Optimizing Whole-mount Confocal Microscopy for Ovules and Seeds
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Introduction
Preparation of ovules and seeds for microscopic imaging is fraught with challenges:
- Ovules are small and enclosed within maternal tissues, making it difficult to achieve the desired orientation during sectioning.
- Ovules can be cleared and imaged in whole-mount with Nomarski optics (1), but these techniques are not often compatible with staining techniques that reveal histochromic information.
- Seed coats pose a particular challenge. Their impermeability hinders proper infiltration of embedding media - internal tissue altogether detached from the seed coat and popping out of sections is a common problem.
- Cleaning of whole-mount seeds is difficult due to the thick cell walls and high pigment or phenolic compound content of seed coats.
- Both ovules and seeds are complex, 3-D structures that are difficult to reconstruct from single images of 2-dimensional planes. (2, 3)

We provide a streamlined approach to preparing ovules and seeds for whole-mount, confocal microscopy. These techniques can:
- collect sub-cellular, histochemical information
- facilitate creation of 3-D reconstructions
- allow imaging as little as 48 hours after sample collection

Small Samples
Entire developing or unfertilized ovules and young seeds of many taxa can be cleared by infiltration with immersion oil.

Case Study: Bunchonchus functional megaspore
A whole-mount immersion-oil treated ovule. Strands of condensed chromatin are seen in the megaspore nucleus (inset) 3-D model of chromatin strands of the same nucleus, with the nucleolar evident

Clearing Procedure:
- Start with stained samples
- Dehydrate through an ethanol series, to 100% ethanol (2-4 hours)
- Incubate in 1:1 ethanol: immersion oil (30 min)
- Incubate in 100% immersion oil (10 min. samples can be stored for multiple weeks)
- Ready for Mounting and Imaging

Approach Overview
How does the quality of optical sections compare to other methods?
- Samples can be stored:
  - indefinitely in 70% ethanol, after fixation or staining
  - for months to years, after embedding in resin
  - for weeks to months, after clearing in immersion oil

Mounting and Imaging
By using combinations of excitation wavelengths and splitting emission ranges into different channels, it is possible to collect additional histochemical information (7) with a single staining procedure.

Case Study: Megaposporogenesis in Nymphaea (Top Row) Confocal optical sections (Bottom Row) Resin-embedded sections, stained with PAS reaction

Fixing and Staining
The type of fixative used to preserve samples can influence how the sample will react to staining and imaging. Combinations of fixatives and stains must be tested to optimize preservation of, and contrast between, structures of interest.

We find that fixation with 4% paraformaldehyde and staining with Feulgen reaction (4) works well for most ovules and seeds.

Case Study: Female Gametophytes in Aquilegia (Far Right) All ovules were cleared and imaged using the same fixation settings. Fixation with 4% paraformaldehyde gives better preservation, but results in too much background when stained with DAPI. When stained with the Feulgen reaction, it is possible to distinguish nuclear material and starch grains. (Right) To verify the fluorescence patterns of starch grains, tissue from the storage tuber of a potato was treated and imaged.

Large or Difficult Samples
Seed coats often prevent imaging of interior tissues, but removing the seed coat or overlying layers can destroy the entire sample (5). Embedding in resin, while adding time to the procedure, allows for clean cuts - even by hand, with an ordinary razor blade.

Case Study: Nymphaea thermarum seeds
(Left) An enlarged view of an entire seed. Because of resin embedding, the integrity of the delicate interior tissues is preserved. (Below) Automated collection of serial optical sections facilitates 3-D modeling of endosperm and embryo development. This series of models shows the changes in volumetric relationships off the seedling's germination throughout seed development.

References and Acknowledgements
(4) Eady, P. and J. USchopka et al. (2000). *Confocal microscopy of whole seeds for analysis of microtubule development in the angiosperm* *Mimulus luteus*.

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