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:

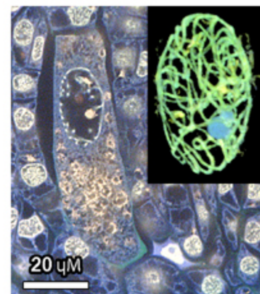
- Ovules are small and enclosed within maternal tissues, making it difficult to achieve the desired orientation during sectioning.
- Ovules can be cleared and imaged in whole-mount with Nomarski optics *(1)*, but these techniques are not often compatible with staining techniques that reveal histochemical information
- Seed coats pose a particular challenge. Their impermeability hinders proper infiltration of embedding media - internal tissue altogether detaching from the seed coat and popping out of sections is a common problem.
- Clearing 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: *Bunchosia* 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 nucleolus evident

Clearing Procedure:

- **Start with stained samples**

1) Dehydrate though an ethanol series, to 100% ethanol (2-4 hours)

2) Incubate in 1:1 ethanol: Immersion oil (30 min)

3) Incubate in 100% Immersion oil (10 min, samples can be stored for multiple weeks)

- **Ready for Mounting and Imaging**

References and Acknowledgements

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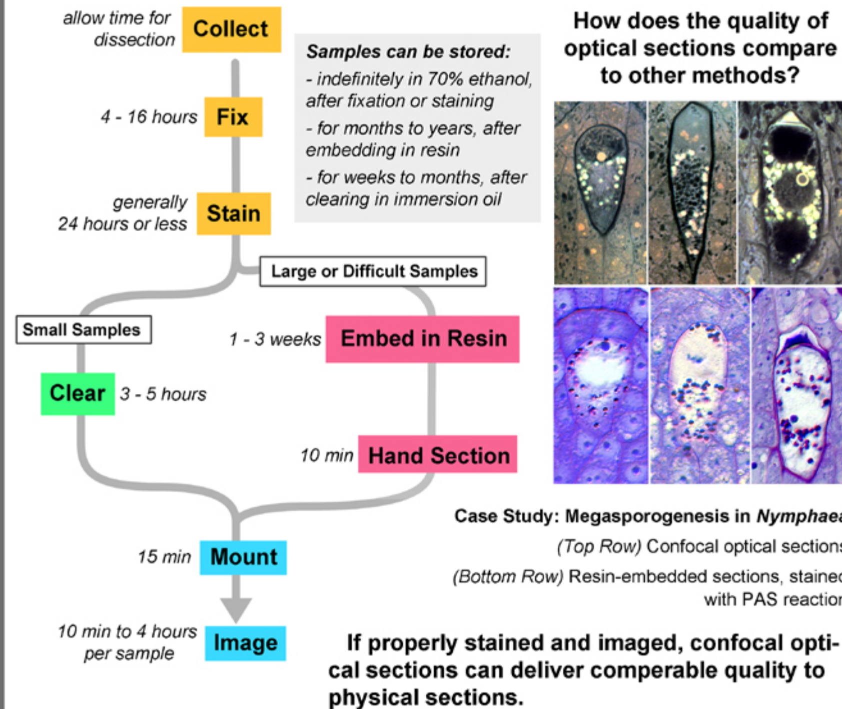
Approach Overview



How does the quality of optical sections compare to other methods?

Case Study: Megasporogenesis in *Nymphaea*

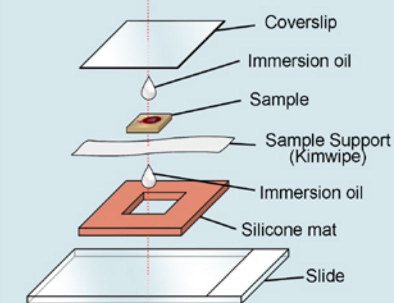
(Top Row) Confocal optical sections

(Bottom Row) Resin-embedded sections, stained with PAS reaction

If properly stained and imaged, confocal optical sections can deliver comperable quality to physical sections.

Mounting and Imaging

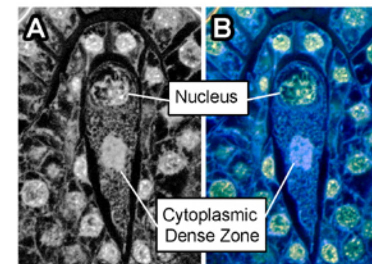
Making Well Slides for Mounting Samples



Whole mount samples need to be placed in well slides to keep from being crushed by the coverslip *(6)*. This mounting system works for immersion-oil cleared samples and for resin-embedded samples.

Image	Channel	Excitation	Detection Range
A	1 (white)	639 nm	500-750 nm
B	1 (blue)	405 & 488 nm	400-588 nm
	2 (green)	405 & 488 nm	588-750 nm
	3 (red)	639 nm	500-750 nm

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: Megaspore Mother Cell in *Nymphaea*

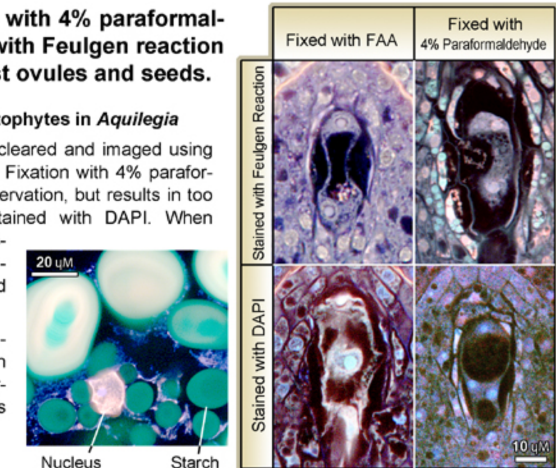
The megaspore mother cell can be characterized by a cytoplasmic dense zone *(8)*. *(A)* In ovules stained and imaged to detect nucleic acids, it is difficult to distinguish the cytoplasmic dense zone from nuclear material. *(B)* However, if additional excitation and emission settings are used, then it is possible to tell cytoplasm from nucleic acids.

(Left) Excitation and detection settings for images A and B.

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 detection 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.

20 μM

Nucleus Starch

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.

Embryo
Endosperm
Perisperm
100 μM

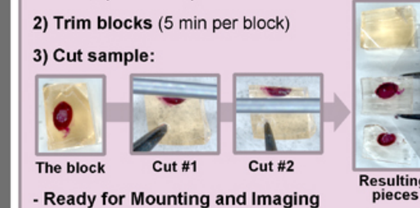
Resin Procedure:

- **Start with stained samples**

1) Infiltrate and embed material in resin of choice, (1-3 weeks)

2) Trim blocks (5 min per block)

3) Cut sample:



- **Ready for Mounting and Imaging**

Case Study: *Nymphaea thermarum* seeds

(Right) An even longitudinal view of an entire seed. Because of resin embedding, the integrity of the delicate interior tissues are 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 between the offspring tissues throughout seed development.