Clear heads:
simplified tissue preparation for studying plant anatomy and development in the Asteraceae

Reid Selby* [1,2], Brannan R. Cliver* [1,2], Zach Meharg[3,4], Alex Harkess[4] & Daniel S. Jones[1,2]

[1] Department of Biological Sciences, Clemson University, Clemson SC, USA
[2] Department of Biological Sciences, Auburn University, Auburn AL, USA
[3] Department of Crop, Soil, and Environmental Science, Auburn University, Auburn AL, USA
[4] HudsonAlpha Institute for Biotechnology, Huntsville AL, USA

Author for correspondence: *dsj2@clemson.edu*
* *These authors contributed equally and share first authorship.*

DOI: http://dx.doi.org/10.53875/capitulum.04.1.02

ABSTRACT

The use of microscopy is instrumental for our understanding of plant anatomy and development. A major bottleneck is that conventional methods for plant tissue preparation can take weeks to complete and can involve hazardous materials that many labs are not equipped to handle. Furthermore, these methods traditionally involve embedding the fixed tissue in embedding substrates such as paraffin or plastic resins, which is both a laborious and time-intensive process. Here we describe a simplified and accessible method that accomplishes tissue fixation and clearing in just three days, minimizes the use of hazardous materials, and eliminates the need for embedding/sectioning. This method also utilizes structural autofluorescence of plant tissues and thus does not require any counterstaining to resolve structure. We demonstrate the utility of this preparation using both epifluorescence and confocal microscopy on examples from the Asteraceae family, making both techniques more approachable to biologists of all backgrounds and thus enabling a richer exploration of plant anatomy and development.

Keywords: anatomy, confocal, development, fluorescence microscopy, morphological diversity, tissue clearing.

INTRODUCTION

Light microscopy is an invaluable tool in studying plant anatomy and development. Specifically, the use of fluorescent microscopy has become an essential part of most plant cell, molecular and developmental biology laboratories' toolkits, complementing the vast number of molecular cloning tools that have come about in the last 30 years (i.e., fluorescent proteins). Most of these applications require transformation as well as live imaging of tissues to visualize fluorescent markers, limiting their utility largely to model systems. This prevalence of fluorescent microscopy does mean, however, that some form of epifluorescence or confocal microscopy is increasingly available. To further facilitate the accessibility of fluorescence microscopy, here we describe a method of plant tissue preparation that is compatible with most sample types and approachable for researchers who are new to microscopy.

A key advantage of this protocol is chemical safety. While eco-friendly and low-risk methods of tissue clearing are growing in popularity, many still involve

Morphology begins with development

Flowering plants display remarkable morphological diversity, which is well-exemplified by Asteraceae capitula. Understanding diversity of form begins with understanding diversity of development.

Lateral view of developed head of Tagetes erecta 'Antigua Orange'
Photo by Reid Selby

harsh chemicals like chloral hydrate and xylenes. This protocol uses methyl salicylate, a common food flavoring agent that smells of mint or wintergreen, making it another useful alternative to more hazardous reagents. The tissue clearing properties of methyl salicylate were first described in an 1866 study comparing clearing agents with high refractive indices (Stieda, 1866). Because the refractive index of methyl salicylate (1.53) is close to glass (1.55), light passing through one medium into the other undergoes minimal distortion (limited diffraction). Methyl salicylate has since been well-described as an effective clearing agent in a variety of tissues (Hériché et al., 2022). In addition to using harsh chemicals, common methods used to prepare tissues are time consuming and can require specialized equipment (e.g., tissue embedding stations). Tissue preparation is still a major bottleneck for biologists working in diverse, non-model plant systems, especially when imaging larger structures at high resolution, which typically require experience and time to create quality sections. This technique is fast, approachable, and requires minimal equipment. To showcase the versatility of this method across sample types, we imaged a variety of tissues from multiple Asteraceae species. This method is particularly effective for imaging stages spanning capitulum development, demonstrating its utility in preparing large samples for detailed imaging. While methyl salicylate is not the only safe method of tissue clearing, it should be strongly considered by both experienced microscopists looking for a fast and easy alternative to other methods, and researchers new to microscopy looking for an approachable technique.

PROTOCOL

This protocol can be broadly categorized into four fundamental steps: dissection, fixation, clearing, and imaging (Figure 1).

Dissection:

While the rest of this protocol can be applied to most tissue types with limited modifications, dissection techniques will vary according to your tissue of interest. For many samples, only a single cut is necessary to both expose the structure for imaging and to allow for the proper penetration of fixing and clearing agents. A clean, flat surface in the same plane as the region being imaged is necessary for the best quality image. A single stroke of a razor blade with minimal downward force is best, relying on slicing rather than chopping. It is worth noting that even the most careful cuts can result in some damage; however, sufficiently cleared tissue enables imaging on a focal plane a few cell layers in, bypassing damage from the cut site. For all images of developing capitula in this paper, we made a single longitudinal cut down the center of the bud with a fresh razor blade. If the structure being imaged is external or near external, such as leaf stomata or root tips, it is possible that limited or no dissection is required. For thicker samples, some additional sectioning may be necessary to allow the fixative and methyl salicylate to properly penetrate the tissue.

Fixation and dehydration:

Immediately after dissecting, place samples in ice cold FAA (2% (w/v) formaldehyde, 5% (v/v) acetic acid, 60% (v/v) ethanol) to fix at 4°C for ~24 hours while rocking—ideally with a nutating mixer, but any form of gentle agitation is suitable. While many fixatives can work with this protocol, we find that using FAA with 2% formaldehyde gently fixes the tissue and preserves cellular integrity well (Johansen, 1940; Jackson et al., 1994). Moreover, using a relatively low starting percentage of ethanol is sufficient for fixation, prevents excessive tissue hardening, and allows for a gentle transition to 70% ethanol in the next step. The day after fixation begins, decant the FAA, and add cold 70% ethanol for at least 30 minutes. Fixed tissue can then be stored in 70% ethanol for several months before continuing to the next step. Storing fixed samples at 4°C may further increase longevity. To prepare tissues for clearing, serially dehydrate them by replacing the 70% ethanol with 80%, then 90%, 95%, and finally 100% twice. Wait 30 minutes between each step of the dehydration. Make sure to use sufficient volumes of FAA and ethanol, such that the sample is never greater than 10% of the total volume. While fixation and dehydration are possible at room temperature, we find that using chilled FAA and ethanol slows dehydration as well as plasmolysis, preventing cellular distortion.

Clearing and imaging:

Once the tissue is fully dehydrated, decant the ethanol and replace it with 100% methyl salicylate. Allow the methyl salicylate to clear the tissue for

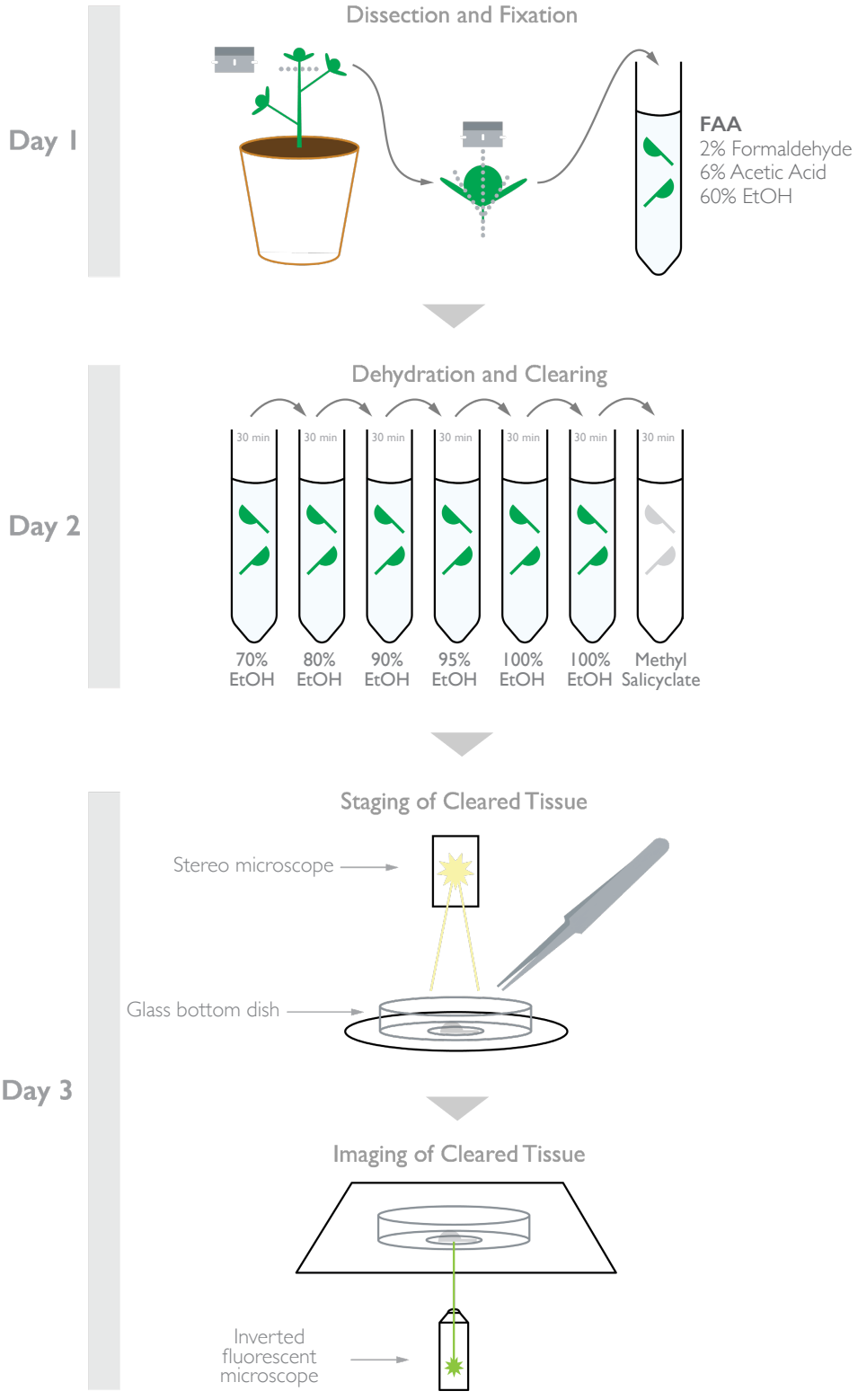

Figure 1. A workflow for imaging with methyl salicylate. This example of fixing and clearing tissue with methyl salicylate is spread across three days. Day 1, the tissue is dissected and fixed overnight in FAA (2% formaldehyde, 5% acetic acid, 60% ethanol). Day 2 the tissue is serially dehydrated by placing in 70% ethanol for 30 minutes, 80% ethanol for 30 minutes, 90% ethanol for 30 minutes, 95% ethanol for 30 minutes, and 100% ethanol twice for 30 minutes each. The tissue is then placed in methyl salicylate overnight. Day 3 involves staging the cleared tissue on a stereo microscope before imaging on an inverted fluorescent microscope.

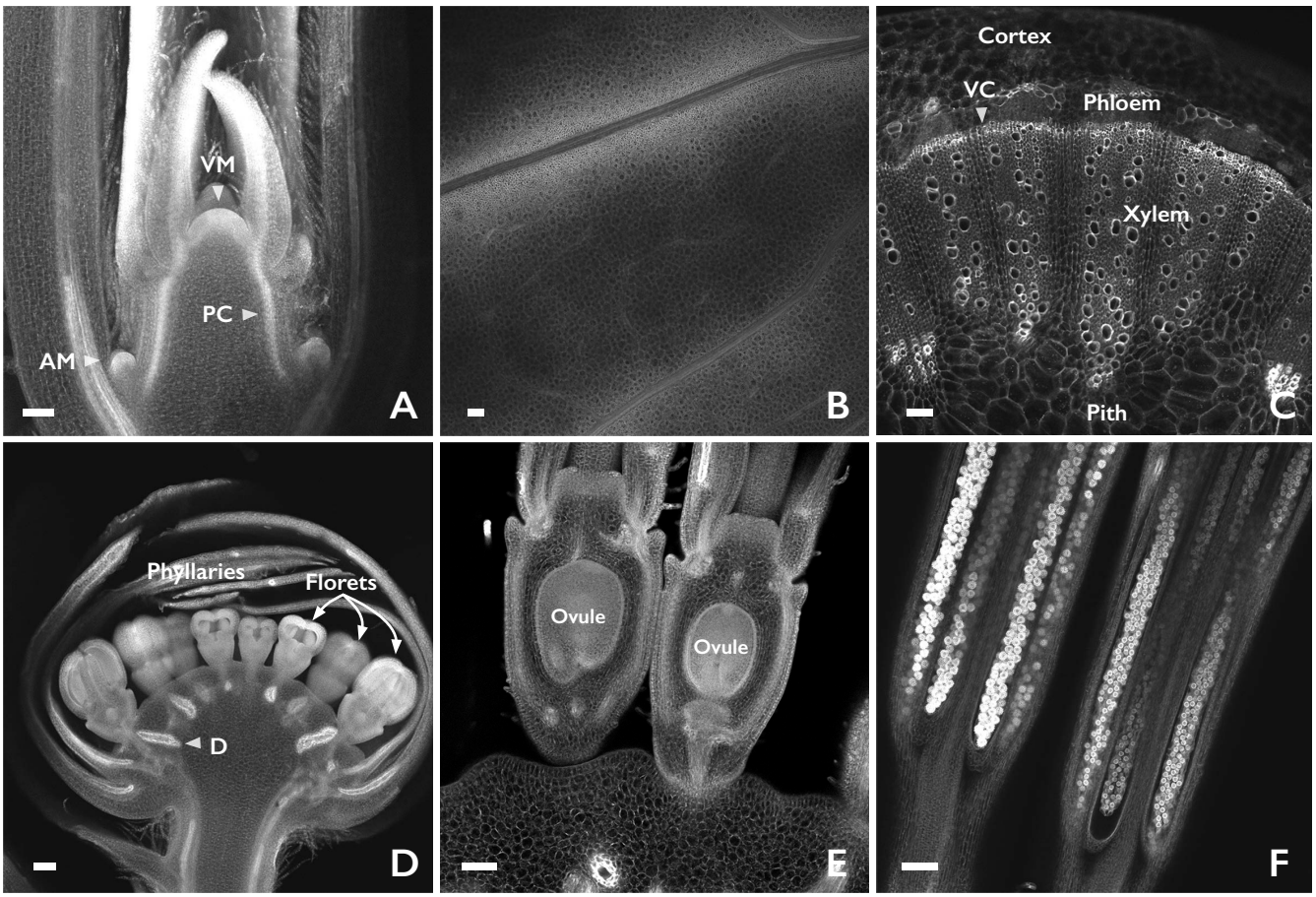

Figure 2. A showcase of tissues from *Jamesianthus alabamensis*. Images were captured on a Nikon A1 CLSM. **A.** A shoot apex with vegetative apical meristem (VM), axillary meristems (AM), and procambium (PC). **B.** An undissected leaf with mesophyll, a major vein, and minor veins. **C.** A cross-section of stem tissue with substantial secondary growth showing cortex, phloem, vascular cambium (VC), xylem, and pith. **D.** A developing capitulum with florets at various stages of floral organ formation, each with developing pappus, corolla, stamens, gynoecia, and ovules; phyllaries surround the developing florets and extracellular ducts (D) are present within the receptacle. **E.** A pair of developing florets with nearly mature ovules. **F.** Anthers containing pollen, just prior to anthesis. Scale bars all represent 100 µm.

at least 24 hours. Samples can be stored in methyl salicylate indefinitely, and many tissues become clearer with time; however, samples stored in methyl salicylate are more fragile than those stored in 70% ethanol. Storing cleared samples at 4°C may increase longevity. When tissues are fully cleared, simply mount samples in methyl salicylate and image. A major advantage of this protocol is that samples can be prepared with minimal dissection, which typically means the only flat surface is often the cut face to be imaged. Therefore, we recommend using a glass bottom petri dish on an inverted microscope, with the cut surface facing downward. To help prevent air bubbles from distorting the image, place a small drop of methyl salicylate on the slide before mounting the sample. The images in this article were captured on either a Nikon A1 confocal laser scanning microscope (CLSM) or an Echo Revolve, then processed in FIJI or ImageJ (Schindelin et al., 2012; Schneider et al., 2021) (see "Considerations in Imaging Method" for details).

METHYL SALICYLATE IS A BROADLY EFFECTIVE CLEARING AGENT

Diverse Tissues:

One of the largest benefits of methyl salicylate as a clearing agent is its ability to clear most tissues. Here we use *Jamesianthus alabamensis* S.F.Blake & Sherff, a rare

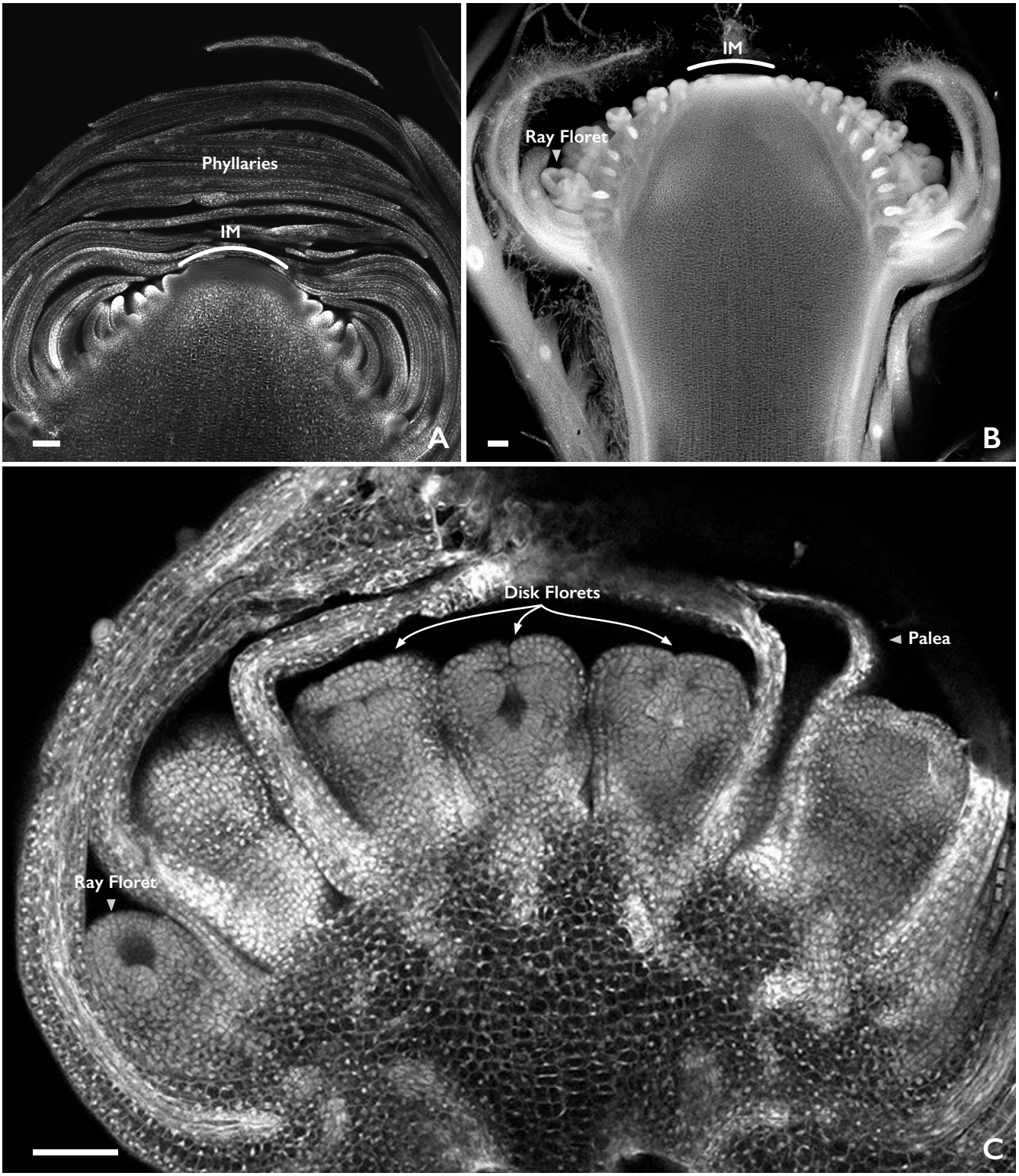

Figure 3. Developing capitula in a variety of species. Images were captured on a Nikon A1 CLSM. **A.** *Dahlia* 'Edna C' with conspicuous phyllaries and unconsumed inflorescence meristem (IM). **B.** *Tagetes erecta* with acropetally maturing florets ranging from visibly zygomorphic ray florets to unconsumed inflorescence meristem (IM). **C.** *Bidens forbesii* already showing distinct corolla morphologies between the zygomorphic ray florets and actinomorphic disk florets, as well as substantial paleae. Scale bars all represent 100 µm.

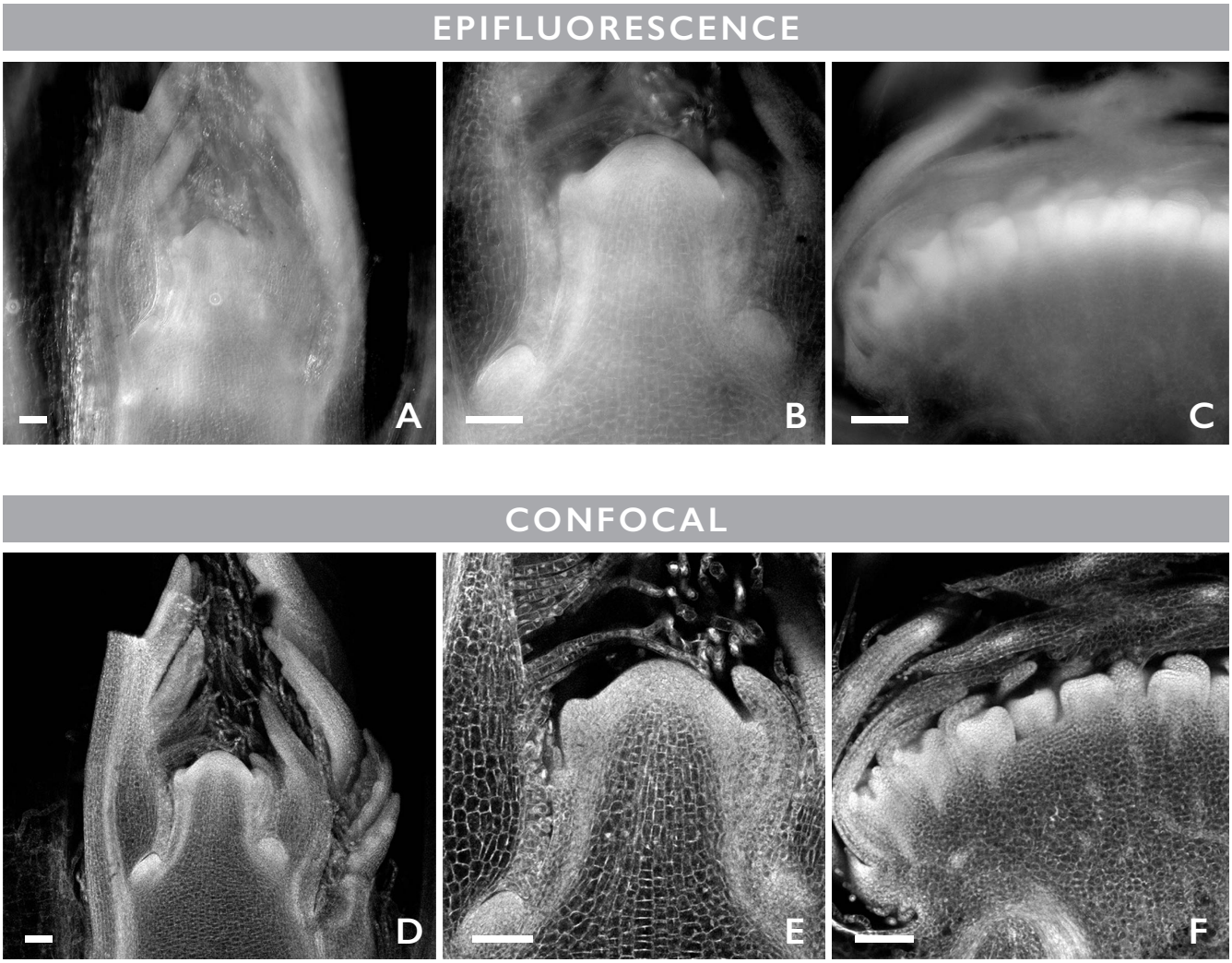

Figure 4. Confocal vs epifluorescence microscopy. Confocal images taken on a Nikon A1 CLSM, and epifluorescence images taken on an Echo Revolve. **A-B.** *Tagetes erecta* vegetative meristem imaged on an epifluorescent microscope. **C.** A developing capitulum from *Bidens* 'Compact Yellow' imaged using epifluorescence **D-E.** *Tagetes erecta* 'Antigua Orange' vegetative meristem imaged on a confocal microscope. **F.** A developing capitulum from *Bidens* 'Compact Yellow' imaged using the confocal microscope. A and D were captured with a 4x objective. B, C, E, F captured with 10x objective. Scale bars all represent 100 µm.

species endemic to freshwater streams and riverbanks in northern Alabama and Georgia, to demonstrate the variety of tissues that can be imaged. Although we focus on the population dynamics and conservation of Jamesianthus alabamensis, the simplicity of methyl salicylate clearing enables a thorough exploration of this rare plant's anatomy and development (Figure 2). The shoot apex with leaf primordia, vegetative shoot meristem, and developing procambial strands (Figure 2A), leaf anatomy (Figure 2B), secondary growth in the stem (Figure 2C), a developing capitulum soon after all floral whorls have been specified (Figure 2D), developing ovules (Figure 2E), and anthers before anthesis (Figure 2F).

Diverse Species:

Methyl salicylate is also effective across diverse species; methods have been described for *Oryza sativa* L., *Arabidopsis thaliana* (L.) Heynh., *Beta vulgaris* L., and more (Zeng et al., 2007; Rodriguez-Leal et al., 2019; Kwiatkowska et al., 2019; Jones et al., 2021; John et al., 2023). In addition to *Jamesianthus alabamensis*, we captured micrographs of developing capitula from multiple additional species (Figure 3). These include two ornamentals; *Dahlia variabilis* (Willd.) Desf. 'Edna C' (Figure 3A), which even while developing can reach almost 4 cm in diameter, and *Tagetes erecta* L. 'Antigua Orange' (Figure 3B), and a capitulum from

Development made clear

While morphological diversity is most obvious in mature structures and organs, variation in form begins at the earliest stages of development. A vegetative shoot apical meristem and developing leaves of *Bidens* 'Compact Yellow' illustrate this well. By coupling this approachable tissue-clearing technique with fluorescence microscopy, we can easily capture the details of meristem and organ primordia morphology.

Image of Bidens 'Compact Yellow' *viewed in two channels (FITC in green and TRITC in red), highlighting the distinct fluorescent properties of naturally occurring compounds. Photo by Daniel S. Jones*

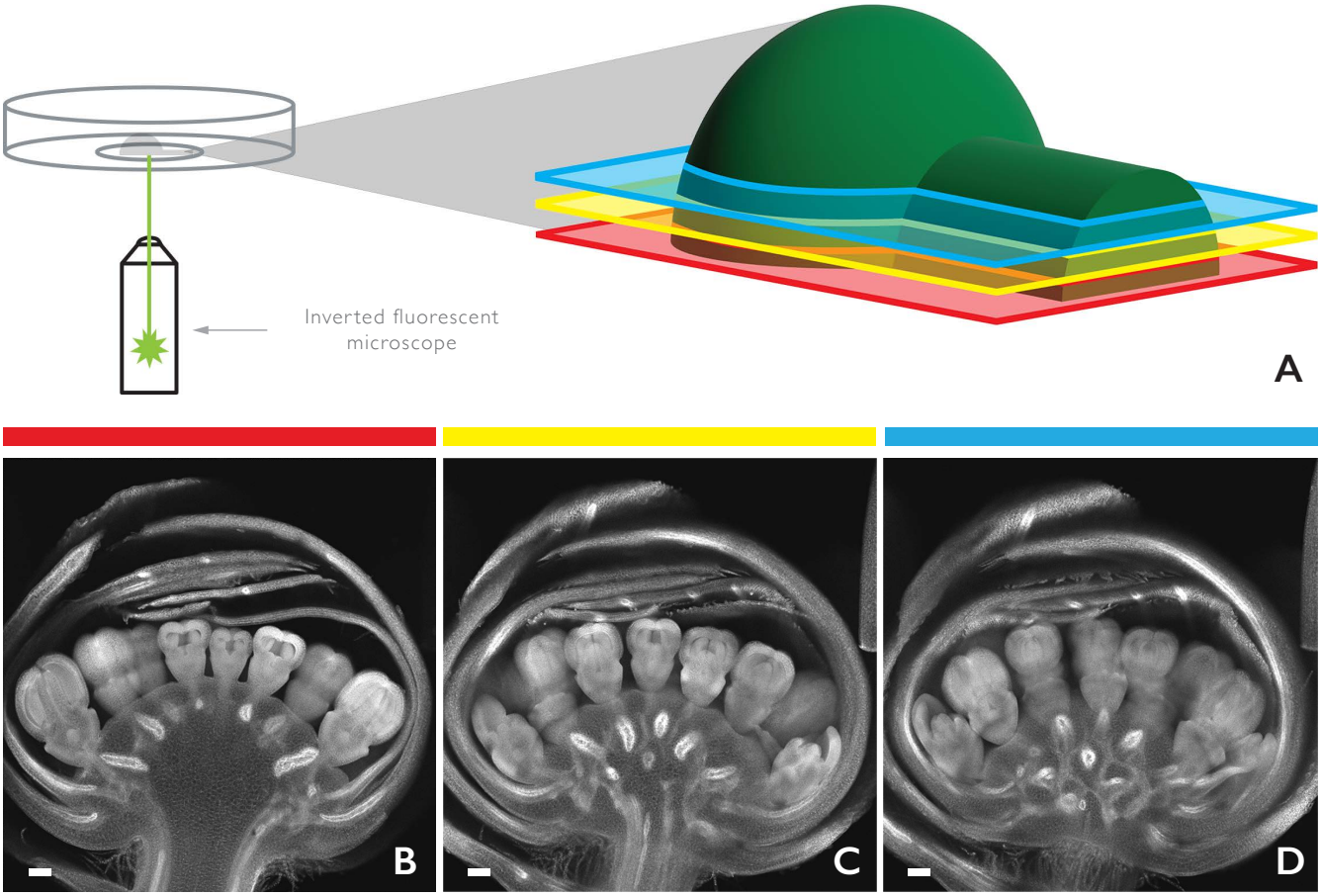

Figure 5. Optical sectioning of *Jamesianthus* ducts. Images were captured on a Nikon A1 CLSM. **A.** An illustration of the optical sections represented in images B-D; colors correspond to relative depth of focus. **B.** An image captured from a focal plane immediately adjacent to the initial physical section. **C-D.** Images captured from focal planes progressively deeper into the cleared capitulum. Scale bars all represent 100 µm.

Bidens forbesii Sherff (Figure 3C), a species endemic to the Hawaiian Islands. The difference in size across all three capitula may cause concerns for image acquisition and quality, especially for the large *Dahlia* Cav. samples. However, we show that even in *Dahlia* (Figure 3A), well-resolved images can be obtained with minimal dissection. Images of *Dahlia variabilis* 'Edna C' and *Tagetes erecta* 'Antigua Orange' (Figure 3A–B) illustrate the broad range of developmental stages that may coexist within a single capitulum. While the center may have unconsumed inflorescence meristem that has not formed any floral primordia yet, floral primordia at the periphery may be well into the development of all floral whorls. This enables a unique snapshot of the progression of floral ontogeny in a single image. In contrast, *Bidens forbesii* (Figure 3C) exhibits a more synchronized developmental pattern, with florets progressing within a similar temporal window. Methyl salicylate clearing enables these rapid, high-contrast comparisons among species and across developmental time points.

CONSIDERATIONS IN IMAGING METHOD

Confocal vs Epifluorescence:

Due to point-scanning and a pinhole aperture blocking out of focus light from detectors (Elliot, 2020), confocal microscopy will always provide more resolution than standard epifluorescence microscopy.

However, epifluorescence is sufficient for many research questions and is typically more accessible and less expensive than confocal microscopy. All images prior to this section used a confocal microscope, but methyl salicylate clearing is suitable for both modes of imaging. To compare both methods, we imaged the same samples using epifluorescence (Echo Revolve) and confocal (Nikon A1 CLSM) microscopes. The same vegetative meristem from *Tagetes patula* L. and the developing capitulum of *Bidens* 'Compact Yellow' (Figure 4A–F) are shown, illustrating not only the increased resolution and contrast achieved through confocal microscopy but also the level of analysis possible using epifluorescence—even with minimal sample preparation.

Optical Sectioning:

The ability to restrict incoming light to a small plane of focus via the pinhole aperture enables a technique in confocal microscopy known as optical sectioning. When dissecting samples, it is nearly impossible to make a cut that is precisely in the same plane you wish to image. Additionally, imaging the same tissue at various depths can provide valuable insight into its three-dimensional structure. Cutting the sample into multiple thin layers (sectioning) addresses both these points and is often achieved by methods such as paraffin embedding, enabling researchers to view multiple slices of the same sample. Thin sectioning can be resource- and time-intensive and reduces the three-dimensional context of structures by physically separating them. Clearing allows optical sections to be taken by adjusting the focal plane of the microscope. The cleared tissue minimizes light diffraction and allows for deeper light penetration. This, combined with the capability of confocal microscopes to omit signal from out-of-focus regions, allows for clear micrographs to be captured from a range of focal planes (Conchello & Lichtman, 2005). Therefore, imaging is not restricted to regions immediately adjacent to the initial cut site. Here we demonstrate this technique by imaging the ducts found within the receptacle of a *Jamesianthus alabamensis* capitulum (Figure 5). While ducts are visible in the plane of focus adjacent to the initial cut site (Figure 5B), the three-dimensional organization of these ducts becomes more apparent when viewed in multiple planes of focus progressing deeper into the tissue (Figure 5C–D).

Leveraging Autofluorescence:

This technique does not require any staining or labeling. Instead, it utilizes the abundance of naturally fluorescent compounds that plants produce. While this autofluorescence can create problems when imagining fluorescent stains or proteins like GFP, it can also be leveraged for stain-free structural analysis. Because FAA is a potent solvent, many autofluorescent molecules (i.e., pigments) will be washed out prior to clearing. However, there is still a considerable amount of autofluorescent material after clearing and fixing, especially in cell walls. There are many different sources of autofluorescence, and each molecule has different excitation and emission wavelengths (Donaldson, 2020). Therefore, it can be difficult to predict the most useful channel or filter to use when imaging with this method. While channels for fluorescein isothiocyanate (FITC) or green fluorescent protein (GFP) often give the best signal; however, this varies between samples. It is best to experiment with different channels or filters to find the optimum for a given sample. It is also possible to combine multiple channels. All the images in this article were captured using a combination of FITC (ex: 488 nm and em: 520 nm) and tetramethylrhodamine isothiocyanate (TRITC) fluorescent filters (ex: 550 nm and em: 580 nm). The levels of each channel were then adjusted and merged using FIJI (ImageJ) to produce a single balanced image. Although colored look-up tables (LUTs) can aid in visualizing multichannel images, grayscale presentation is preferable for structural analysis.

CONCLUSIONS

This simplified method offers an accessible, fast, and safe alternative to conventional tissue clearing techniques. While fluorescent counterstains remain necessary for some applications, leveraging autofluorescence from plant cell walls provides an in-depth look at developing structures with minimal treatment post-fixation. While this method is especially suitable for imaging capitula, methyl salicylate's ability to clear a variety of tissues makes this method broadly applicable. Additionally, the increasing availability of fluorescence microscopy necessitates accessible protocols. Our hope is that this method will lower the barrier to entry for fluorescence microscopy and open avenues of research previously unconsidered by those studying the Asteraceae and beyond.

ACKNOWLEDGEMENTS

We thank Dr. Matt Knope and the Native Hawaiian Plant Conservation group for sending *Bidens forbesii* seeds, and the Auburn University Research Instrumentation Facility. B.C, R.S and D.J are funded by an NSF IOS-PGRP # 2214474 as well as startup funds from Clemson and Auburn University. Z.M is funded by the American Dahlia Society. A.H. is funded by NSF IOS-PGRP CAREER grant #2239530.

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