *Original Sample*, from a soil sample (1a), or from an artifact (1b). Gloves (non-starched) should be worn and changed between samples to avoid contamination.

1a. Soil Preparation.

- Dry Soils.
- Sieve through 0.5 mm mesh.
- Weigh as accurately as possible. Aim for 5-10 g (more for soils with higher clay content).
- Place soils in 50 ml tubes. These will be your *Original Soil Samples*.
- Add defloculant (e.g., 0.1% alconox), filling vials to 20 ml.
- Put all *Original Soil Samples* on shaker for several hours*.

<p>*After leaving vials on shaker, you can place 1 drop of the solution on a microscope slide to see whether particles are coming together. If they are, return vials to shaker.</p>
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1b. Artifact Residue Preparation.

- Create *Sediment 1* by thoroughly scrubbing artifact with a clean, wet toothbrush.
- Wash all resulting water and soil into a 50 ml tube. This will be *Original Sediment 1*.
- Create *Sediment 2* by submerging artifact in a water filled sonicator, and running for >10 minutes (artifact may be submerged in a suspended, sealed plastic bag, or placed in a glass beaker).
- Sediment 1* and *Sediment 2* samples will likely exceed 50 ml. To fit them into 50 ml tubes, simply centrifuge, decant, and refill as necessary. Final decant should leave as little water as possible in sample.
- If you are planning to weigh your samples to calculate density, place all *Original Sediment 1* and *Original Sediment 2 Samples* in a furnace at 40° C (100° F). Once dried (24+ hours), weigh and record sample weights.
- *If you are not planning to weigh your samples, you can either refill them with distilled water for Clay Removal (step 2), or transfer them to 15 ml tubes for Starch Flotation (step 3). (Clay removal is only necessary for dirty/opaque samples – most artifact residues will be translucent).

2. Clay Removal. The purpose of this step is to clean your *Original Sample* before flotation. Small particles of soil removed in this step will ultimately result in cleaner slides, which will aid in your identification of starch and phytoliths. (If your *Original Sample* is reasonably clear, particularly with artifact residues, this step may not be necessary). Gloves should be worn to avoid contamination.

- Shake/vortex vials vigorously (when turned upside down, there should be no soil clumped in the bottom).
- Centrifuge vials for approximately 2 minutes at 1000 rpm.
- Using a syringe or pipette, remove the upper water column, being careful not to disturb the soil clumped at the bottom.
- Refill vial with distilled water.
- Repeat entire process (shake, centrifuge, syringe, refill...) until your *Original Sample* is translucent (this may take 5+ times). After the final removal of the upper water column, do not refill with distilled water.

3. Starch Flotation. This step will generate a new set of samples, so that each *Original Sample* will have a paired *Starch Sample*. Gloves should be worn to prevent contamination, and to limit contact with heavy liquid (non-toxic). Remember to keep heavy liquids covered at all times (even with simple saran wrap) to avoid evaporation, as this could increase the specific gravity.

- Transfer *Original Samples* from 50 ml tubes to 15 ml tubes, using a squeeze bottle of distilled water to carefully wash material from the edges of the 50 ml tube into the 15 ml tube. If the 15 ml tube fills up before you have transferred all material, simply centrifuge (2 minutes at 3000 rpm) and decant to create more space. The 15 ml vial should be labeled exactly the same as the 50 ml vial (*Original Sample #*).
- Decant all water from 15 ml tubes, leaving compact soil in the base.
- Add ~4ml of 1.6 sg heavy liquid*.
- Shake/vortex vigorously (when turned upside down, there should be no soil clumped in the bottom).
- Centrifuge for 1 minute at 3000 rpm.
- Rinse material clinging to sides back into solution by gently tipping the sealed tube back and forth.
- Centrifuge for 3 minutes at 3000 rpm.
- Add another ~4ml of 1.6 sg heavy liquid.
- Shake/vortex vigorously.
- Centrifuge for 1 minute at 3000 rpm.
- Rinse material clinging to sides back into solution by gently tipping the sealed tube back and forth.
- Centrifuge for 3 minutes at 3000 rpm.
- Decant *Original Sample* into a new 15 ml tube, labeled as the corresponding *Starch Sample* (including relevant sample number or notes).
- Fill *Starch Sample* with distilled water, so that the vial now contains ~8 ml of 1.6 sg heavy liquid and ~7 ml of distilled water.
- Shake/vortex *Starch Sample* vigorously.

<p>*To prepare 100 ml of 1.6 sg solution, use 31 ml of LMT (2.95 sg) and 69 ml of distilled water (1.0 sg).</p>

- Centrifuge *Starch Sample* for 3 minutes at 3000 rpm.
- Decant *Starch Sample* into a container of used heavy liquid (for recycling*).
- Add another ~4 ml of 1.6 sg heavy liquid to *Original Sample*.
- Shake/vortex vigorously.
- Centrifuge for 1 minute at 3000 rpm.
- Rinse material clinging to sides back into solution by gently tipping the sealed tube back and forth.
- Centrifuge for 3 minutes at 3000 rpm.
- Decant *Original Sample* into corresponding *Starch Sample*.
- Fill all *Original Sample* vials and *Starch Sample* vials with water.
- Shake/vortex vigorously.
- Centrifuge all vials for 3 minutes at 3000 rpm.
- Decant all vials into a container of used heavy liquid.
- Set aside decanted *Original Sample* vials for Chemical Digestion (step 4).
- Label a corresponding 2 ml vial for each 15 ml *Starch Sample* vial, including all relevant information.
- Weigh and record the weight of each empty 2 ml vial, including sample number. This weight will be used in Slide Mounting (step 6).
- Use small disposable pipettes to transfer *Starch Samples* from 15 ml to 2 ml vials. Remember to label and use only 1 pipette for each sample to avoid contamination. If the 2 ml tube fills up before you have transferred all material, simply centrifuge (2 minutes at 3000 rpm) and remove supernatant (with pipette) to create more space.
- Place open 2 ml vials in a furnace at 40° C (100° F) until dry (12+ hours).

*Note that by adding ~7 ml of water to ~8 ml of 1.6 sg heavy liquid, you are reducing the overall specific gravity within the vial to ~1.2 sg, meaning that the starches (~1.4 sg) are now on the bottom.

4. Chemical Digestion. In this step, you will remove organics from your original sample in preparation for Phytolith Flotation (step 5). Removing organics will ultimately create cleaner slides. Because phytoliths are silica, they will withstand the harsh chemicals used in this step. However, any organic materials you wish to recover (i.e., starch or pollen) must be removed prior to this step, or they will be destroyed. Proper protection (heavy gloves, goggles, lab coats, closed shoes) should be worn throughout this step, and all chemicals should be kept under a fume hood. Remember to properly dispose of all hazardous chemicals in appropriately labeled containers.

*To make dilute HCl (1M): Mix 86 ml of concentrated HCl (11.6 M) with enough distilled water to make 1 liter. Store in a wash bottle.
 *To make Strong Acid: Carefully mix equal portions (50 ml per sample) of concentrated hydrochloric and nitric acid in a beaker. Make fresh for each session.

- Place a pot of water on hot plate under the fume hood and heat to ~90° C (194° F).
- Add a few ml of dilute hydrochloric acid* to *Original Sample* vials and place in hot water bath for ~10 minutes. (Remember these vials should not contain excess water).
- Slowly add strong acid* to sample. The more carbonates there are in the sample, the stronger the reaction will be. If a sample looks red, it is reacting strongly; if a sample looks yellow, it is not. Leave vials in bath for at least 90 minutes (longer if they are reacting). A glass rod can be used to stir up contents within vial. Bubbles indicate that a reaction is still happening.
- Fill tubes with warm water and centrifuge for 2.5 minutes at 2500 rpm in order to rinse acids. Decant and repeat three times. Remember to shake/vortex vigorously between refilling/centrifuging, and remember to decant into an appropriately labeled disposal container.
- Add ~5 ml of household bleach to *Original Sample* vials. Bathe in hot water for 5 minutes only. (If samples do not look very dirty, skip this step and move onto hydrogen peroxide).
- Add ~5 ml of hydrogen peroxide (27-35% strength). Bathe in hot water (lids off) for 20-90 minutes.
- Fill *Original Sample* vials with distilled water. Centrifuge for 2.5 minutes at 2500 rpm to rinse chemicals, and decant into an appropriately labeled disposal container. Repeat twice, remembering to shake/vortex vigorously between refilling/centrifuging.

5. Phytolith Flotation. This step will generate a new set of samples, so that each *Original Sample* will have a paired *Phytolith Sample*. This step essentially mirrors Starch Flotation (step 3), except that the heavy liquid will have a higher specific gravity. Gloves should be worn to prevent contamination, and to limit contact with heavy liquid (non-toxic). Remember to keep heavy liquids covered at all times (even with simple saran wrap) to avoid evaporation, as this could increase the specific gravity.

- Add ~4ml of 2.3 sg heavy liquid* to *Original Samples* (which should have been decanted in the previous step to remove all excess water).
- Shake/vortex vigorously (when turned upside down, there should be no soil clumped in the bottom).
- Centrifuge for 1 minute at 3000 rpm.
- Rinse material clinging to sides back into solution by gently tipping the sealed tube back and forth.
- Centrifuge for 3 minutes at 3000 rpm.
- Add another ~4ml of 2.3 sg heavy liquid.
- Shake/vortex vigorously.
- Centrifuge for 1 minute at 3000 rpm.
- Rinse material clinging to sides back into solution by gently tipping the sealed tube back and forth.
- Centrifuge for 3 minutes at 3000 rpm.

<p>*To prepare 100 ml of 2.3 sg solution, use 67 ml of LMT (2.95 sg) and 33 ml of distilled water (1.0 sg).</p>

- Decant *Original Sample* into a new 15 ml tube, labeled as the corresponding *Phytolith Sample* (including relevant sample number or notes).
- Fill *Phytolith Sample* with distilled water, so that the vial now contains ~8 ml of 2.3 sg heavy liquid and ~7 ml of distilled water.
- Shake/vortex *Phytolith Sample* vigorously.
- Centrifuge *Phytolith Sample* for 3 minutes at 3000 rpm.
- Decant *Phytolith Sample* into a container of used heavy liquid (for recycling*).
- Add another ~4 ml of 2.3 sg heavy liquid to *Original Sample*.
- Shake/vortex vigorously.
- Centrifuge for 1 minute at 3000 rpm.
- Rinse material clinging to sides back into solution by gently tipping the sealed tube back and forth.
- Centrifuge for 3 minutes at 3000 rpm.
- Decant *Original Sample* into corresponding *Phytolith Sample*.
- Fill all *Original Sample* vials and *Phytolith Sample* vials with water.
- Shake/vortex vigorously.
- Centrifuge all vials for 3 minutes at 3000 rpm.
- Decant all vials into a container of used heavy liquid.
- Set aside decanted *Original Sample* vials.
- Label a corresponding 2 ml vial for each 15 ml *Phytolith Sample* vial, including all relevant information.
- Weigh and record the weight of each empty 2 ml vial, including sample number.
- Use small disposable pipettes to transfer *Phytolith Samples* from 15 ml to 2 ml vials. Remember to label and use only 1 pipette for each sample to avoid contamination. If the 2 ml tube fills up before you have transferred all material, simply centrifuge (2 minutes at 3000 rpm) and remove supernatant (with pipette) to create more space.
- Place open 2 ml vials in a furnace at 100° C (212° F) until dry. If any starch samples are in the furnace, keep temperature to 40° C (100° F).
- If desired, *Original Sample* vials (15 ml) can be similarly transferred to 2 ml vials and dried for Slide Mounting (step 6).

*Note that by adding ~7 ml of water to ~8 ml of 2.3 sg heavy liquid, you are reducing the overall specific gravity within the vial to ~1.7 sg, meaning that the phytoliths (~1.9 sg) are now on the bottom.

6. Slide Mounting. This step describes how to make a standardized mount for microscope slides. A standardized mount allows one to estimate the density of starch grains/phytoliths within a sample without mounting the entire sample. This process saves time, but also preserves some of your sample for future slides (as slides tend to dry out over time). To make a non-standardized sample, simply

ignore the calculations described below. When making slides, one should wear gloves to avoid contamination. All materials are nontoxic.

6a. Mounting Starch.

- Weigh each 2 ml *Starch Sample* vial and subtract the weight of the vial itself (as recorded in step 3).
- Add corn syrup to *Starch Sample* in a 0.05:1 ratio. (Assuming that 0.05 ml of corn syrup weighs 0.0695 g, you should add 0.695 of corn syrup for every mg of extract in the starch vials). Place vial on scale (secured in styrofoam platform) and slowly add corn syrup using a syringe or pipette until desired weight is reached. This most likely will not be exact, so be sure to record the final weight/volume added.
- Use a toothpick to thoroughly stir mixture (several minutes). You can reduce air bubbles by centrifuging samples for 3 minutes at 3000 rpm.
- Use a toothpick to spread a thin layer of your prepared standardized mount onto a glass slide, remembering to stay within the size boundaries of a cover slip.
- Apply a cover slip, slowly laying it down from left to right to avoid bubbles.
- Seal the cover slip to the slide using nail polish. Apply to corners last, to allow air bubbles to escape.

6b. Mounting Phytoliths.

- Weigh each 2 ml *Phytolith Sample* vial and subtract the weight of the vial itself (as recorded in step 5).
- Add immersion oil to *Phytolith Sample* in a 0.05:1 ratio. (Assuming that 0.05 ml of immersion oil weighs 0.0462 g, you should add 0.0462 g of immersion for every mg of extract in the starch vials). Place vial on scale (secured in styrofoam platform) and slowly add immersion oil using a syringe or pipette until desired weight is reached. This most likely will not be exact, so be sure to record the final weight/volume added.
- Use a toothpick to thoroughly stir mixture (several minutes). You can reduce air bubbles by centrifuging samples for 3 minutes at 3000 rpm.
- Use a toothpick to spread a thin layer of your prepared standardized mount onto a glass slide, remembering to stay within the size boundaries of a cover slip.
- Apply a cover slip, slowly laying it down from left to right to avoid bubbles.
- Seal the cover slip to the slide using nail polish. Apply to corners last, to allow air bubbles to escape.

This laboratory procedure is written by Kristin Hoppa and is based on procedures used by Deborah Pearsall¹ at University of Missouri, Columbia, and Rob Cuthrell² at University of California, Berkeley.

¹ Chandler-Ezell, K. and D. M. Pearsall. 2003. "Piggyback' Microfossil Processing: Joint Starch and Phytolith Sampling from Stone Tools." *The Phytolitharien* 15:2-8.

² Cuthrell, Rob. 2012. Personal Communication, July 2012. University of California, Berkeley