Non-Toxic Microtechnique Protocols
For Plant Material

Ted Kinsman
Rochester Institute of Technology
Rochester, New York

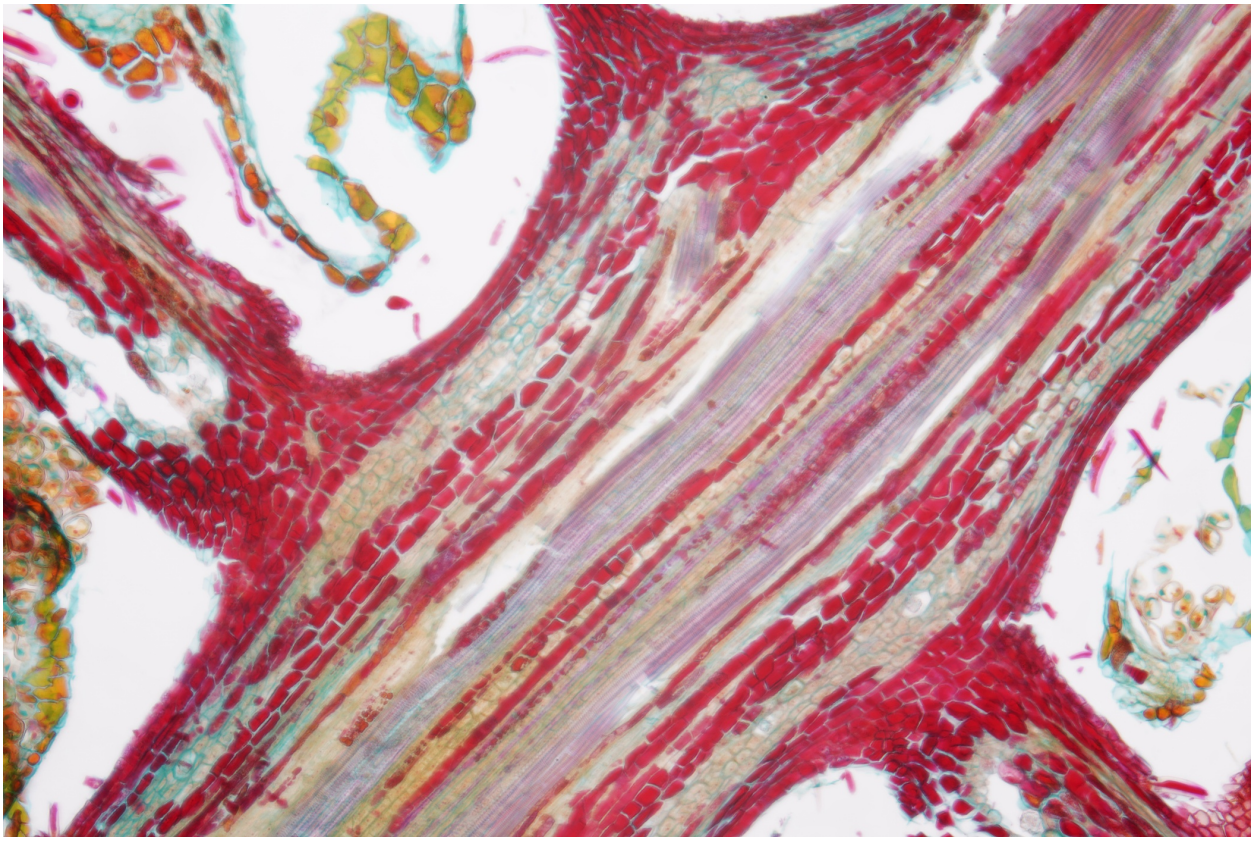

Fig. 1: The stem of a Hazelnut catkin flower stained with the Wacker Solution. (40-minute stain time with a thickness of 12 micrometers. The images were captured at 20x magnification using an Olympus BX50 microscope and is a focus stack of 10 images combined in Helicon Focus software.

Introduction:

Plant microtechnique involves collecting plant material and preparing it for inspection under a microscope. The process can be divided into stages: fixation, embedding in paraffin, staining, and mounting. Each of these steps has details that are crucial to the overall success of the procedure.

There are numerous procedures used to prepare plant material for microscopic study. These processes, which have been utilized for centuries contain toxic chemicals like formaldehyde and

xylene which are not suitable for modern use. This research focuses on creating a procedure suitable for college-level students with minimal exposure to toxic substances. After testing out many different procedures, I settled on this protocol. This procedure will explain the rationale behind each step in the standard process.

Each sample of plant material can have a procedure optimized for its different cell types. After running several samples, the process can be optimized for a particular specimen type. Stain color results depend on various factors such as the plant specimen, sample thickness, stain time, stain concentration, and stain temperature. This procedure serves as a starting point for the process.

In this article, I will cover two different staining procedures: the use of two simultaneous stains and the use of three simultaneous stains.

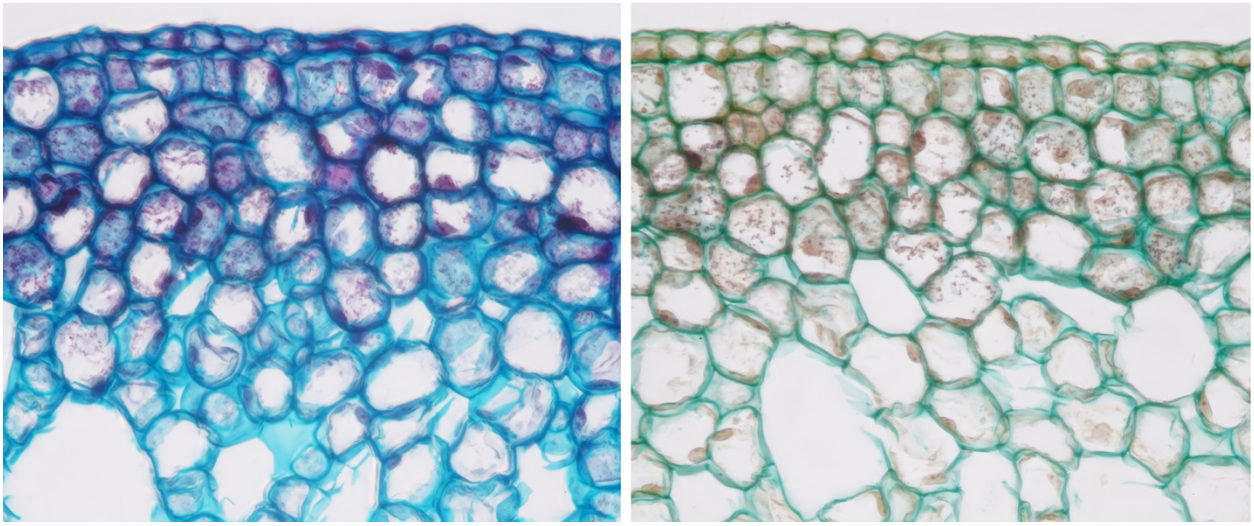

Fig. 2: Comparison of Different Stain Combinations: On the left is Alcian Blue and Saffron O Staining Solution (20-minute stain time), while on the right is Wacker stain containing Rhodamine B/Acriflavine/Alcian Blue Solution (30-minute stain time). The samples were stained at room temperature (20°C). Each image depicts a similar section of the stem of a skunk cabbage flower (Symplocarpus foetidus), with a thickness of 12 micrometers. The images were captured at 20x magnification using an Olympus BX50 microscope. To facilitate comparison between stains, both images were captured with the same camera settings, and no color or level adjustments were made to the images. Additionally, each image was focus-stacked from 10 individual images using Helicon Focus software.

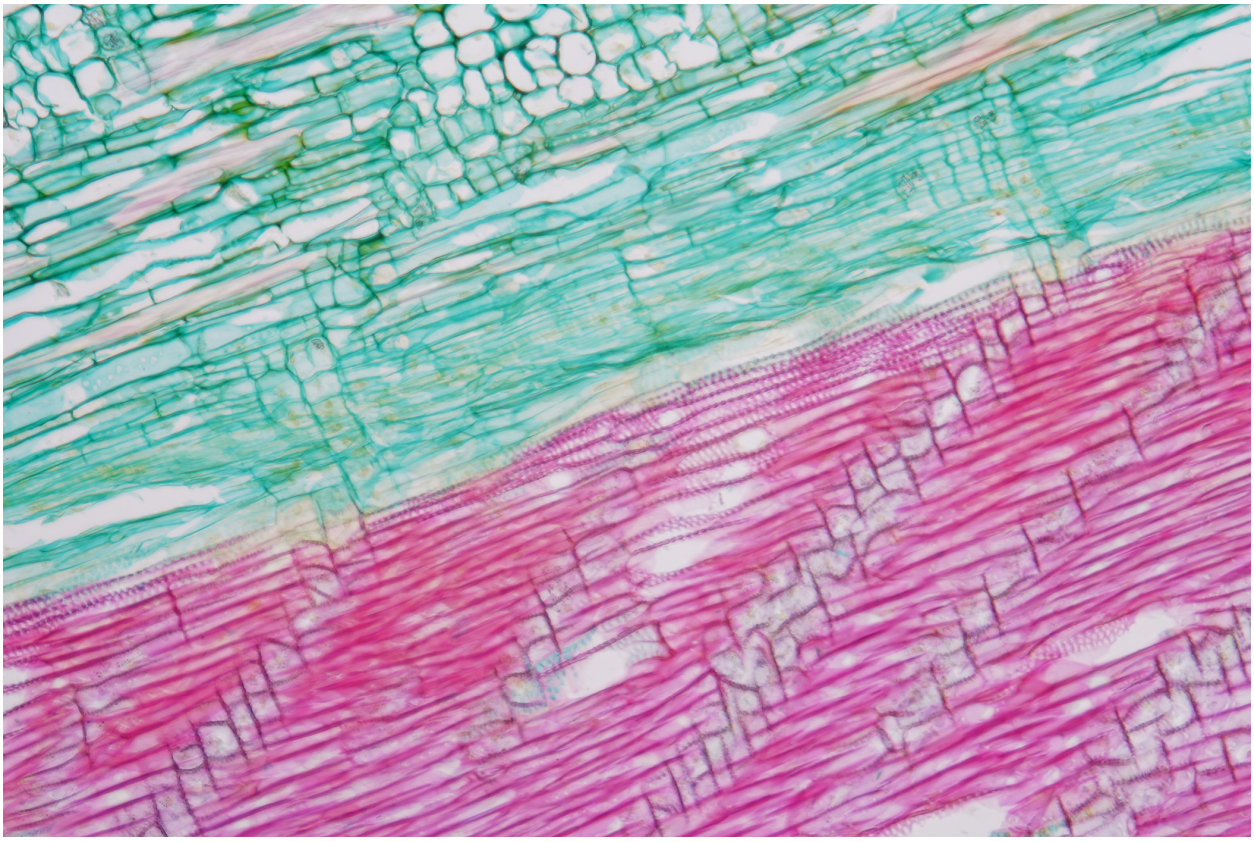

Fig. 3: Wacker stain containing Rhodamine B/Acriflavine/Alcian Blue Solution (30-minute stain time) of a section of Cannabis stem (12 um thickness). The center cell structures readily absorb the Rhodamine B. Image taken with a 20x UPlanSApo Olympus objective on an Olympus BX50 microscope. Image stacked from 10 images with Helicon Focus software.

Collecting Specimens of the right size:

The size of the specimen is of particular importance. If the specimen is too large, the chemicals may not adequately reach the center or may require a significant amount of time to do so. Conversely, if the sample is too small, it becomes difficult to handle.
I prefer to collect and cut a specimen that is no larger than 2 mm in distance from the surface. This means that a plant stem is cut to a thickness of 4 mm. Even if the diameter of the stem is larger than 10 mm, there is no point in the volume farther than 2 mm from the surface. This distance allows the chemicals to reach the cells in the center of the specimen within a reasonable amount of time, as outlined in this protocol.

Fig. 4: Samples of tomato stem are placed in a histology cassette prior to placing in the Acetic Acid, and Alcohol fixative (AA) solution.

The Fixative:

Fixation is the process of halting all living organisms in a sample. The role of a fixative is to strike a balance between arresting all biological processes while preserving the physical size of the cells.

The primary function of the fixative is to terminate cellular activity and stabilize the chemistry to ensure that biological processes cease completely, and the specimen remains immune from bacterial and fungal contamination throughout the procedure. It's crucial to note that incorrect fixation steps can alter the size of the specimen, affect its hardness, and complicate microtome cutting. These issues vary depending on the sample, necessitating optimization of processes for each unique specimen.

One of the traditional fixatives is a combination of Formaldehyde, Acetic Acid, and Alcohol, commonly known as FAA. However, this fixative is no longer recommended due to the presence of formaldehyde, which can cause sensitivity issues. Additionally, current regulations prohibit formaldehyde use with students.

In my research, I've found an acceptable alternative to FAA is a mixture of 30% Acetic acid and 70% Isopropyl Alcohol (AA). After conducting tests with sections of tomato stems, I observed no discernible difference between FAA and AA in terms of their efficacy as fixatives. I've replaced Ethanol Alcohol with Isopropyl Alcohol since it is soluble in both paraffin and Histo-Clear chemicals used in subsequent steps.

Thin specimens should be immersed in the fixative for a minimum of 12 to 24 hours, while larger specimens may require up to a week. To ensure thorough penetration and interaction of the fixative with the sample, place it under a vacuum for at least 12 hours. This vacuum can be easily created using a hand pump, readily available for home use in fermentation processes like making sauerkraut. These canning vacuum hand pumps are designed for wide and regular mouth canning jars. There are even a number of battery-operated pumps on the home canning / fermenting market now.

A vacuum bell jar will also work for this process. When pulling a vacuum with a mechanical pump do not allow the fixative to boil out of the container.

Fixative AA
30% Acetic Acid
70% isopropyl alcohol

One of negative aspects of the use of alcohols in this process is that the alcohols specifically dissolve chlorophyl from the specimen. Chlorophyl is a strongly fluorescing material found in green plants and emits red light when stimulated by short wave blue light. This fluorescence can easily be observed by using a blue laser pointer of 405 nm wavelength.

After the fixation process, the specimen is washed for a minimum time of 2 to 3 hours. This process removes the Acetic acid from the specimen and neutralizes the pH of the sample.

Non-Toxic Plant Microtechnique

Fig. 5: The full microtechnique protocol is displayed in a block diagram.

Transition to paraffin wax:

The role of mounting a specimen in paraffin wax is to allow the delicate cells of the specimen to be supported by the wax as the specimen is sliced into thin sections in a microtome.

Once the specimen has been in the fixative for a minimum of 12 to 24 hours the transition to paraffin can start taking place after washing in water to neutralize the pH of the acetic acid.

A sequence of steps of Isopropyl alcohol is used to remove all the water (dehydrate) and prepare the specimen for an environment free of water. Since the fixative AA has 70% alcohol in the formula a series of the following steps will work. Note: for delicate specimens the steps can be modified to 10% Isopropyl steps.

25% Isopropyl Alcohol and 75% Water – 2 hours.

50% Isopropyl Alcohol and 50% Water – 2 hours minimum (this time depends on the size of the sample) is okay to run longer – I typically let each of these steps run over night (12 hours or more) If the sample reaches equilibrium – there will be no change so longer the better.

75% Isopropyl Alcohol and 25% Water – 4 hours minimum (longer the better)

100% Isopropyl Alcohol – longer the better. 6 Hour for very small specimens and several days for larger specimens.

Paraffin is melted in a 50% solution of Isopropyl alcohol by weight and the samples are submersed for a minimum of 12 hr. large specimens will require more time. This can be disconcerting since each specimen is unique. Plant material like flowers going to seed will take substantially more time than other specimens. When in doubt cut thinner samples and increase the soak time of this preliminary saturation of paraffin.

100% paraffin is used for the next step. A soak of a minimum of 8 hours is recommended for large samples and a shorter time for smaller samples. One process I often use is to include a sacrificial specimen (or two) that is included in the paraffin imbedding process. These samples are cut open and inspected to see if the paraffin has reached the center of the sample.

When the paraffin has reached the center of the samples it is time to mount the sample in a block of paraffin. To keep track of small specimens and to mount specimens a sample cassette is used. The cassettes come in several sizes and colors, use a cassette that matches the mounting block of your microtome.

Before the sample can be mounted small paraffin containers can be made by folding stiff paper. I fold containers out of paper and use tape to hold the containers in shape. Once made the containers can be used dozens of times. Professional paraffin casting containers made from stainless steel can be found on the internet for a few dollars each. These stainless-steel molds are very convenient to use.

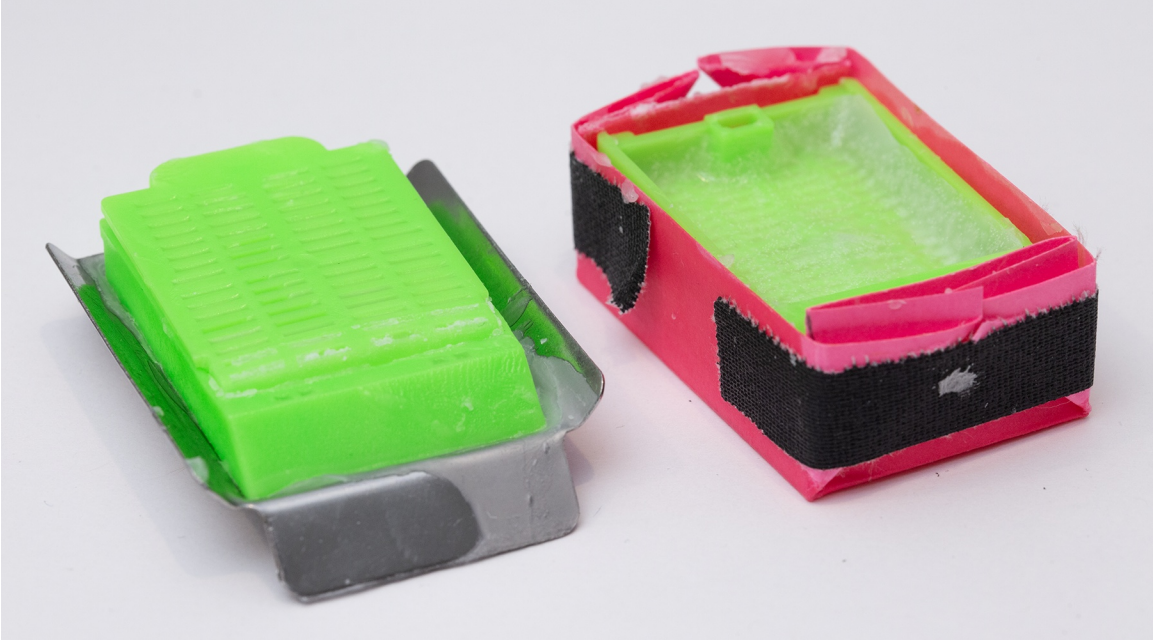

Fig. 6: A professional made stainless-steel mold on the left and a traditional paper box mold on the right. The paper mold is often convenient when the specimen is an odd shape. The stainless-steel mold is pressured on the sides and the paraffin will separate from the metal. The paper easily peeled away from the paraffin and can be reused several times.

To mount the specimen a small layer of paraffin is poured into the container and before the wax can set up, the specimen in placed in the correct position and more paraffin is added. A preheated cassette is placed on top of the paraffin mold and topped off with molten wax.

Place the block in a refrigerator to cool, but not the freezer. Blocks cooled too quickly will crack, and blocks cooled too slowly at room temperature will be hard to cut due to the formation of large paraffin crystals in the wax. Once the specimen in mounted in the paraffin block the specimen will keep for years.

Note: the paraffin crystals are easy to observe under a polarized light microscope.

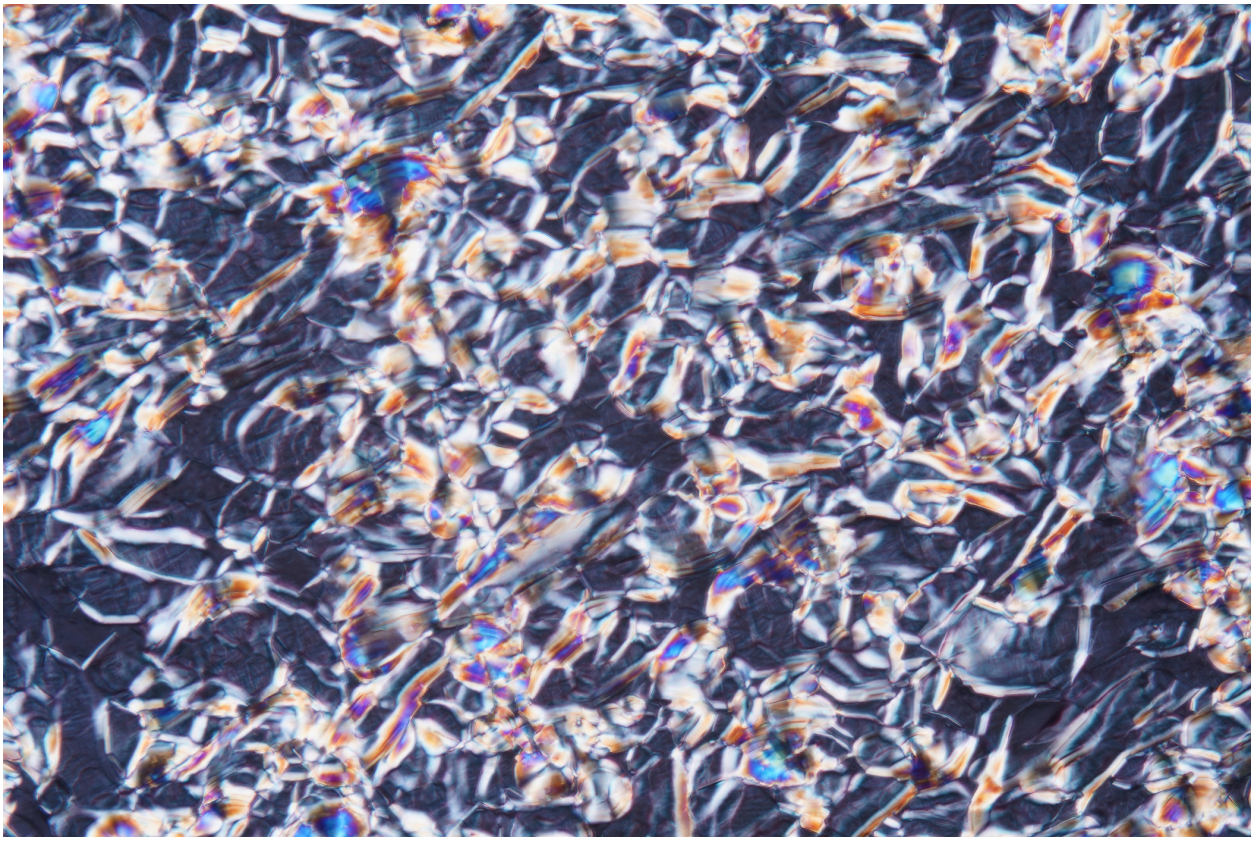

Fig. 7: The crystals formed in paraffin are clearly seen in polarized light at 10x magnification. Field of view in the horizonal direction is 950 um.

Many complex shaped samples will not stay on an uncoated microscope slide for the duration of the microtechnique process. These troublesome specimens will need either a homemade adhesion layer or the use of pre-charged glass microscope slides. It is useful to test your samples to determine is you can get away with uncoated slides. Coated slides cost more but are a lot easier than making your own adhesion solution and are readily available from several manufactures. Most plant samples I have prepared do not require additional adhesion. Basic uncoated glass microscope slides work well. Always run a few test samples on uncoated slides to see if the use of uncoated slides can save money in the process.

The thin sheet of plant material imbedded in wax is shaved off the block with a microtome. 12um thick specimens are the standard for plant specimens, although with practice specimens can be cut much thinner.

There are numerous books on using microtomes: Please see references 1,2,3, and 4.

The reality of the microtome is the more experience you have on the device the better you will be. Practice, Practice, Practice! Only experience will tell you when to replace a blade, when to clean wax off the back side of the blade, how to shape a mounted specimen for the best cutting, and how to carefully pull a ribbon from the blade.

To give an overview of the process please see this video I made for my students;
https://www.youtube.com/watch?v=zwzI3D_fru0

Thin specimens are easier to image under a microscope with minimum use of focus stacking procedures. Thicker specimens take the stains more and can give richer colors. Thin specimens can also be stained longer to achieve similar results. These variables are all sample dependent. Some samples just do not want to cut very thin. Overall, I have found the sample thickness of 12 um to work well for most plant specimens.

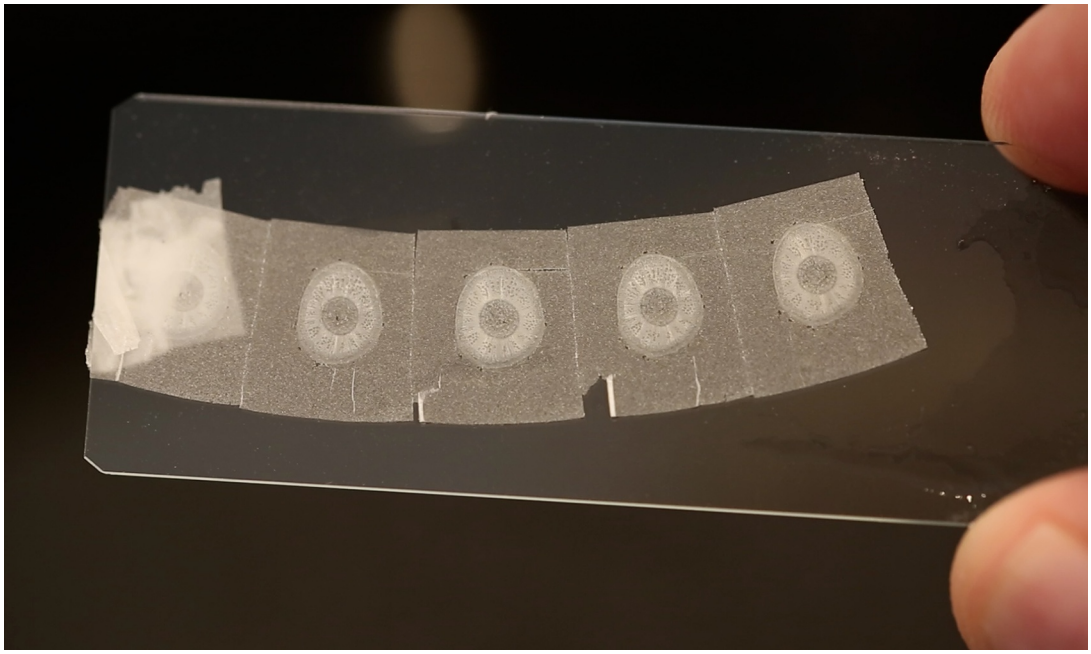

Fig. 8: A paraffin ribbon of tomato stems is displayed on the microscope slide after the floating procedure in the water bath.

A 12um thick sample will go through the following dewaxing steps twice as fast as a 24um sample – keep your wax thickness in mind as you refine your process for your specific sample type.

Dewaxing: Dissolving the Paraffin from the Sample.

The Dewaxing process: Removing the embedding paraffin wax:
In the old days xylene was the standard, but due to modern health concerns this chemical is no longer allowed for use with students and has been replaced. A suitable replacement is Histo-Clear a company name for the substance d-limonene. This substance is a byproduct of the manufacture of orange juice and is extracted by pressing orange peels. In the United States this substance is often sold under the brand name “Goo Be Gone” as a liquid for removing labels and sticky residue on surfaces. Histo-Clear is also an excellent solvent for cleaning paraffin off

microtomes and other lab equipment. Histo-Clear has a pleasant odor of oranges. In a pinch "Goo Be Gone" can be used to dewax slides since it is available in most hardware stores.

The thickness of the microtome slice is very important here -the thinner the slice the quicker the dewaxing process. My plant specimens are cut at 12 um - a thickness larger than many other labs. Samples of 1cm^2 are common so the time referenced in this article might be substantially larger than thinner, smaller samples.

After paraffin slices are made and adhered to the microscope slide by floating in warm 42C water. The slides are baked overnight in an incubator at 40C. The baking step removes all the water from the sample.

The samples can be left in this state for an extended time. I often make several hundred slides and will keep them for weeks as I use them to optimize the staining steps. Keep the slides covered to keep room dust off the surfaces until ready for the staining steps.

Dewaxing:

Place the slides containing plant/paraffin in a bath of pure Histo-Clear for 20 minutes before moving the slides to a second container of clean Histo-Clear for another 20 minutes.
These pure Histo-Clear baths will remove all the paraffin from the sample just leaving the thin slice of plant material on the microscope slide.

The next set of steps takes the sample that has been in pure Histo-Clear to water solubility in several steps. The following steps slowly add water back to the sample. These steps are necessary since the stains used here are water soluble.

Note: The Histo-Clear is only soluble in Isopropyl alcohol, so ethanol is not suitable for this process.

The following steps will bring the sample to water solubility.

100% Isopropyl Alcohol – min of 5 min.

75% Isopropyl Alcohol / 25% Water – min of 5 min.

50% Isopropyl Alcohol / 50% Water – min of 5 min.

25% Isopropyl Alcohol / 75% Water – min of 5 min.

100% Water - min of 3 min. (if your stain solution has a 25% alcohol, this step is often skipped)

Each, of these steps should be allowed to reach equilibrium and this is dependent on the thickness and area of the specimen. Samples should be able to reach equilibrium in 5 min, but if

the specimen is left in the solution for longer times will not be a problem – unless there are adhesion problems and the samples fall off the slides. In this case -keep the steps as short as possible and try not to jiggle the samples as they are moved from solution to solution.

The sample is now in a state where all solvents have been extracted from the tissue and the cells are hopefully in the same position and are the same size they were when the plant was alive. The difference now is the cells are emersed in water and this state is called specimen hydration.

The sample is now prepared for the staining solution.

I have two favorite staining solutions – a simultaneous double dye of Alcian Blue and saffron O, and a triple simultaneous stain solution invented by Robin Wacker in 2006. These simultaneous stains save a lot of time and steps in the staining process. These are my favorites, but there are thousands of different stains that give great results for specific applications.

Alcian Blue and saffron O Staining Solution as developed by I. Shtein (see reference 5)
To mix this Alcian Blue / Saffron O stain two solutions are made:

Solution A is 1% Alcian Blue in distilled water.
This solution is made by adding 1 gram of alcian blue stain to 100ml of distilled water

Solution B is 1% Saffron O in 50/50 ethyl alcohol and distilled water.
This solution is made by adding 1gram of the Saffron O to a 50/50 solution of ethyl alcohol and distilled water

Final mix for staining:

Add two parts solution A to one part solution B.

Stain for 20 min to 45 minutes depending on sample. Keep the stain bath at room temperature. Higher stain penetration and deeper color can be achieved by elevating this staining temperature. Keep in mind that the staining times and temperatures are highly dependent on the nature of your botanical samples. Adjust the time and temperature for results that are good for your sample.

Wacker 7 stain (Dornberg VII staining) invented by Robin Wacker (see reference 6)

To simplify the triple staining process, Wacker found a combination of stains that work together. Over the years different microscopists have modified the procedure to take into consideration modern stains. Here is the 7^{th} modification of the Wacker stain process.

Solution A: Rhodamine B Solution
1 gram of rhodamine B dissolved in a 50% solution of ethanol and water.
2ml Acetic Acid

Solution B: Acriflavine Solution
1 gram acriflavine dissolved in 100 ml of water.
2ml Acetic Acid

Solution C: Alcian Blue Solution
0.2 gram to 100ml of water
2ml Acetic Acid

Final Wacker Stain Solution:
6 parts Solution A (Rhodamine B solution)
1 part Solution B (Acriflavine Solution)
4 parts Solution C (Alcian Blue Solution)

Stain microscope slides for 30 min to 90 minutes depending on desired saturation of color in specimen.
Note: Every specimen will take the stain in a different way depending on cell types.

Preparation for Permanent Mounting

After staining the slides are washed for 30 seconds in water and repeated for a total of three washes of a total time of 90 sec.

At this stage the slides need to move to 50% isopropyl alcohol/ water for 30 sec to 1 min.

The slides then are moved to 100% isopropyl alcohol to remove the last amount of water, this final isopropyl bath also differentiates the different stains. It is important to keep this step short since the stains can be completely removed from the sample with extended submersion times. I like to keep slides in this final isopropyl bath for 60 seconds. If this bath gets contaminated the final mounting medium will not have good adhesion.

The slides are now moved from the isopropyl bath to a bath of Histo-clear for a minimum of 10 minutes. This step allows the specimen to absorb the Histo-clear which has the same solubility as the permanent mounting medium.

The slides are now ready to be mounted with a few drops of Acrytol Mounting medium and sealed with a cover slip. The finished slides are weighted down with a 50gram mass and let dry for several days. The amount of Acrytol Mounting medium used is dependent on the specimen and the size of the cover slip. Some specimens like lichens like to absorb the mounting medium and will expand in size requiring more medium than other samples.

The finished slides are labeled with adhesive paper that has been laser printed with the specimen information. The finished slides are kept in a slide box so that the slides are kept horizontal in storage.

In this state the slides can last centuries.

I hope you give this procedure a try.

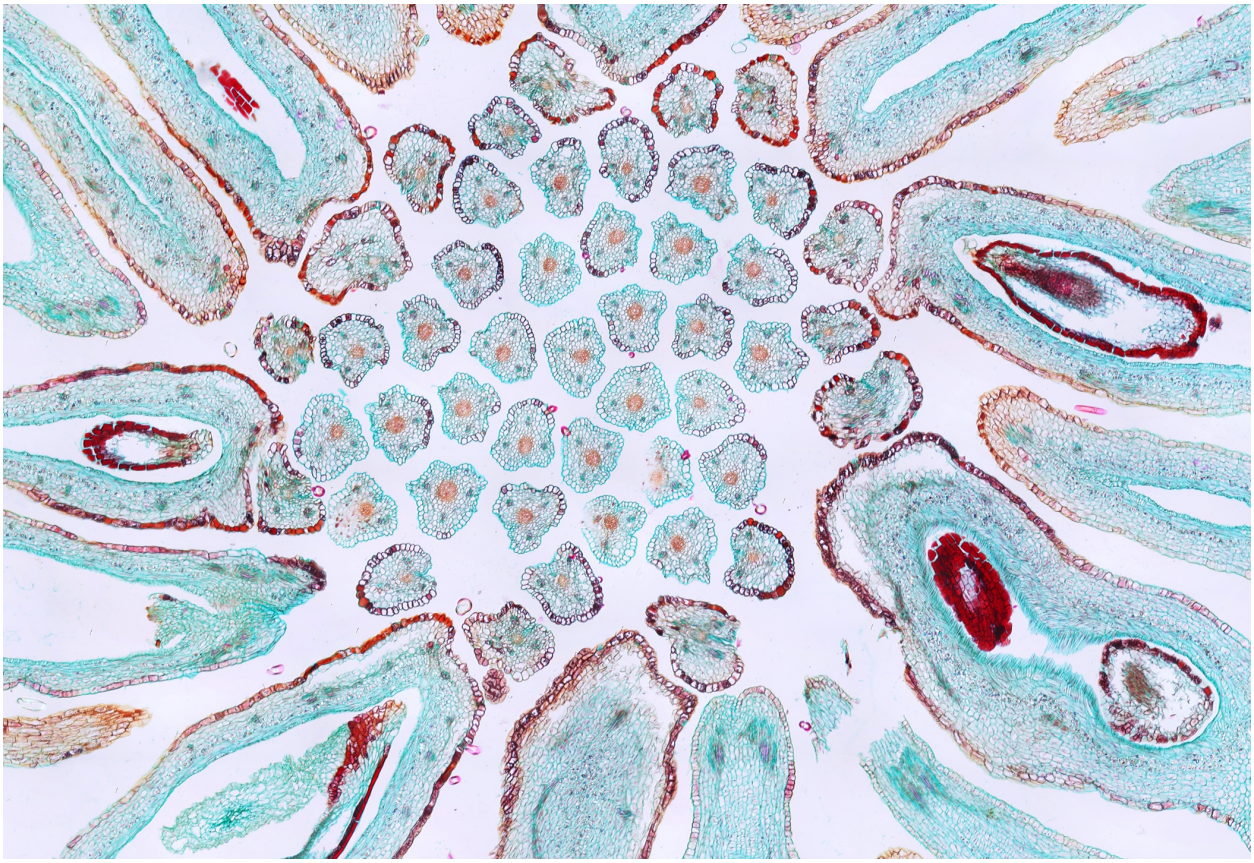

Fig. 9: A section of the rose bud stained with the Wacker Solution. (30-minute stain time with a thickness of 12 micrometers. The images were captured at 10x magnification using an Olympus BX50 microscope and is large panoramic image with a field of view of 3.5mm in the horizonal direction.

*Fig. 10: A section of the two poinsettia buds (*Euphorbia family*) stained with the Wacker Solution. (30-minute stain time with a thickness of 12 micrometers. The images were captured at 10x magnification using an Olympus BX50 microscope. This image has a horizontal field of view of 4mm.*

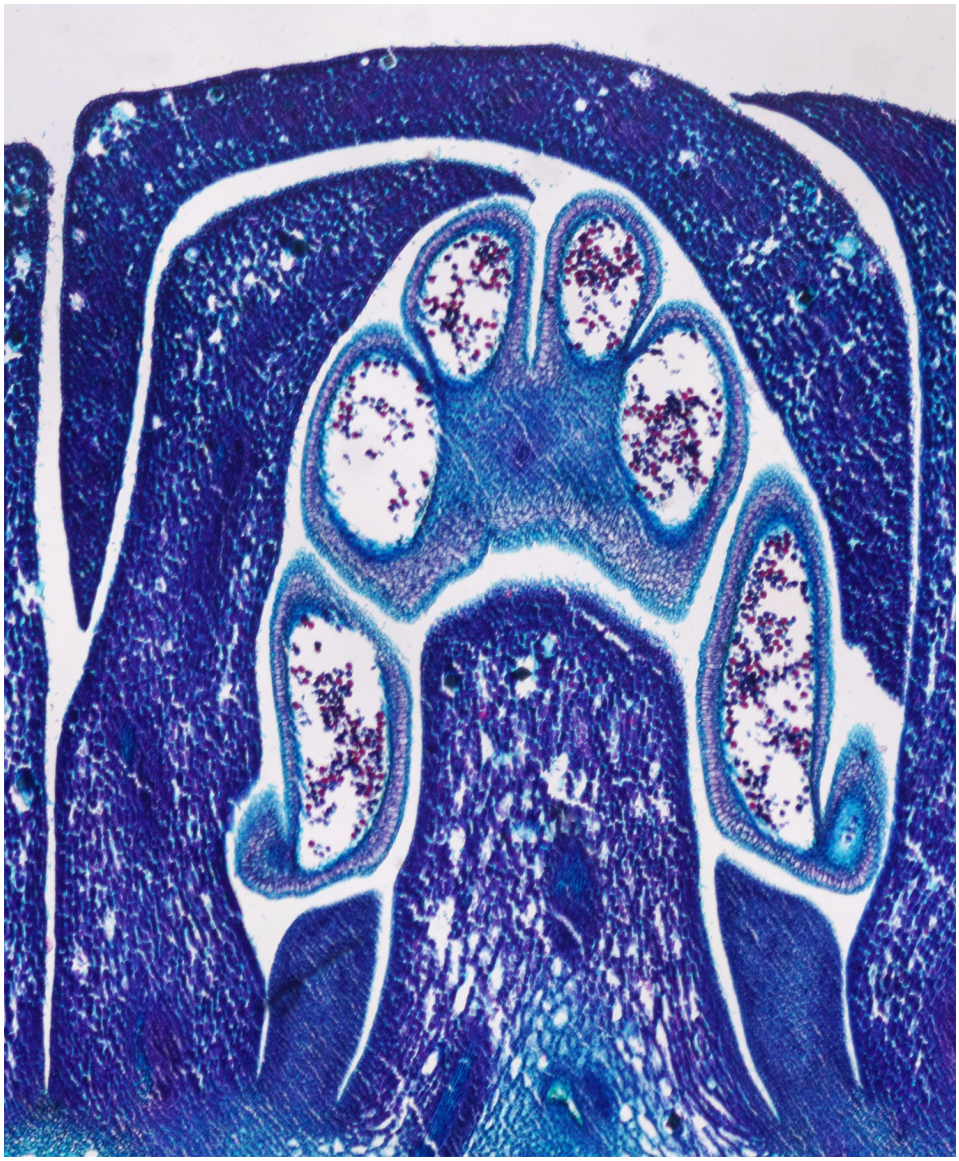

Fig. 11: A section of the flowering structure of a skunk cabbage flower (Symplocarpus foetidus) stained with the Alcian Blue / Saffron O Staining Solution. (20-minute stain time with a thickness of 12 micrometers. The images were captured at 10x magnification using an Olympus BX50 microscope and is a combination of 20 images using the panoramic software in Adobe Photoshop.

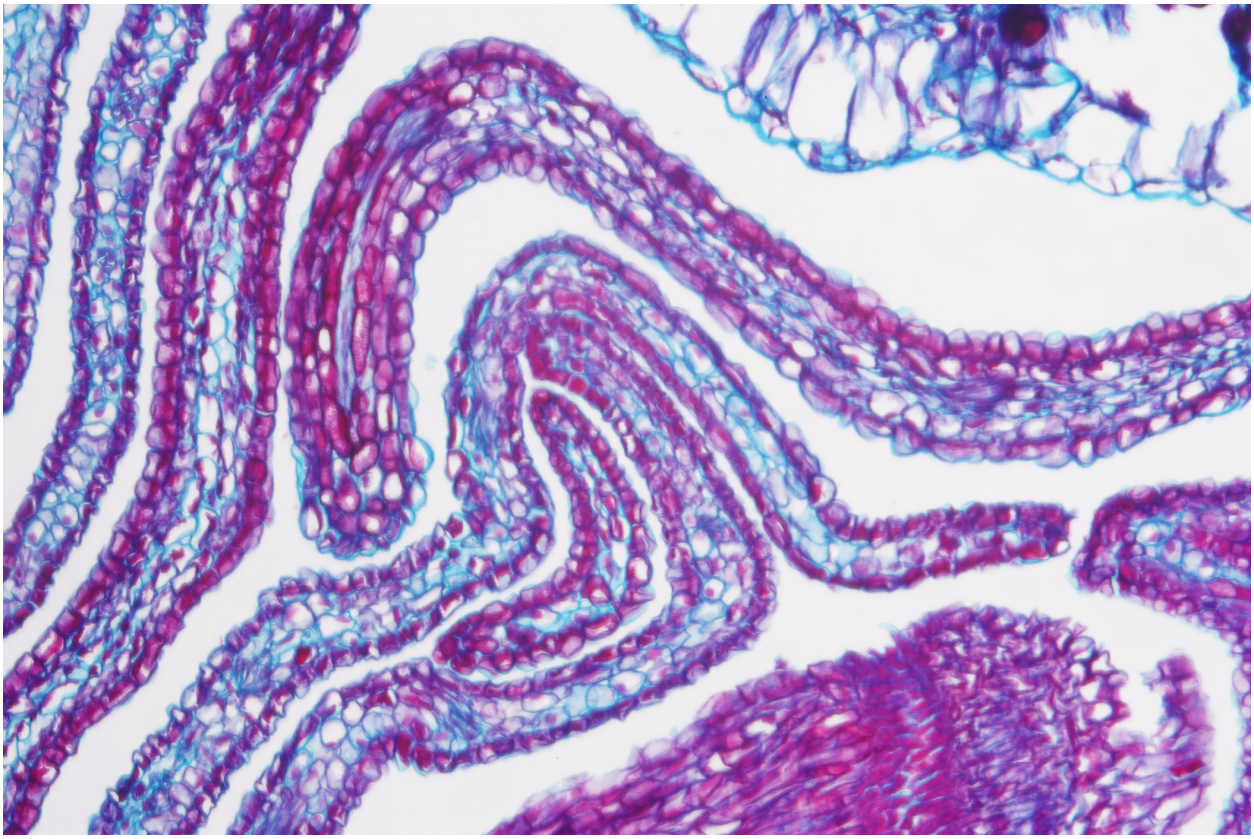

Fig. 12: A section of the unopened bud of a camellia flower stained with the Alcian Blue / Saffron O Staining Solution. (20-minute stain time with a thickness of 12 micrometers. The images were captured at 20x magnification using an Olympus BX50 microscope and is a combination of 10 images using Helicon Focus software.

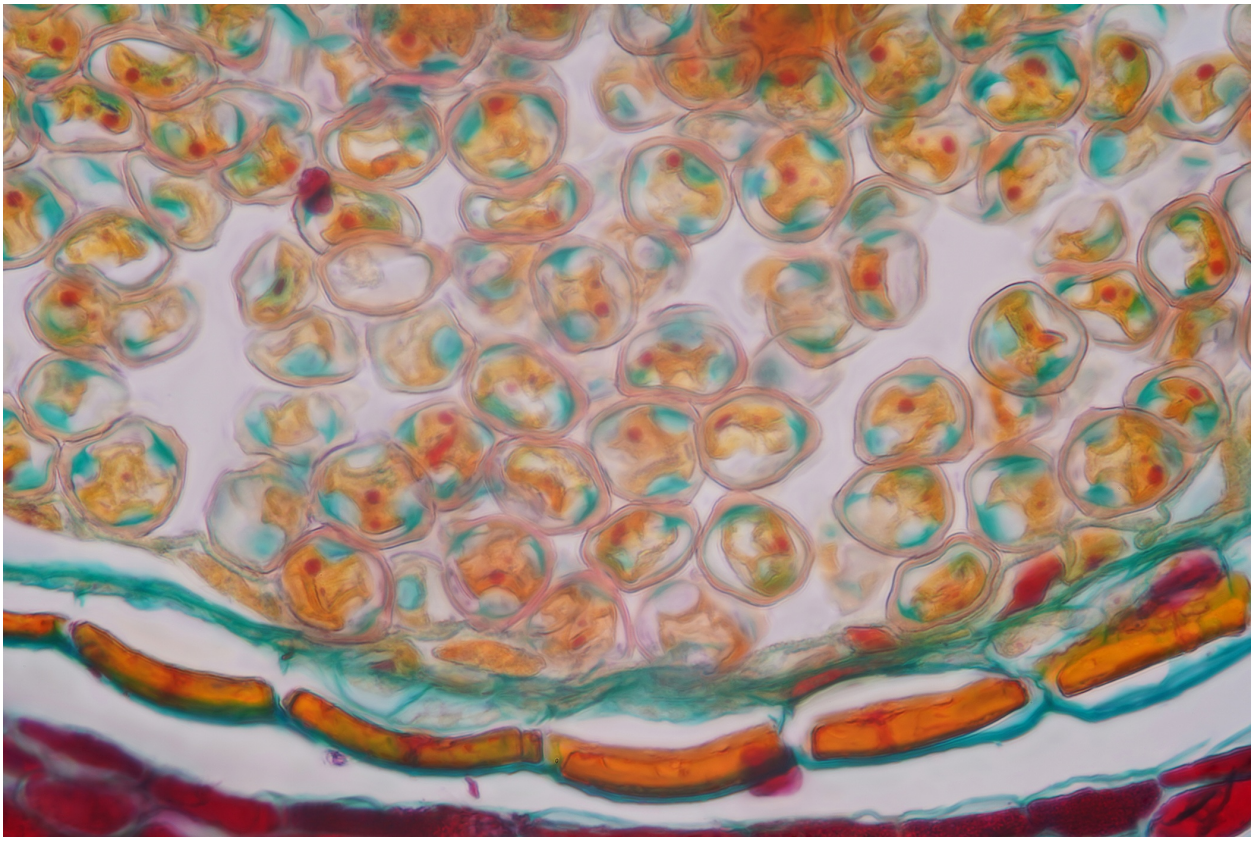

Fig. 13: The pollen of a Hazelnut catkin flower stained with the Wacker Solution. (40-minute stain time with a thickness of 12 micrometers. The images were captured at 40x magnification using an Olympus BX50 microscope and is a focus stack of 10 images combined in Helicon Focus software.

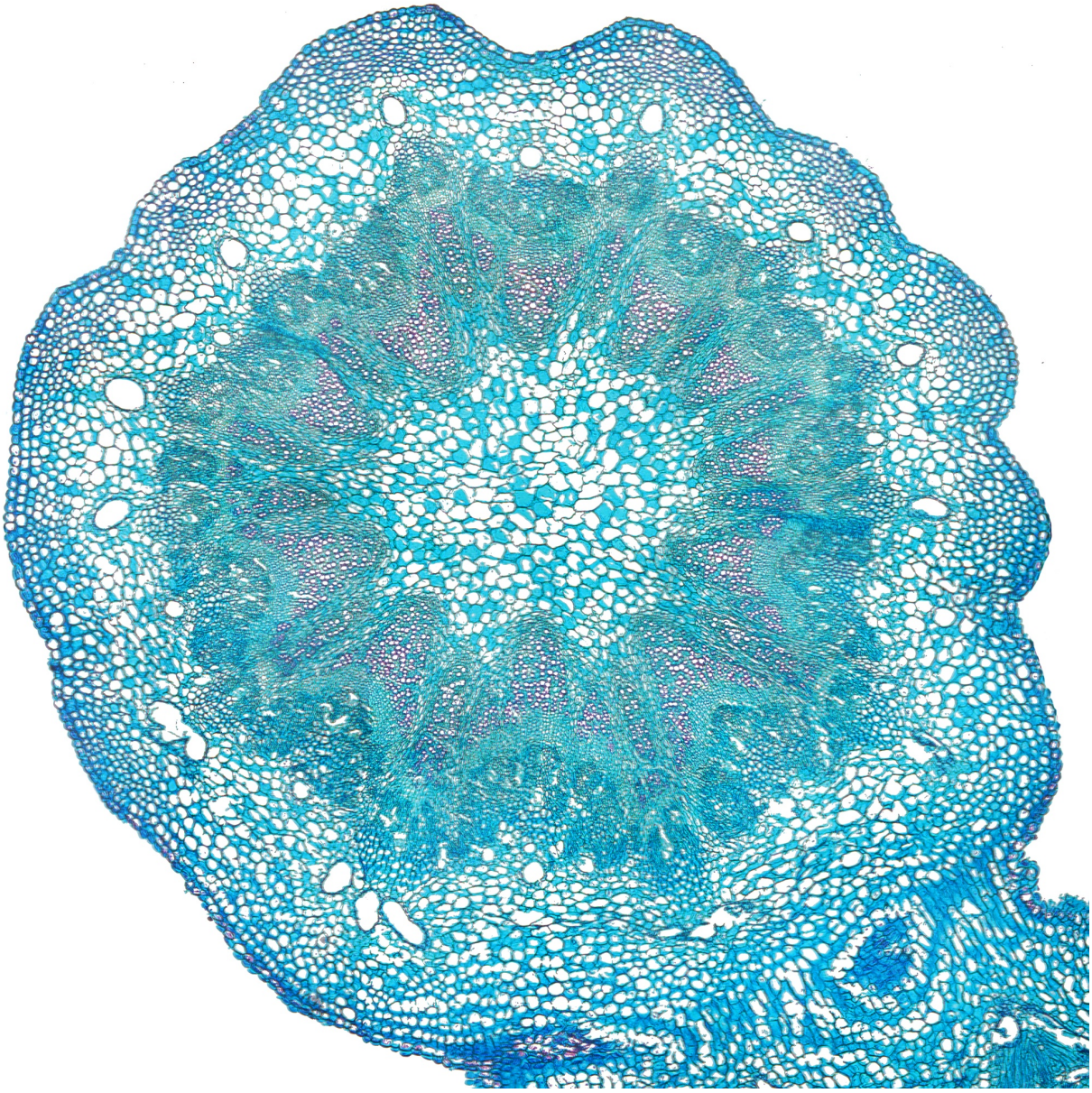

Fig. 14: Queen Anne's Lace stem (Daucus carota) stained with the Alcian Blue / Saffron O Staining Solution. (20-minute stain time with a thickness of 12 micrometers. Field of view in the horizontal direction is approximately 2.5mm.

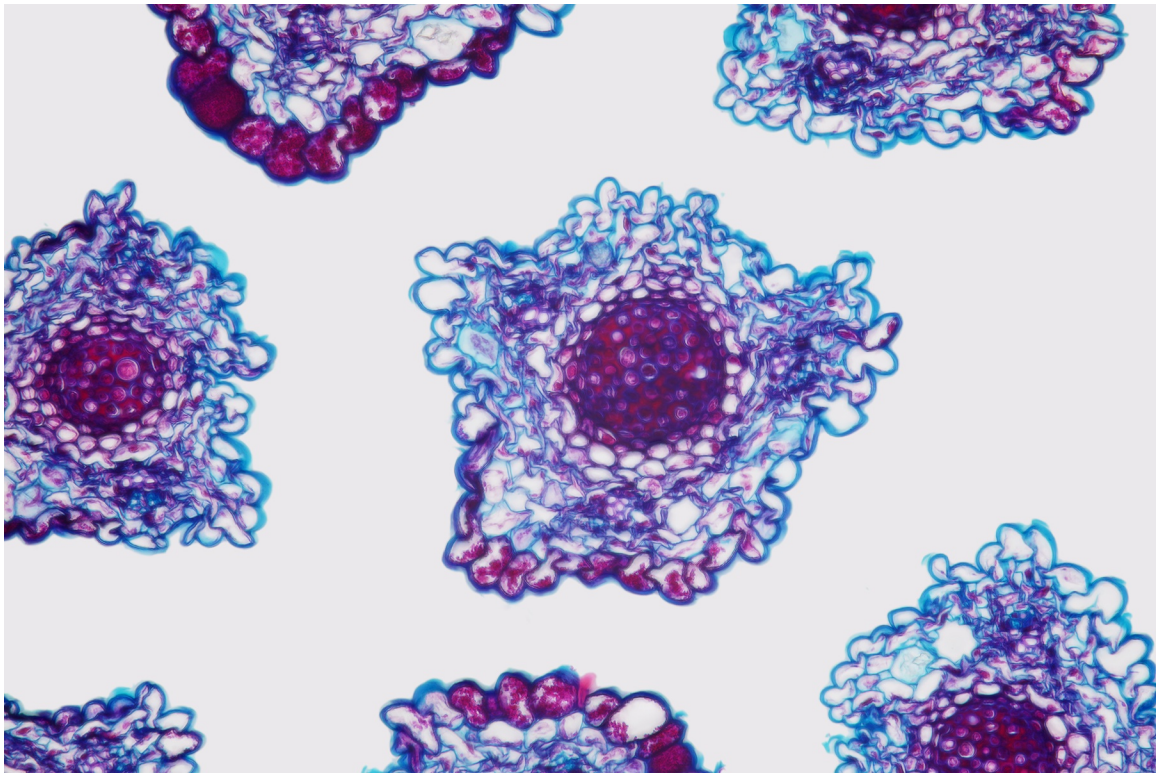

Fig. 15: The center of the calyx from a ripe tomato stained with the Alcian Blue / Saffron O Staining Solution. (20-minute stain time with a thickness of 12 micrometers. The images were captured at 40x magnification using an Olympus BX50 microscope and is a combination of 10 images using Helicon Focus software.

Please direct correspondence to:

Ted Kinsman Rochester Institute of Technology, Rochester, New York emkpph@rit.edu

References:

1. Romeis, Erwin. Mikroskopische Technik. 19. Auflage. Springer Spektrum, 2015.
- Note: 19th Edition in German.
- Page 107 for isopropyl/paraffin technique.

2. Chamberlain, C.J. Methods in Plant Histology. University of Chicago Press, 4th ed., 1932.

3. Johansen, D.A. Plant Microtechnique. New York: McGraw Hill, 1940.

4. Ruzin, S. Plant Microtechnique and Microscopy. Oxford University Press Inc, USA, 1999.

5. Wolberg, Shunamit, Mor Haim, and Illana Shtein. "Simple differential staining method of paraffin-embedded plant sections with safranin-alcian blue." IAWA Journal 44, no. 2 (2023): 170-175.

6. Wacker, Robin, Rolf-Dieter Müller, and Jörg Weiß. "Wacker für alle - neue Simultanfärbungen auf Basis der W3A Färbung." 17.06.2011. [Online]
- Available at:
https://mikroskopie-bonn.de/_downloads/MKB_Artikel_W3Asim_I_und_II_RDM_WSS_110619_1.pdf

7. Müller, Rolf-Dieter. "Dörnberg VII (W-Asim III) - a new botanical coloring." September 26, 2019. [Online]
- Available at: https://www.mikroskopie-bonn.de/bibliothek/botanische_mikrotechnik/296.html

8. Non-Toxic MicroTechniques by Ted Kinsman (video)
https://www.youtube.com/watch?v=zwzI3D_fru0

With special thanks to:

Illana Shtein, for answering all my questions about her article and pointing me to many good references.

Published in the March 2024 issue of *Micscape* magazine.

www.micscape.org