Chapter 9

SPECIAL METHODS

Presented in this chapter are a few important, but unrelated, techniques that may be useful to the plant and animal biologist. Some are presented mainly as a historical connection to systematic botany (*e.g.*, microincineration, preserving color in whole mounted specimens, and macerations), while others are old techniques that have found new uses in the molecular world. Clearing, for example, has become extremely useful in screening embryo and ovule mutants, and the several methods for beaching tissue presented here are useful for treating tissues destined for *in situ* hybridization. Keep in mind that these are just a few of the myriad useful techniques found in the old literature.

TISSUE CLEARING

Plant cells and tissues contain a variety of pigments and inclusions (ergastic substances) that render them opaque, or at best translucent. For a nonobscured view of internal structures of the plant organ the surrounding tissue must first be made transparent without destroying internal cell or tissue organization. Tissue clearing is useful for studying the vascular anatomy of plant organs as well as elucidating the three-dimensional organization of plant organs. There are many methods of clearing tissues using organic or aqueous chemicals. For example, Lersten(1967) lists 31 methods, all variations on the same theme. Herr (1971; 1972; 1982; 1985) describes the processes of tissue clearing as belonging to two distinct classes. In one class, clearing involves the removal of protoplasmic components, usually through the use of harsh chemicals (*e.g.*, NaOH). Chemically fixing the tissue beforehand is, of course, unnecessary since all cytoplasmic components are removed by the clearing procedure. Indeed, it usually is easier to clear dried, unfixed material rather than fixed material remaining in the fixative, presumable because chemicals of the fixative in some manner more closely bind to protoplasmic components.

In the second class of clearing procedures, chemical agents are not designed to remove cytoplasm, but rather to modify the refractive indices of cytoplasmic components so that they all become more-or-less equal and thus transparent. Difficulties emerge if the tissue contains a large amount of light-absorbing ergastic substances (*i.e.*, tannins); however the use of a bleaching agent (*e.g.*, Stockwell's bleach, *Bleaching tissues*, this chapter) before clearing may help.

The general protocol for clearing plant tissues is as follows. Fixed or non-fixed tissues are treated with one or more clearing solutions (usually NaOH, chloral hydrate, BB-$4^{1/2}$, etc.) over a period of several days. Following clearing, the tissues are then artificially stained using methods similar to staining tissue sections. Acid stains in concentrations from 0.1 to 1.0 mg/ml are effective for differential staining of cytoplasmic components. Basic dyes usually do not stain cleared tissues. Safranin O is one common stain used since an elucidation of vascular anatomy is often the goal.[64] Subsequent to clearing and staining, the tissues can then be mounted on conventional glass microscope slides or large glass plates (whole leaves, for example). The following are two examples of clearing techniques where either cytoplasm is removed through the use of caustic chemicals or maintained but made transparent by the addition of a clearing solution. Specimens (especially leaves) should be dried before clearing with NaOH.

[64]Safranin O stains lignified cell walls and stains the xylem and fibers of the vascular bundles.

CLEARING TISSUES WITH NaOH AND CHLORAL HYDRATE

Adapted from the methods of Arnott (1959) and Brady *et al.* (1965).

1. Treat tissue in 5% NaOH at RT to 37°C one to several days or until the tissue clears and no more pigment is removed.
2. Rinse briefly in DI water. Be careful as the tissues are quite fragile at this step.
3. If dark areas persist, bleach 2–5 min in full strength commercial bleach (Clorox or other commercial sodium hypochlorite solutions). Longer times can damage tissue. Stockwell's solution is acceptable, but takes much longer. Rinse in three changes of DI. (See notes following regarding bleach and staining properties.)
4. Treat tissue with chloral hydrate (CH) solution (saturated[65]; 250 g/100 ml DI) at RT to 37°C until material is transparent. This usually takes several hours to days. Tissue may be stored in chloral hydrate solution.
5. Wash 2× in DI water 15 min each, then overnight in DI water to remove the last traces of chloral hydrate.
6. Stain 20–30 min in 1% Safranin O solution in methyl cellosolve:95%EtOH:DI water, 2:1:1 (Johansen, 1940). Longer times will overstain.
7. If material is overstained, destain 2–3 min with acidic alcohol (95% EtOH + 0.5% picric acid).
8. Dehydrate in 100% EtOH.
9. Although usually not necessary or recommended because it adds little and wastes time, you may counterstain with Fast Green FCF. Johansen's Fast Green FCF solution should work fine (0.25% FG in 1:1:1 100% EtOH:methyl cellosolve:methyl salicylate) or use 0.25% FG in 100% EtOH. Stain a few minutes—to be determined empirically.
10. Rinse in EtOH:methyl salicylate:xylene (1:1:1) to destain. Two solution changes in a few seconds to minutes is adequate.
11. Transfer to 1:1 100% EtOH:xylene and rinse for a few minutes.
12. Transfer to xylene for a few minutes to remove precipitated stain.
13. Transfer to fresh xylene. Examine staining and either destain more (step 7), restain (step 6), or continue.
14. Transfer to xylene and store until mounting. Xylene will harden the sample; thus be careful when manipulating it at this point so as not to cause damage.
15. Mount with Permount, Merkoglas, etc., on conventional glass microscope slides or between thin glass plates (*e.g.*, whole leaves). It may be necessary to place a small lead weight on the coverslip or top glass plate during drying at 42°C. Drying will take a very long time (months in the case of large specimens).

Clearing Notes

- For delicate materials use 2.5% NaOH instead of the usual 5%.
- Prior to mounting, tissues must be placed in 100% EtOH for a sufficient time to ensure complete dehydration before transferring in xylene.
- To restore the staining properties of tissues:
    1. Soak for 15 min in 10% benzoyl peroxide in acetone.
    2. Wash in 2:3 xylene:acetone.
    3. Wash in 100% EtOH.

[65]Prepare chloral hydrate solution by filling the new reagent bottle with DI. Allow the chloral hydrate crystals to dissolve overnight.

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