Chapter 1

Histological Analysis of the *Arabidopsis* Gynoecium and Ovules Using Chloral Hydrate Clearing and Differential Interference Contrast Light Microscopy

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Abstract

The use of chloral hydrate optical clearing paired with differential interference contrast microscopy allows the analysis of internal structures of developing plant organs without the need for paraffin embedding and sectioning. This approach is appropriate for the analysis of the developing gynoecium or seedpod of the flowering plant *Arabidopsis thaliana* and many other types of fixed plant material. Early stages of ovule development are observable with this approach.

Key words Ovule, Chloral hydrate, Nomarski optics, Ovary, Seed development, Seedpod

1 Introduction

The gynoecium of the flowering plant *Arabidopsis thaliana* is the female reproductive organ and the site of development of the ovules, the precursors to the seeds. Early stages of ovule development take place within the developing gynoecial tube. During development, the observation of ovules and other internal gynoecial structures is obscured by overlying tissues.

One approach to visualize internal structures of the gynoecium is histological sectioning of the material. This involves imbedding the material in paraffin or plastic resin and cutting thin sections on a microtome. Although results can be quite good with sectioning, the process is time-consuming. An alternative to sectioning is the use of a clearing agent to increase the transparency of the overlying tissue. Optical clearing is often paired with an optical sectioning technique such as the use of differential interference contrast (DIC) microscopy also known as Nomarski microscopy [1]. This allows the researcher to image optical sections of the specimen without having to physically section the material. Thus internal structures can often be visualized without the physical removal of the overlying tissue (Fig. 1). This

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Fig. 1 Differential interference contrast (DIC) microscopic image of a optically cleared gynoecium of *Camelina sativa*. Internal structures, including two rows of developing ovules, can be easily visualized without physically sectioning the gynoecium. Scale bar represents 100 μm

procedure is particularly useful for characterize morphological changes that result from experimental treatments or environmental conditions or are associated with a specific genotype. Here we present our protocol for the analysis of *Arabidopsis* gynoecial structures using chloral hydrate (*see* **Note 1**) as a component of Hoyer's media [2].

2 Materials

1. A compound microscope with differential interference contrast (DIC) optics.
2. Glass scintillation vials (20 ml capacity).
3. Forceps.
4. Dissection scope.
5. Glass slides.
6. Coverslips.
7. Eppendorf tubes.
8. Fixative solution: 90% ethanol and 10% glacial acetic acid.
9. Hoyer's solution: 70% chloral hydrate w/v, 4% glycerol, and 5% gum arabic. To prepare 20 ml of Hoyer's solution, use a

50 ml conical tube to mix 8 ml of distilled water H_2O, 2 ml of 40% glycerol, 14 g of chloral hydrate, and 1 g of gum arabic. Tightly seal the conical tube with parafilm and allow it to rock or nutate for several hours to ensure complete dissolution. Do not heat the solution. Then adjust final volume to 20 ml with distilled water. Spin in a centrifuge (100 × *g*, 5 min) to remove precipitate. Store supernatant at room temperature in a sealed tube. The solution will last at least 6 months.

10. 90% Ethanol.

3 Methods

3.1 Fixation and Initial Clearing

1. Fix inflorescences or other plant material for 2 h at room temperature in fixative solution (90% ethanol; 10% glacial acetic acid). We typically fix in glass scintillation vials. About ten *Arabidopsis* inflorescences can be fixed together in one 20 ml scintillation vial (*see* **Note 2**).
2. Remove fixative and wash the fixed tissue with 10–20 ml of 90% ethanol for 30 min. Rock or nutate to the wash tissue.
3. Remove first wash and repeat this 30 min. Wash with fresh 90% ethanol. Fixed tissue samples can be stored for long periods of time (months) in 90% ethanol in tightly capped scintillation vials if desired.
4. Transfer a single inflorescence to a clean Eppendorf tube containing approximately 500 µl of Hoyer's solution (*see* **Note 3**). When transferring the tissue, touch the tissue to a Kimwipes briefly to remove excess ethanol before transferring into Hoyer's solution. However, do not allow the tissue to dry completely, as that can alter the morphology of epidermal structures. Some ethanol will inevitably be transferred into the Hoyer's solution, but this will be diluted in subsequent steps.
5. Ensure that the Hoyer's solution is covering the tissue completely. Allow the tissue to soak in Hoyer's solution for several hours or overnight.

3.2 Dissecting and Mounting the Tissue

1. Transfer the tissue to fresh Hoyer's solution in a clean Eppendorf tube.
2. Under a dissection scope, in a drop of Hoyer's solution, dissect the sample with forceps. Often it is best to dissect the sepals, petals, and stamens away from the central gynoecium to allow best imaging of the gynoecium. As a single inflorescence has many flowers, it is good to dissect the inflorescence in stages and transfer smaller subsections to a fresh drop of Hoyer's on a clean glass slide to continue dissection (*see* **Note 4**).
3. Once plant organs are dissected from the inflorescence, proceed to mounting sample.

3.3 Mounting Gynoecia in Hoyer's Solution

1. Place a fresh drop (approximately 75 μl) of Hoyer's solution on a clean glass microscope slide. Transfer dissected gynoecium with forceps into the drop of Hoyer's on the slide. Carefully lay a coverslip over the gynoecium allowing Hoyer's to flow to the edge of the coverslip as the coverslip sinks down. For very small or delicate samples, "bridges" made from smaller coverslips can be used to support the main coverslip if needed to prevent sample from being crushed. Typically this is not necessary.
2. Allow slide to lay flat for 12–24 h to allow it to dry. Hoyer's solution will harden over this time and generate a permanent mounted specimen. There is no need to seal the edge of the coverslip as the Hoyer's will self-seal as it hardens and dries (*see* **Note 5**).

3.4 Examine the Gynoecium Under DIC Optics Using Koehler Illumination

Proper viewing of samples requires DIC or Nomarski optics to generate contrast in the otherwise clear samples. This requires proper microscopic equipment as well as proper adjusting of the microscope illumination source (i.e., to obtain Koehler illumination).

1. To set the illumination source for optimal Koehler illumination, rack up the condenser for the light source.
2. Put a sample on the microscope and focus your microscope on the sample with the 10× objective.
3. Close the lamp field diaphragm to limit the illuminated area to its smallest extent.
4. Raise or lower the condenser so that the diaphragm is in focus. The edge of the diaphragm should appear crisp through the eyepiece.
5. Use the condenser centering screws to center the condenser diaphragm if necessary.
6. Now open up the lamp field diaphragm so that the illuminated area just fills the area to be imaged (i.e., open up the diaphragm so that the edge of the diaphragm is still visible at the outer edge of the viewing area but is outside of the frame of the area that will be captured photographically).
7. Adjust the contrast of the image using the condenser diaphragm to achieve best image (*see* **Notes 6**, **7** and **8**).

4 Notes

1. Note that chloral hydrate is a controlled substance in the United States [3]. Thus state and federal regulations require registration of users to purchase, store, and use. See drug regulatory diversion site [http://www.deadiversion.usdoj.gov/] to obtain federal approval. As chloral hydrate is a sedative and a central nervous system depressant at high doses, wear gloves when handling Hoyer's or chloral hydrate and wash hands

after using. Do not eat of drink while using chloral hydrate. Visikol™ is a nonregulated alternative for chloral hydrate [4]. However, I have not yet tested Visikol™ as a replacement for chloral hydrate in my lab.

2. We usually fix many samples and then keep the tissue in 90% ethanol in parafilm-sealed glass scintillation vials for storage until ready to dissect and mount. Mesh screens can be useful for "catching" tissue when removing liquid from scintillation vials.
3. Gynoecia can be cleared directly in Hoyer's without a prefix, but these samples will eventually dissolve or deteriorate in the Hoyer's. Thus, we prefer to prefix the tissue and allow for the generation of permanent mounted specimens for long-term storage and imaging. For *Arabidopsis* inflorescences, we do not typically apply vacuum infiltration to allow penetration of fix. With other sample types, this may be more critical depending on thickness of the sample and amount of trapped air in the intracellular spaces. If using vacuum infiltration to enhance penetration of the fixative, it is best to pull the vacuum slowly and release the vacuum slowly so as to not burst sensitive cells. If needed, apply vacuum for about 10 min and then release it for 10 min. Repeat this cycle as needed.
4. There are many variant recipes for Hoyer's [2, 5, 6] and in our hands they all seem to clear well. Higher percentages of gum arabic seal faster when drying, but recipe is probably a matter of personal preference.
5. Dissecting gynoecia from the inflorescence is often done in a serial fashion (i.e., first put the inflorescence in a drop of chloral hydrate on the first slide and then pick off a few flowers of the appropriate stage and move those to a second slide with fresh drop of Hoyer's). This helps to ensure that any ethanol is well diluted in Hoyer's and generates the clearest samples for viewing. As you dissect, you may see the ethanol still leaving the tissue. Dissection of the sample in a drop of Hoyer's can be difficult under a dissection scope due to the reflection of the illumination light off the surface of the Hoyer's solution. Adjustment of the angle of the illumination or illuminating the sample from below can help with this issue. Opening the gynoecial tube by pinching with a forceps or cutting with a razor blade the apex or base (top or bottom) of the gynoecium allows better penetration of Hoyer's into center of the gynoecial tube. This is perhaps more important with older developmental stages. This is typically not needed with younger developmental stages or with many mutants since they are often not sealed up tightly into a closed tube. Allowing the Hoyer's to penetrate the gynoecial tube allows you see the ovules better through the valve without having to tear apart the gynoecium. If viewing of the internally located ovules during late developmental stages is the objective, more dissection may be required. If desired, one of the gynoecial valves (ovary walls) can be removed with forceps to

expose the ovules and image the more mature ovules. If dissected samples are small, consider using a smaller-sized Eppendorf tube during the initial Hoyer's clearing step. With small samples, it can be difficult to pick up the sample in the bottom of the Eppendorf tube with the forceps and shallower or wide-mouthed tubes may be more appropriate.

6. Gynoecia may not be completely cleared when first mounted. The final clearing and penetration of Hoyer's into the gynoecial tissues appears to happen within the first 12–24 h after mounting. After several hours, as the Hoyer's starts to dry, it may become apparent that more Hoyer's needs to be added to the sample. This will be visible as an air bubble starts to form near the edge of the cover slip. If this occurs, simply add a small amount of Hoyer's carefully under the edge of the coverslip and allow the slide to continue to dry for several more hours.
7. DIC microscopy requires the microscope to be equipped with a polarizing filter (polarizer), a condenser prism, an objective prism, and an analyzer (See Murphy and Davidson for additional details [1]). The nature and positioning of these components can vary on different microscopes; however, a few general tips are worth mentioning here to help you adjust the scope for the best-quality images. Firstly, each objective is often fitted with a different objective prism. These are paired with a particular condenser prism. Thus one should ensure that the correct condenser prism is selected for each objective lens. Selection of the condenser prism is often controlled by a "wheel" that the user turns to select different condenser prisms. Secondly, fine tuning of the contrast of the image can be achieved by adjusting the orientation/angle of the objective prism. This is typically achieved by turning a small knob or set screw that is located on the objective lens. Finally, the orientation of the sample will also dramatically alter the image due to the directionality of the polarized light source. One can adjust the image by rotating the sample using a rotating stage, if the microscope is equipped with this feature. Rotating the stage can dramatically alter the image and is a useful parameter to adjust for optimal image quality.
8. A summary of the different floral stages of *Arabidopsis* floral development can be found here in Smyth et al. [7]. A description of the stages of *Arabidopsis* ovule development can be found here [8]. A description of *Arabidopsis* gynoecial morphology can be found here [9].

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