

## Leaf Clearing Protocol to Observe Stomata and Other Cells on Leaf Surface

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**[Abstract]** In this protocol, leaves are cleared and fixed in an ethanol and acetic acid solution, and mounted in Hoyer's solution. The cleared leaves are imaged under differential interference contrast (DIC) microscope. This protocol is beneficial for studying stomata, hair cells, and other epidermal cells in plants.

**Keywords:** Leaf clearing, Stomata, DIC microscopy, Hoyer's solution

**[Background]** There are multiple ways to observe stomata and other epidermal cells such as hair cells on plant leaf surface. Traditionally a clear nail polish or wood glue is applied to the leaf surface and let dry. The leaf is peeled and observed under the microscope. Alternatively, scotch tape is applied to the leaf and removed to observe an imprint of the leaf surface. These traditional methods can be used for thicker leaves that are sturdy but the images are generally not of the highest quality. Small and delicate leaves such as *Arabidopsis* leaves require a more advanced method. A fresh *Arabidopsis* or *Brachypodium* leaf may also be observed directly under the microscope; however, the thickness and pigments in the leaf pose difficulties in viewing the stomata and other epidermal cells clearly. This protocol describes a method clearing of leaves for visualizing stomata including other epidermal cells and obtaining good quality images for publishing in peer-reviewed journals (Anderson, 1954).

### Materials and Reagents

1. Multi-well plates (Greiner Bio One International, catalog number: 665102)
2. Parafilm (Bemis, catalog number: PM996)
3. Microscope slides, 25 x 75 x 1.0 mm (Fisher Scientific, catalog number: 12-550-A3)
4. Cover slips (Fisher Scientific, catalog number: 12-544D)
5. Aluminium foil
6. *Arabidopsis* or *Brachypodium* leaf  
*Note: You may cut the Brachypodium leaf into 1 cm pieces to fit into wells.*
7. Ethanol (Sigma-Aldrich, catalog number: 459844)
8. Acetic acid (Sigma-Aldrich, catalog number: AX0077-1)
9. Potassium hydroxide (KOH) (Sigma-Aldrich, catalog number: 221473)
10. Gum Arabic (Sigma-Aldrich, catalog number: 30888-1KG)
11. Chloral hydrate (Sigma-Aldrich, catalog number: C8383)
12. Glycerol (Sigma-Aldrich, catalog number: G5516-1L)

*Note: Sterile is not required.*

13. Distilled water
14. Clearing solution (see Recipes)
15. 1 N KOH (see Recipes)
16. Hoyer's solution (see Recipes)

### **Equipment**

1. Fume hood
2. Forceps (Fisher Scientific, catalog number: 22-327379)
3. Microscope (Leica, model: Leica DM6 B)
4. 1 L glass beaker (Sigma-Aldrich, catalog number: Z169161)
5. Magnetic stirrer (Thermo Fisher Scientific, Thermo Scientific™, catalog number: S194615)

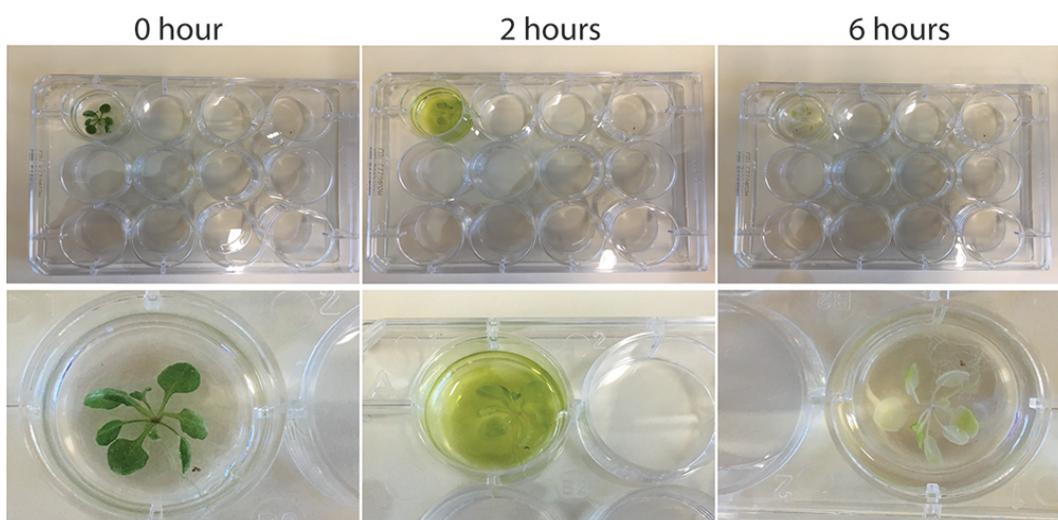
### **Software**

1. MS Excel  
(<https://support.office.com/en-us/article/STDEV-function-51fecaaa-231e-4bbb-9230-33650a72c9b0>)

*Note: Calculate Standard Deviation in MS Excel.*

### **Procedure**

1. Fill multi-well culture plate wells with 500 µl-1 ml clearing solution (work in a fume hood to avoid the smell of acetic acid, see Recipes). Place the whole seedling or a leaf (about 1 cm) in the clearing solution and seal the plate with Parafilm (Figure 1). Leave the tissue in clearing solution overnight at room temperature. Younger seedlings (2-4 d old) only need a few hours to clear. For bigger leaves (6 d old or more), change the clearing solution every 4 h until the leaves become transparent (for faster clearing, otherwise overnight incubation is fine). You may keep the leaves in clearing solution for about a week.



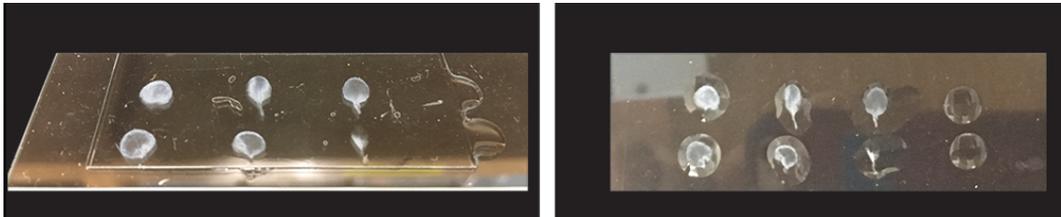
**Figure 1. Leaf clearing and fixing.** The ethanol:acetic acid solution fixes the tissue and the tissue becomes transparent.

2. Remove the clearing solution and refill the wells with 500  $\mu$ l-1 ml 1 N KOH (see Recipes) until the tissue becomes transparent (Figures 2C-2D).  
*Note: Usually takes 30 min, you may leave them longer (5-6 h) in 1 N KOH but overnight incubation is not advised.*
3. Remove 1 N KOH and refill the wells with 500  $\mu$ l-1 ml water to wash the cleared leaves (one wash is enough) and remove excess KOH.
4. Take a clean microscope slide and place 2 rows of Hoyer's solution (see Recipes) drops (~10  $\mu$ l each for *Arabidopsis* cotyledons). You may put 4-6 drops per row. Thicker leaves require more Hoyer's solution (Figure 2E).



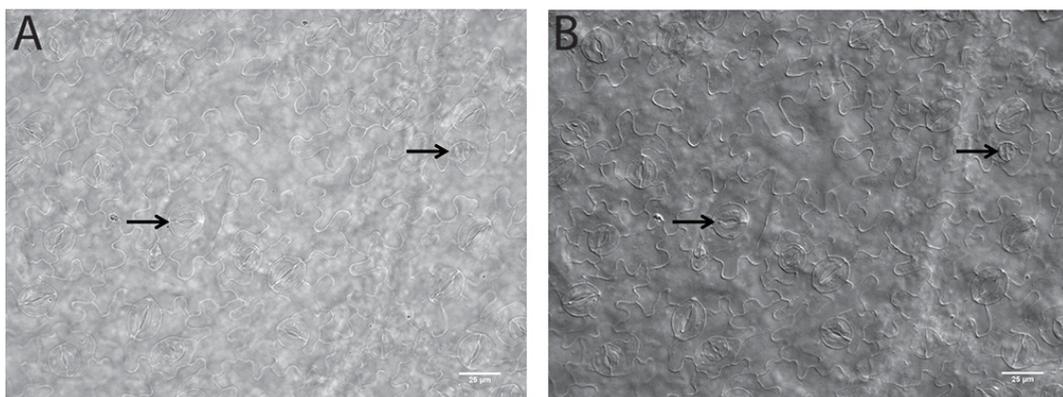
**Figure 2. Washing the tissue.** Cleared tissue (A, B) is washed in 1 N KOH (C, D). Tissue is washed in water (E, top slide) and placed in Hoyer's solution (E, bottom slide).

- Place the seedling on a cover slide and dissect out the cleared leaves (you may use magnifying glasses), so that you have at least 2 leaves (cotyledons or true leaves) and hold them with abaxial side up using forceps (Figure 3).



**Figure 3. Slide preparation.** Individual leaves form a precipitate when they are placed in Hoyer's solution.

- Place the leaves in Hoyer's solution droplets (Figure 3).  
*Note: KOH reacts with Hoyer's solution to form a precipitate that can guide you to place one leaf in each droplet. The precipitate will dissolve away quickly for young seedlings and may require more time for larger leaves.*
- Repeat the above steps for 3-4 seedlings per slide.
- Gently place coverslip and press to flatten the leaf for better visualization (Figure 3).
- Leave the slides to dry overnight at room temperature. You may image the young seedlings in 3-4 h but longer drying is advised for bigger leaves (1-5 days).
- Apply additional Hoyer's solution on the edges of the coverslip to fill any air bubbles formed during drying period.
- Image your cleared seedling under a microscope with DIC filter and document the phenotype (Figure 4).



**Figure 4. Same leaf imaged with Brightfield or DIC filters.** DIC image (B) gives better resolution than Brightfield (A) to observe different cell types. Arrows: stomata (young and mature).

## Data analysis

Different cell types e.g., young vs. mature stomata (pores on leaf surface); pavement cells vs. stomata; or the number of hair cells per surface area can be measured through the images obtained in this protocol (Figure 5). Typically at least three leaves ( $N = 3$ ) are observed for each line. E.g.,

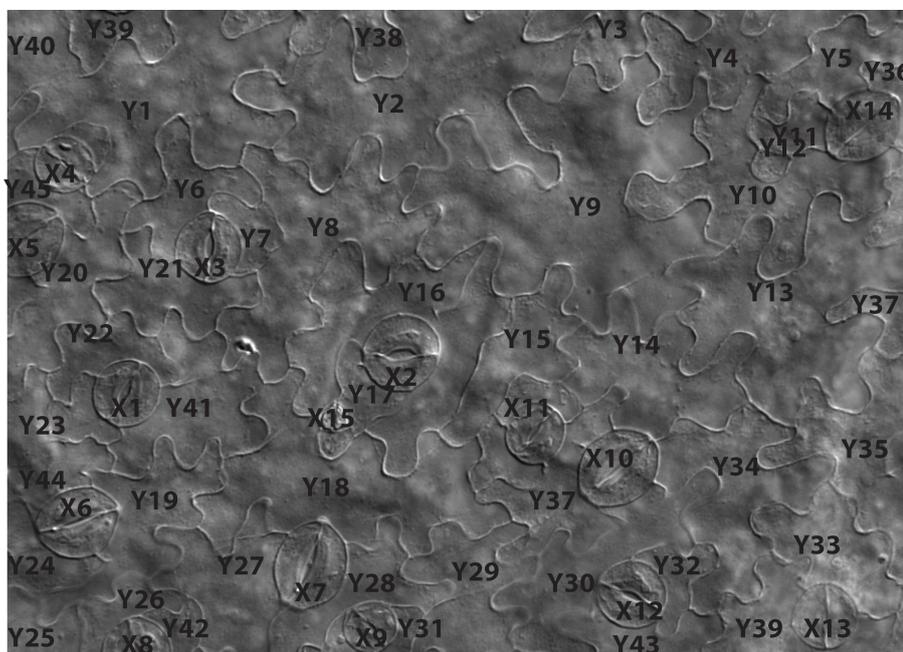
Count No. of stomata = X1, X2, X3

Count No. of pavement cells = Y1, Y2, Y3

Average X and Y values respectively.

Calculate Stomatal Index:  $X/(X + Y)$

Calculate standard deviation ([Calculate Standard Deviation in MS Excel](#)) and standard error (standard deviation/ $\sqrt{N}$ ) for each cell type and plot in a graph.



Number of stomata(X)=15  
 Number of pavement cells (Y)=45  
 Stomatal index= $15/(15+45)=0.25$

**Figure 5. Quantification of stomata.** Number of stomata (X) and pavement cells (Y) are counted to calculate stomatal index.

## Recipes

1. Clearing solution  
 7:1 solution of 95% ethanol:acetic acid  
 7 ml 95% ethanol + 1 ml acetic acid

## 2. 1 N KOH

56.11 g KOH in 1 L water

*Note: The dissolution of KOH in water is exothermic, hence the solution will get warmer and it is important to add KOH slowly.*

## 3. Hoyer's solution (prepare in a fume hood)

- a. Add 15 g of Gum Arabic to 25 ml of H<sub>2</sub>O in a 1 L glass beaker (cover the beaker with aluminium foil)
- b. Heat to 60 °C
- c. Stir overnight on a magnetic stirrer in the fume hood at room temperature
- d. Add 200 g of chloral hydrate until dissolved
- e. Add 20 ml of glycerol and mix

*Notes:*

- i. Store the solution at room temperature for about a year.*
- ii. Pellets appear after long storage but they do not cause any problems in tissue preparation or imaging.*

### **Acknowledgments**

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### **References**

1. Anderson, L. E. (1954). [Hoyer's solution as a rapid permanent mounting medium for bryophytes.](#) *Bryologist* 57(3): 242-244.