PROTOCOL FOR RESTORATION OF HERBARIUM SPECIMENS FOR SEM AND SERIAL PARAFFIN SECTIONING METHODS

1. After thoroughly cleaning plants of soil and other debris, place them in a 1% solution of Tween 20 (or other detergent). Cap the vial and and allow the specimen to soak in this solution for 24 hours.

2. Rinse 3 times in dH_2O, allowing 1 hour for each rinse.

3. If the specimens are thick-walled, clear by placing in a 1.75% solution of KOH for 24 hours. The KOH softens the cell wall and makes it more permeable to the chloral hydrate solution. Fragile plants like *Fossombronia* do not require this clearing stage.

4. Rinse 3 times in dH_2O, allowing 1 hour for each rinse.

5. Replace the last water rinse with a saturated solution of chloral hydrate (=250 gms chloral hydrate in 100 ml dH_2O). Allow the specimens to remain in this solution from 4 to 7 days, depending on specimen texture. The chloral hydrate solution will slowly infiltrate the cells of the specimen, swelling them back to their original sizes and shapes. For large, dense plants 7 days is required to infiltrate all of the cells of the specimen, but for small, fragile specimens, like *Fossombronia*, this may be accomplished in 4 days. When the specimens are fully infiltrated, they will appear fairly clear and flexible.

6. Rinse 3 times in dH_2O, allowing 1 hour per rinse.

7. The samples are now ready for fixation. Specimens that are to be embedded in paraffin for sectioning are fixed for 24 hours in weak chromo-acetic acid. This fixative is mixed as follows: 2.5 ml of 10% aqueous chromic acid, 5 ml of 10% aqueous acetic acid, 92.5 ml dH_2O. Specimens to be prepared for SEM study may be fixed in the same way, or they can be fixed in a solution of 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.2 for 3 hours at room temperature, rinsed 3 times in dH2O, and post-fixed in 2% buffered osmium tetroxide for 3 hours at room temperature. There is less cell collapse in fragile tissues during drying and coating of SEM samples with the latter fixation regime.

8. Following either type of fixation, the samples are then rinsed 3 times, allowing 1 hour per rinse, and dehydrated appropriately. For paraffin sectioning, dehydration proceeds from 10% ETOH to 25% ETOH to the TBA series as described in the paraffin-sectioning protocol. For SEM preparations, dehydration begins with 10% ETOH and proceeds through a graded ethanol series to 100% ETOH. Specimens are critical point dried directly from 100% ETOH, using CO_2 as the transition fluid. Dried specimens are mounted and coated according to standard SEM protocols.