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Optical clearing of plant tissues for fluorescence imaging

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TITLE:

Optical Clearing of Plant Tissues for Fluorescence Imaging

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KEYWORDS:

Optical clearing, Plant tissue, Deep imaging, Fluorescent protein, Chemical dye, Fixation.

SUMMARY:

Here, a method is described for making plant tissues transparent while maintaining the stability of fluorescent proteins. This technique facilitates deep imaging of cleared plant tissues without physical sectioning.

ABSTRACT:

It is challenging to directly observe the internal structure of multi-layered and opaque plant specimens, without dissection, under a microscope. In addition, autofluorescence attributed to chlorophyll hampers the observation of fluorescent proteins in plants. For a long time, various clearing reagents have been used to make plants transparent. However, conventional clearing reagents diminish fluorescent signals; therefore, it has not been possible to observe the cellular and intracellular structures with fluorescent proteins. Reagents were developed that can clear plant tissues by removing chlorophyll while maintaining fluorescent protein stability. A detailed protocol is provided here for the optical clearing of plant tissues using clearing reagents, ClearSee (CS) or ClearSeeAlpha (CSA). The preparation of cleared plant tissues involves three steps: fixation, washing, and clearing. Fixation is a crucial step in maintaining the cellular structures and intracellular stability of fluorescent proteins. The incubation time for clearing depends on the tissue type and species. In *Arabidopsis thaliana*, the time required for clearing with CS was 4 days for leaves and roots, 7 days for seedlings, and 1 month for pistils. CS also required a relatively

short time of 4 days to make the gametophytic leaves of *Physcomitrium patens* transparent. In contrast, pistils in tobacco and torenia produced brown pigment due to oxidation during CS treatment. CSA reduced the brown pigment by preventing oxidation and could make tobacco and torenia pistils transparent, although it took a relatively long time (1 or 2 months). CS and CSA were also compatible with staining using chemical dyes, such as DAPI (4',6-diamidino-2-phenylindole) and Hoechst 33342 for DNA and Calcofluor White, SR2200, and Direct Red 23 for the cell wall. This method can be useful for whole-plant imaging to reveal intact morphology, developmental processes, plant-microbe interactions, and nematode infections.

INTRODUCTION:

Visualization of cellular structures and localization of proteins in living organisms is important for clarifying their functions *in vivo*. However, since the living body is not transparent, it is challenging to observe the internal structure of living organisms without dissection. Especially, in the case of plant tissues, which are multi-layered with cells of different shapes, the index mismatch caused by their structure and the presence of light-absorbing pigments is problematic. For example, plant leaves have a complex structure that allows them to efficiently utilize the light that enters their bodies for photosynthesis[1], whereas the structure also causes refractive index mismatch, making them difficult to observe. However, leaves have many light-absorbing pigments, such as chlorophyll, which emit strong red fluorescence and brownish pigments are produced by oxidation[2,3]. These pigments also hinder whole-mount fluorescence microscopy observations in plants. Therefore, for observing the internal structure of plants, decolorization and fixation by alcohol and clearing using chloral hydrate have been used for a long time to eliminate the refractive index mismatch and autofluorescence[4,5]. These conventional methods have been adopted for many years, but they have the drawback of eliminating the fluorescence of fluorescent proteins at the same time[6,7]. This is problematic since fluorescent proteins have become indispensable in current fluorescent imaging.

Therefore, ClearSee (CS) and ClearSeeAlpha (CSA) have been developed as optical clearing reagents for plant tissues. Both reagents reduce chlorophyll autofluorescence while maintaining the stability of fluorescent proteins[7,8]. CSA is particularly useful when brown pigments are produced owing to tissue oxidation. Using these clearing reagents, it is possible to observe the cellular structure and protein localization inside the plant body without physical sectioning.

PROTOCOL:

1. Preparation of clearing solutions

1.1. To prepare CS solution, dissolve 10% (w/v) xylitol, 15% (w/v) sodium deoxycholate, and 25% (w/v) urea in distilled water on a magnetic stirrer.

NOTE: Sodium deoxycholate powder should be weighed in a draft chamber as it easily floats in the air. CS can be stored at room temperature in dark for more than 1 year.

1.2. To prepare CSA solution, add sodium sulfite (50 mM final concentration) to the CS

solution obtained above.

NOTE: Add sodium sulfite to the CS solution right before use as the reducing agent gets deactivated easily.

2. Preparation of the fixative solution

2.1. Transfer 40 mL of sterilized water into a conical tube and add 2 g of paraformaldehyde. Add 200 µL of 2 N NaOH to increase the pH of the solution. After closing and sealing the tube with parafilm, incubate at 60 °C with occasional inversions until everything is dissolved.

2.2. After cooling the solution to room temperature, add 5 mL of 10x phosphate-buffered saline (PBS) to adjust the pH to 7.4. Add sterilized water to make up the volume to 50 mL.

NOTE: A freshly prepared fixative solution is preferred. The solution can also be stored at -30 °C for several months.

3. Fixation of samples

3.1. Immerse plant samples in the fixative solution in a microtube (**Figure 1A**), ensuring that the volume of the fixative solution is more than five times the sample volume.

3.2. Seal the microtube with a parafilm and make holes using a needle. Do not leave the tube open owing to a risk of sample spillage during vacuum decompression.

3.3. Place the microtube in a desiccator and slowly adjust the degree of vacuum (˜690 mmHg) so that bubbles appear gradually from the samples (**Figure 1B**–**C**). Turn off the vacuum pump after evacuating the desiccator. Leave the microtube undisturbed for 30 min at room temperature.

3.4. Vent the desiccator carefully to prevent disturbing the samples. Turn on the vacuum pump again and turn it off after evacuating the desiccator. Leave the microtube undisturbed for 30 min at room temperature.

NOTE: Care should be taken to prevent damage to the samples while venting the desiccator. The penetration of the fixative solution into the samples is enhanced by two vacuum treatments. Further vacuum treatment helps to penetrate the fixative solution into thicker samples.

3.5. Open the desiccator carefully without bumping the fixative solution in the microtube. Using a micropipette, remove the fixative solution and add 1x PBS. After storing for 1 min, replace the old PBS with new 1x PBS (**Figure 1D**).

4. Clearing

4.1. After removing PBS, add five times the sample volume of the clearing solution.

4.2. Seal the microtube with parafilm and make holes using a needle. Place the samples in the desiccator, evacuate as in step 3.3, and turn off the vacuum pump. Leave the microtube undisturbed for 60 min at room temperature.

4.3. Open the desiccator gently. Close the microtube with parafilm and store it at room temperature in the dark to avoid photobleaching of fluorescent proteins. Invert the microtube every 1–2 days to accelerate the clearing process.

4.4. When the clearing solution turns green, replace it with new clearing solutions until the solution remains colorless (**Figure 1E–H**).

[Insert Figure 1 here]

5. Chemical dye staining

5.1. To the microtube, add Hoechst 33342 (final concentration of 10 µg/mL) for nuclear staining or Calcofluor White (final concentration of 1 mg/mL) for cell wall staining and wait for 1 h. After removing the dye solution, wash the sample with a fresh clearing solution for 1 h.

NOTE: Overnight staining and washing can improve fluorescent dye penetration into tissues and reduce background fluorescence. Various fluorescent dyes are compatible with the CS solution such as Basic Fuchsin[9] (lignin), Auramine O[9] (lignin, suberin, and cutin), Nile Red[9] (suberin), Direct Yellow 96[9], Direct Red 23[9], and SR2200[10,11] (cell walls).

6. Observation

6.1. Cut silicone rubber sheet with a razor blade to prepare a frame for the spacer (**Figure 2A**).

NOTE: Adjust the thickness of the silicon rubber sheet according to the thickness of the sample. As samples treated with clearing solution are soft, they will be damaged if they are covered directly with the cover glass.

6.2. Place the silicone frame on cover glass (e.g., 25 x 60 mm) (**Figure 2B**). Place the treated samples within the frame and add ~100 µL of clearing solution to remove any bubbles in the frame. Cover with another cover glass (18 x 18 mm or 24 x 24 mm) to prevent evaporation of the clearing solution (**Figure 2C**).

6.3. Observe the samples under a fluorescent microscope. After observation, return the samples to the clearing solution taken in a microtube and store at room temperature in the dark.

[Insert Figure 2 here]

REPRESENTATIVE RESULTS:

CS can clear leaves of various species (**Figure 3A–H**). It is difficult for the CS solution to penetrate a rice leaf because the leaf surface is covered by cuticular wax in this plant. However, after extracting the cuticular wax by dipping in chloroform for 10 s, CS could clear the rice leaves (**Figure 3H**). CS could not, however, penetrate *Chamaecyparis obtusa* leaves which are less permeable to CS (**Figure 3I,J**). In *Chrysanthemum* leaves, brown pigmentation induced by polyphenol oxidation was observed in the CS-treated leaves (**Figure 3K,L**). Similarly, Tobacco and torenia pistils showed brown pigmentation during the CS treatment (**Figure 3M,O**). As the sodium sulfite component in CSA prevents polyphenol oxidation owing to the reducing effect, CSA could clear tobacco and torenia pistils without any brown pigmentation (**Figure 3N,P**).

Figures 4B shows that CS treatment reduced the pale green color of the *Arabidopsis* H2B-mClover leaf (bright field) and enhanced the fluorescence intensity of H2B-mClover compared with PBS incubation (**Figure 4A**). In addition to flowering plants, CS is also applicable to moss plants (**Figure 4C**); after 4 days of CS treatment, the fluorescence of H2B-mRFP was clearly detected for the entire gametophore with reduced chlorophyll autofluorescence. **Figures 4D,E** show 3D reconstruction images of the H2B-mClover pistil in *Nicotiana benthamiana* cleared using CSA. The sample depth was 440 μm. As the depth-coded maximum intensity projection image shows, CSA allows for deep imaging of challenging tissues, such as tobacco pistils.

CS and CSA were also compatible with fluorescent dye staining. **Figure 5** shows that CS could simultaneously be used to observe the fluorescent protein (H2B-mClover) and organic fluorescent dye staining (Calcofluor White). After 3D reconstruction from the z-stack images, any section could be observed.

FIGURE AND TABLE LEGENDS:

Figure 1: Procedure for CS treatment. (**A**) *Arabidopsis* seedling in 4% PFA (Paraformaldehyde) solution. (**B**) The sample is placed into a desiccator. (**C**) The seedling is fixed under a vacuum. (**D**) The seedling is soaked in the PFA solution after vacuum treatment. (**E**) Resulting 3-day clearing solution-treated seedling. Note the green color of the clearing solution. (**F**) The clearing solution is replaced 3 days after treatment. (**G**) Resulting 5-day clearing solution-treated seedling. (**H**) The clearing solution is replaced 5 days after treatment. Scale bars: 1 cm (**A**,**C–H**) and 5 cm (**B**).

Figure 2: Sample preparation for microscopic observation. (**A**) Cut a 0.2 mm thick silicone sheet into a frame. (**B**) Put the silicone sheet frame onto the cover glass. (**C**) Place the sample treated with clearing solution (marked by dotted border) within the frame and cover it with a cover glass. Scale bars: 5 mm.

Figure 3: Optical clearing of leaves and pistils using clearing solutions. (**A–L**) Fixed leaves of various species were incubated in PBS (**A**,**C**,**E**,**G**,**I**,**K**) or CS (**B**,**D**,**F**,**H**,**J**,**L**) for 8 days and CS (**M**,**O**) or CSA (**N**,**P**) for 2 days. (**A**,**B**,**O**,**P**) *Torenia fournieri*, (**C**,**D**) *Nicotiana tabacum*, (**E**,**F**) *Cucumis sativus*, (**G**,**H**) *Oryza sativa*, (**I**,**J**) *Chamaecyparis obtusa*, (**K**,**L**) *Chrysanthemum morifolium*, (**M**,**N**) *Nicotiana benthamiana*. Scale bars: 1 cm.

Figure 4: Fluorescence imaging of tissues treated with clearing solutions. (**A**,**B**) UBQ10pro::H2B-mClover leaves of *Arabidopsis thaliana* were treated with PBS (**A**) or CS (**B**) for 3 days. (**C**) H2B-mRFP leafy gametophores of *Physcomitrium patens* were treated with CS for 4 days. The nuclei were labeled with H2B-mRFP (green). The CS treatment reduced chlorophyll autofluorescence (magenta). The H2B-mRFP signal in the apical region was clearly observed for both merged images of H2B-mRFP and autofluorescence or bright field. (**D**,**E**) The UBQ10pro::H2B-mClover stigma of *Nicotiana benthamiana* was treated in CSA for 1 month. Maximum-intensity projection (**D**) and depth-coded maximum-intensity projection (**E**) were generated from 88 z-stack images at 5 µm intervals. Scale bars: 100 µm. Images were taken using wide-field (**A**,**B**), confocal (**C**), and two-photon excitation (**D**,**E**) microscopy.

Figure 5: Fluorescent dye staining is compatible with CS. (**A**) CS-treated leaves observed by two-photon excitation microscopy with 950 nm excitation. Cell wall is stained with Calcofluor White (cyan). Nuclei are labeled with UBQ10pro::H2B-mClover (yellow). The yz (**B**) and xz (**C**) images are cross-sections at the position indicated by the white dashed lines in (**A**). Scale bar: 100 µm.

Figure 6: Transparency of sodium deoxycholate. (**A**) Colors of various 15% sodium deoxycholates (listed in **Table of Materials**). (**B**) Fluorescence spectrum of each 15% sodium deoxycholate with 380 nm excitation.

DISCUSSION:

This method consists of fixation, washing, and cleaning. Fixation is a critical step in this protocol. If the fluorescent protein is not observed after PFA fixation, it will not be observed after treatment with clearing solution. The penetration of PFA solution into tissues is critical, but high vacuum treatment is not recommended because it can destroy the cell structure. Vacuum conditions and fixation periods should be optimized for each type of tissue and species. It is recommended to check fluorescent proteins even after fixation. Although samples were usually fixed for 30–60 min at room temperature, they can be fixed at 4 °C for a longer time (overnight or more).

As shown in **Figure 6A**, some sodium deoxycholates had a pale-yellow color when dissolved. Such sodium deoxycholate solutions showed strong autofluorescence in the 400–600 nm region after excitation at 380 nm (**Figure 6B**). This autofluorescence prevents optical clearing and fluorescence imaging. Users should check the color of sodium deoxycholate solution as the quality of the reagent might differ owing to purity, lot-to-lot variation, or other reasons.

The clearing solutions used here have high concentrations of sodium deoxycholate, which could destroy the membrane structure. The plasma membrane marker (RPS5Apro::tdTomato-LTI6b) was observed even after CS treatment[7]. However, it might be better to reduce the concentration of sodium deoxycholate, depending on the structure and tissue of interest. Indeed, images with improved clarity were obtained for *Arabidopsis* pistils with modified CS, in which the concentration of sodium deoxycholate is reduced by half; however, reduced concentrations of sodium deoxycholate required prolonged treatment times (e.g., 1 month for *Arabidopsis* pistils).

CS can reduce red autofluorescence (>610 nm) to remove chlorophyll in treated samples. However, 500–600 nm range autofluorescence (yellow to orange) remained even in CS-treated samples[7]. This autofluorescence is thought to be derived from the cell wall and other cellular components, such as lignin[12,13]. Therefore, it is difficult to make tissues, such as stems with developed secondary walls, completely transparent by CS treatment.

Several clearing reagents besides the ones used here have been developed to observe fluorescent proteins in plants using fluorescent microscopy[14–17]. Compared with these methods, CS and CSA remove chlorophyll and reduce autofluorescence, making the plant tissues more transparent. Recently, Sakamoto et al. developed an improved method, iTOMEI, for fixation, detergent clearing, and mounting to adjust the refractive index mismatch[18]. In *Arabidopsis* seedlings, iTOMEI cleared the tissue within 26 h.

CS is applicable to a wide range of plant species, such as *Arabidopsis thaliana*, *Physcomitrium* patens[7], *Chrysanthemum morifolium*, *Cucumis sativus*, *Nicotiana benthamiana*, *Nicotiana tabacum*, *Torenia fournieri*[8], *Allium ochotense*[19], *Astragalus sinicus*[20], avocado[21], barley[22], *Brassica rapa*[23], *Callitriche*[24], *Eucalyptus*[25], maize[26], *Marchantia polymorpha*[27], *Monophyllaea glabra*[28], *Orobanche minor*[29], petunia[30], rice[31], *Solanum lycopersicum*[32], soybean[33], strawberry[34], wheat[35], and *Wolffiella hyalina*[36]. For thicker tissues, CS can also make the vibratome sections transparent[37,38]. This method allowed studies of the cellular structure and gene expression patterns in plants[37,38]. Moreover, nematode infections[20,39], fungal infections, and symbiosis[19,40,41] were also observed deep inside the CS-treated tissues. Thus, this method is useful for whole tissue imaging from micro- to macro-scales and could help to discover novel interactions among various cells, tissues, organs, and organisms.

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DISCLOSURES:

ClearSee is commercialized (Fujifilm Wako Pure Chemical Corporation, Japan). Nagoya University holds a patent (JP6601842) on the clearing reagent ClearSee. D. Kurihara is the patent inventor. The authors declare no competing financial interests.

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Figure 1

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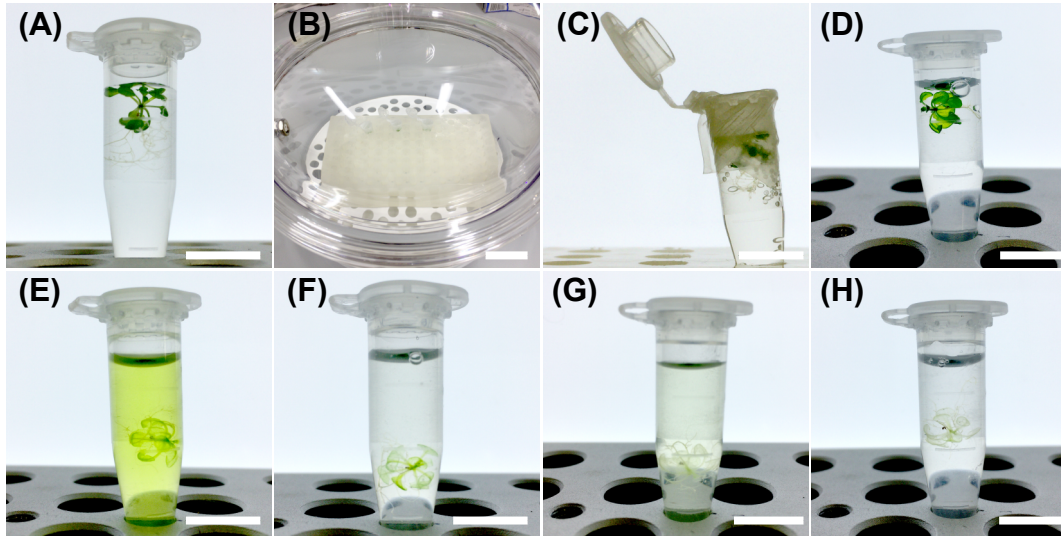

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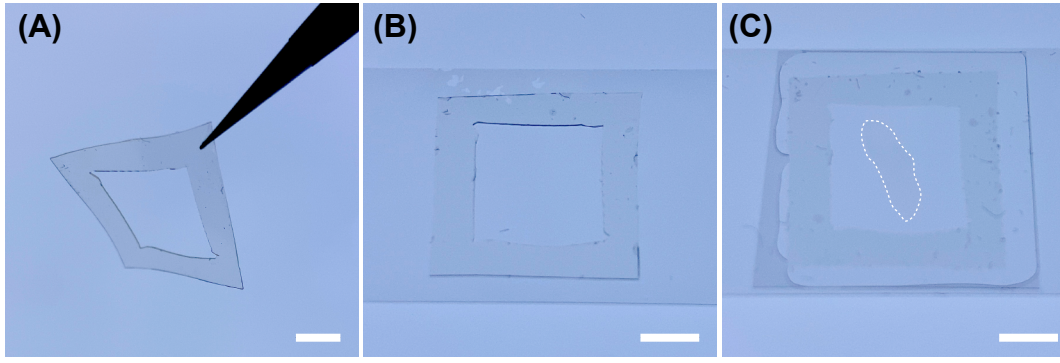

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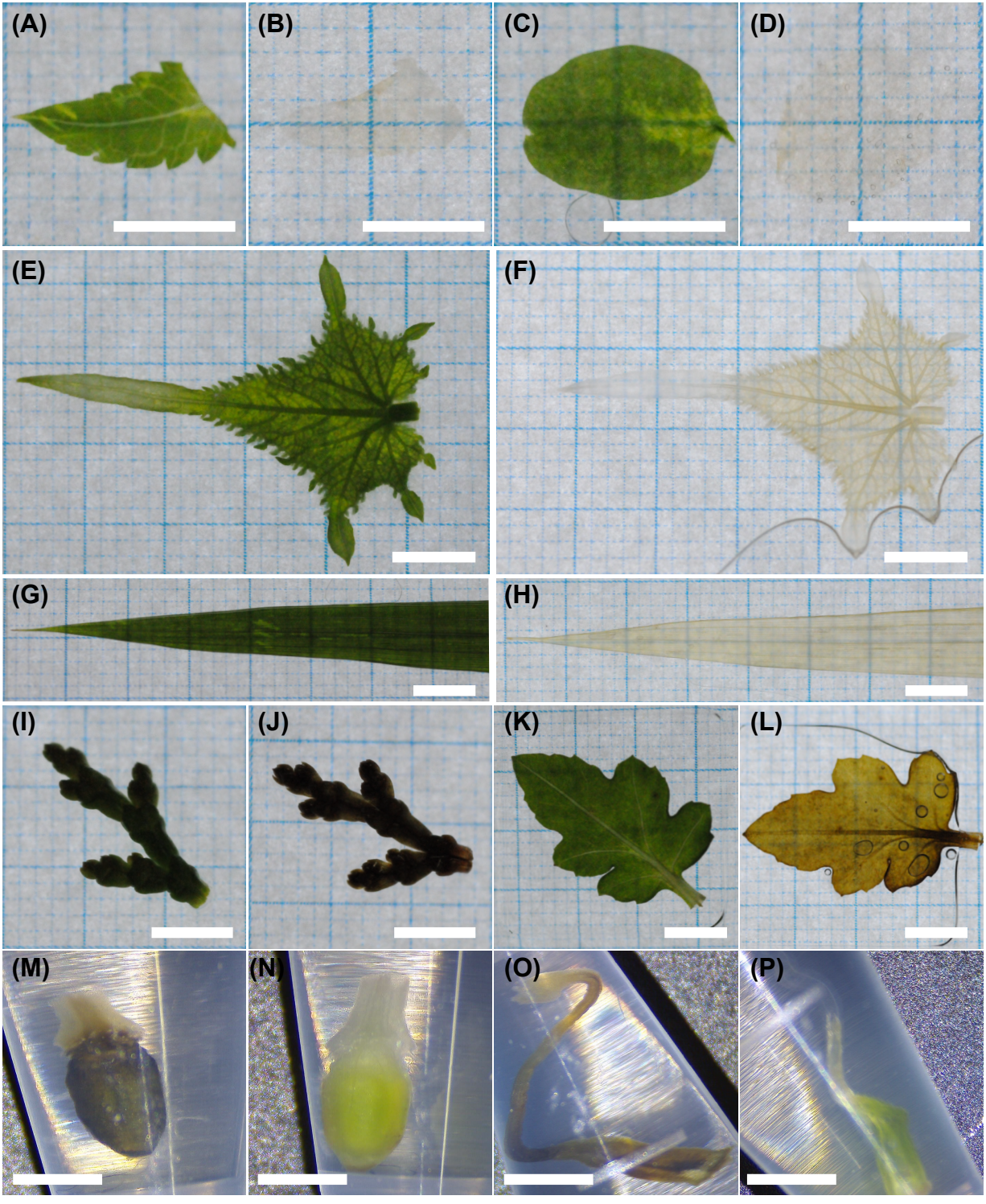

Figure 4

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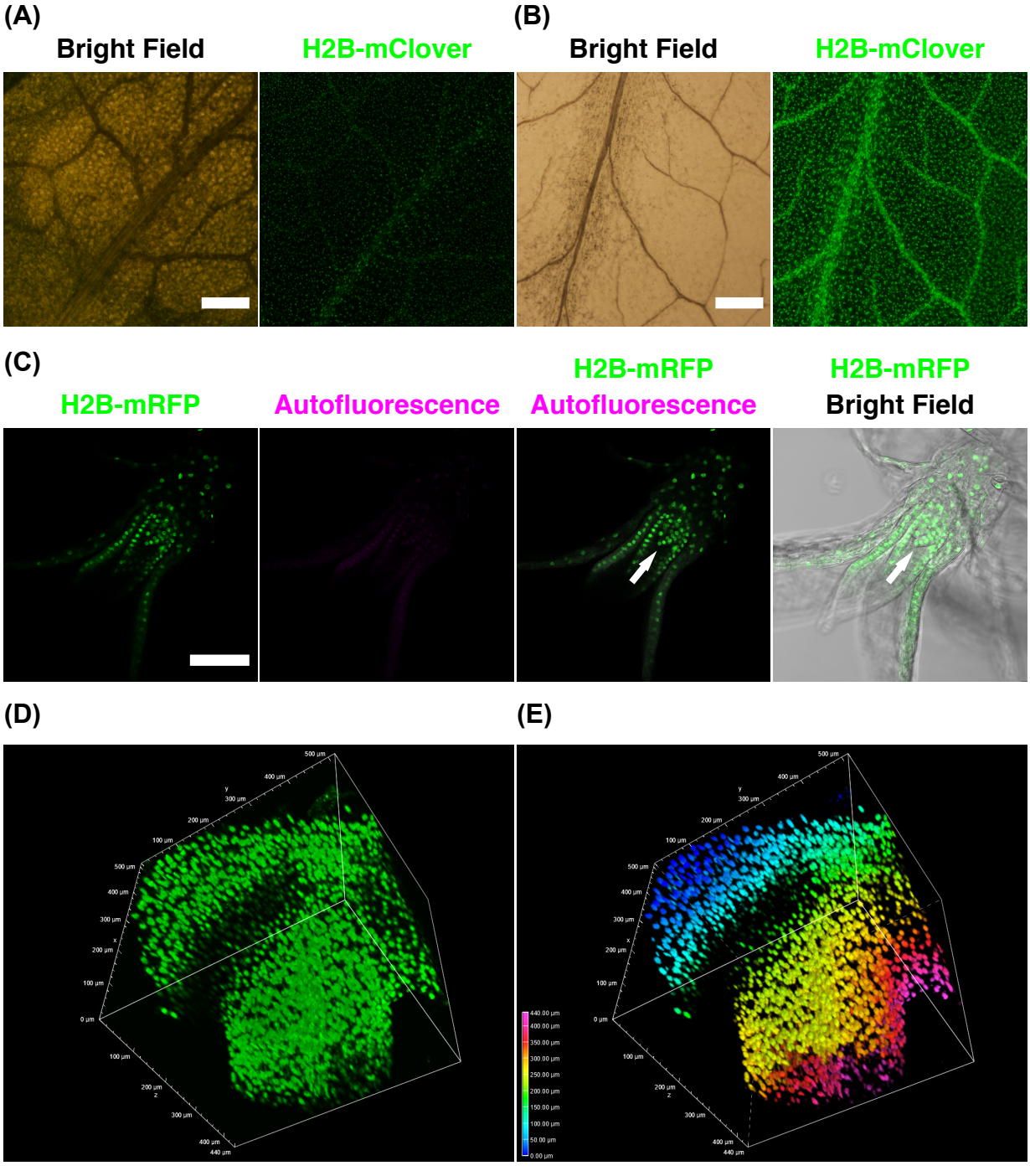


Figure 5

Click here to access/download;Figure;Figure5_R2.pdf

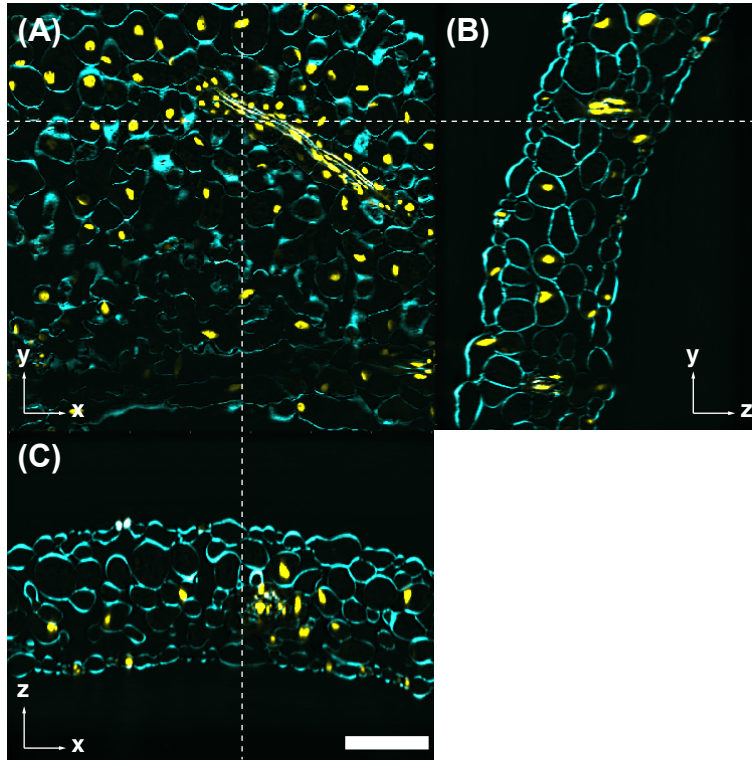
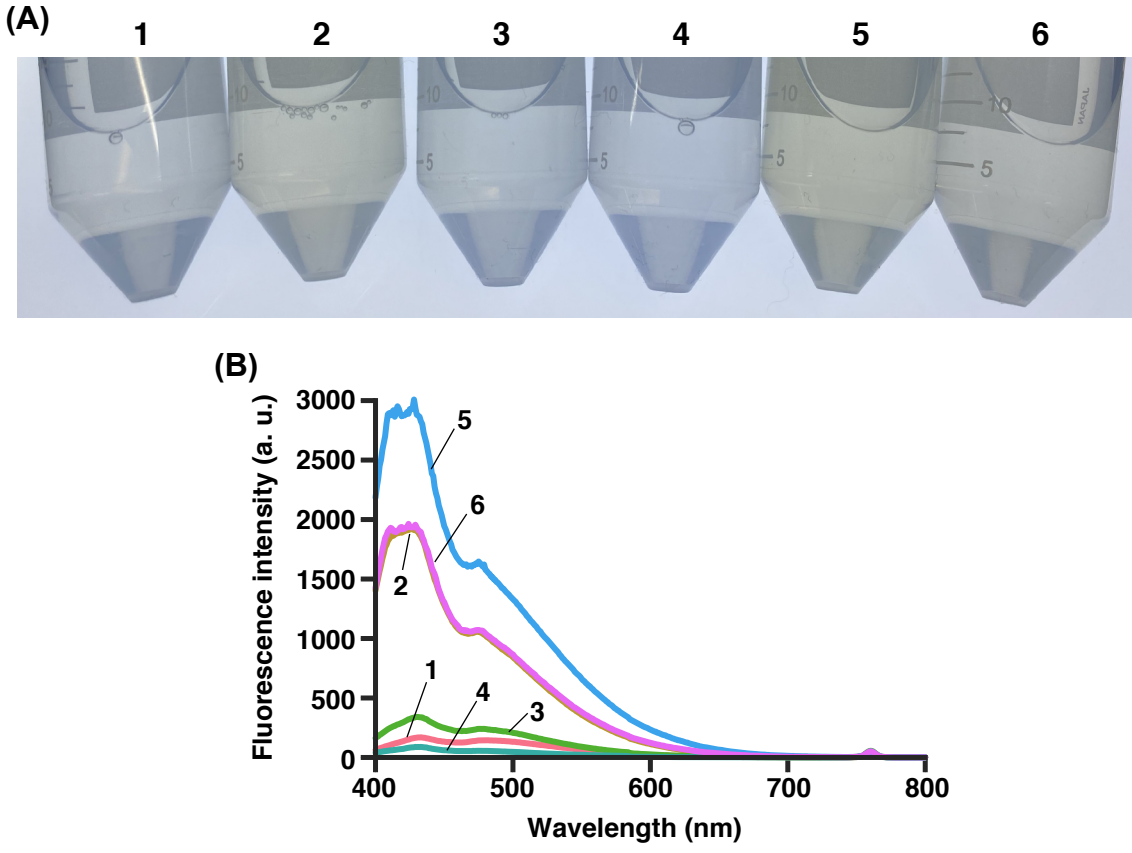


Figure 6

Click here to access/download;Figure;Figure6_R2.pdf



Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.

Response: We double-checked the revised manuscript.

2. To avoid using a commercial name in the title, please revise the title to "Optical clearing of plant tissues".

Response: We changed the title to "Optical clearing of plant tissues for fluorescence imaging"

3. Please revise the following lines to avoid overlap with previously published work: 112-113, 118, 119-128, 130-131, 140-148, 172, 197-198, 236-237.

Response: We revised the text to avoid overlap.

4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: ClearSee, ClearSeeAlpha Obviously, as you have optimized your protocol with ClearSee and certain instruments/reagents/software, you will need to mention them, but include just the bare minimum information (e.g., name of product) in the manuscript and come up with a generic term to refer to the product (include this in the comments column in the Table of Materials) after the first mention so that you don't keep repeating commercial terms throughout the paper. Comparison of your equipment or software to other commercially available ones is allowed in the discussion, but without unnecessary repetition of these names and only for scientific discussion.

Response: Although ClearSee solution is commercially available, the researchers can make ClearSee solution themselves according to this manuscript. ClearSee is also a technique name, such as CUBIC, so we used ClearSee in our manuscript.

5. Consider moving the composition of ClearSee from lines 85-96 to the Table of Materials or into a note after step 1.1. The Protocol should contain only action items that direct the reader to do something.

Response: We moved the composition of ClearSee to the Table of Materials.

6. However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

Response: We moved the description about draft chamber to the next step (new 1.1).

7. Please cite all figures and tables in the text in order.

Response: We cited all figures and tables in the text in order.

8. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please ensure you answer the "how"question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the

protocol action. Please ensure the inclusion of specific details (e.g., button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

Response: We checked each step.

9. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep, and note in the protocol section. Please use Calibri 12 points and one-inch margins on all the side. Please include a ONE LINE SPACE between each protocol step and then HIGHLIGHT up to 3 pages of protocol text for inclusion in the protocol section of the video.

Response: We adjusted it.

10. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations: a) CRITICAL STEPS within the protocol b) Any modifications and troubleshooting of the technique c) Any LIMITATIONS of the technique d) The significance with respect to existing methods e) Any future applications of the technique

Response: a) Critical step is Lines 267–274.

b) Troubleshooting is Lines 267–280.

c) Limitation is Lines 282–289.

We also added about the remained autofluorescence in ClearSee-treated samples as follows:

ClearSee can reduce red autofluorescence (> 610 nm) to remove chlorophyll in treated samples. However, 500–600-nm range autofluorescence (yellow to orange) remained even in ClearSee-treated samples. This autofluorescence is thought to be derived from the cell wall and other cellular components, such as lignin. Therefore, it is difficult to prepare tissues such as stems with developed secondary walls completely transparent by ClearSee treatment.

d) The significance with respect to existing methods are Lines 297–302.

e) Future applications are Lines 304–314.

11. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source (ITALICS). Volume (BOLD) (Issue), FirstPage–LastPage (YEAR).] For 6 and more than 6 authors, list only the first author then et al. Please include volume and issue numbers for all references, and do not abbreviate the journal names. Make sure all references have page numbers or if early online publication, include doi.

Response: We modified the all references.

12. Please add all items (plasticware, glassware, buffers, solvents, equipment, software etc) in the Table of Materials so that it serves as a handy reference for users to get everything ready for the protocol. Please sort the Materials Table alphabetically by the name of the material.

Response: We sorted the order of the material.

Point-by-Point Response to Reviewer #1

We thank you for your kind reviewing and helpful comments.

Minor Concerns

Point 1

It would be nice to have a separate 1-page step-by-step protocol as supplementary file or as a separate figure that could be downloaded and used as bench protocol. This could include a short paragraph with the ClearSee recipe on top, followed by the step-by-step guide. In addition to the video, this print-out would be a perfect companion for inexperienced users in the lab.

Response: Thank your for your kind suggestion. We will make the step-by-step protocol in the future.

Point 2

In the figure legend of figure 6, I would recommend to list the suppliers and Cat. of the various Sodium deoxycholate batches used (so far only listed in Table of Materials). This way, the figure can be read and understood without searching for the information in a separate table.

Response: Thank you for your advise, but the editor said that "JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents."

Point-by-Point Response to Reviewer #2

We thank you for your kind reviewing and helpful comments. As with your advice, we will improve our manuscript with the following points.

Minor Concerns

Point 1

Figure 1 lacks serial number (A-H), and the first middle picture"Fixation under vacuum"is not very clear. The Figure 1 legends lacks a description of the sample changes during the transparency process, which can be added appropriately.

Response: According Reviewer #2 advice, we modified figure 1 legend.

Point 2

The resolution of M–P in Figure 3 is very poor, making it difficult to read them. This aspect must be improved.

Response: We don't think the resolution of M–P is poor, because the readers can compare the colors of ClearSee– or ClearSeeAlpha–treated samples (M, O are brown, N, P are not brown).

Point 3

Line 194-195, there is no description of A in the Figure 4 result description, which need to be added.

Response: We modified the sentence as follows:

"Figures 4B shows that ClearSee treatment reduced the green color of the Arabidopsis leaf (bright field) and enhanced the fluorescence intensity of H2B-mClover compared with PBS incubation (Figure 4A). "

Point 4

The Latin name of all species in the references listed in the manuscript should be italic.

Response: We corrected them.

Point 5

Scale bars should be added in Fig. 1, 2.

Response: We added them.