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Title: Characterizing Stomatal and Epidermal Traits Using Peels, Clearing, and AI-Based Image Analysis

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Author Questionnaire

1. We have marked your project as author-provided footage, meaning you film the video yourself and provide JoVE with the footage to edit. JoVE will not send the videographer. Please confirm that this is correct.

✓ Correct

2. Microscopy: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **No**

3. Software: Does the part of your protocol being filmed include step-by-step descriptions of software usage? **YES**

4. Proposed filming date: To help JoVE process and publish your video in a timely manner, please indicate the proposed date that your group will film here: **06/01/2026**

When you are ready to submit your video files, please contact our Content Manager, [Utkarsh Khare](#).

Current Protocol Length

Number of Steps: 19

Number of Shots: 35

Introduction

Videographer: Obtain headshots for all authors available at the filming location.

INTRODUCTION:

~~REQUIRED: Summarize your research focus and the main questions it aims to answer.~~

- 1.1. **Liisa Kübarsepp**: The study focuses on identifying optimal methods for high-throughput image analysis to measure various epidermal traits, most importantly stomatal traits.
 - 1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

~~Describe the research gap or limitation in existing methods and how this protocol addresses them.~~

- 1.2. **Liisa Kübarsepp**: Manual epidermal image analysis is very time-consuming and greatly limits the number of samples that can be included in studies.
 - 1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

~~Describe the research contexts or systems in which this protocol can be applied.~~

- 1.3. **Liisa Kübarsepp**: The approach we demonstrate can be applied in various contexts from climate-change research to plant breeding.
 - 1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

CONCLUSION:

~~What does this protocol allow researchers to study or measure?~~

- 1.4. **Mait Rungi**: The protocol provides a simple and efficient way to obtain epidermal impressions and to measure stomatal and epidermal traits from these images using AI-based tools.

- 1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera *Suggested B roll: 2.4.1.*

~~How can future studies build on this work? Describe the questions or research directions that can be investigated next.~~

- 1.5. **Liisa Kübarsepp**: This protocol can be used in any plant research topic that requires data on stomatal and epidermal traits.
 - 1.5.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

Videographer: Obtain headshots for all authors available at the filming location.

Protocol

2. Preparation of Stomata Impression Peels for Imaging

Demonstrator: Riccardo Scodeller

- 2.1. To begin, take cleaned leaves of *Fraxinus excelsior* and *Taraxacum officinale* [1]. Using a small brush, apply a thin, even layer of transparent, non-extendible nail polish to the central portion of the leaf target area [2].
 - 2.1.1. WIDE: Talent places the container containing the leaves on the working bench.
 - 2.1.2. Talent applying a thin, even layer of transparent nail polish to the central portion of a leaf with a small brush.
- 2.2. Allow the nail polish to dry completely, but avoid over-drying to prevent tissue discoloration [1].
 - 2.2.1. Shot of the coated leaf resting while the nail polish dries at room temperature.
- 2.3. Once dry, place a strip of transparent tape onto the coated area, starting at one edge of the nail polish and gradually laying it across the surface [1]. Gently press the tape to ensure good adhesion, avoid air bubbles or creases, and wear nitrile gloves to avoid fingerprints [2].
 - 2.3.1. Talent applying a strip of transparent tape onto the dried nail polish coating, starting at one edge and laying it across the leaf surface.
 - 2.3.2. Talent gently pressing the tape onto the coated area while wearing nitrile gloves.
- 2.4. Carefully peel away the tape together with the nail polish film to obtain the epidermal impression [1].
 - 2.4.1. Talent peeling the transparent tape away from the leaf together with the nail polish film.
- 2.5. Place the tape sticky side down onto a clean microscope slide [1]. Avoid folds or overlaps while mounting the tape on the slide [2]. Then image the slides under the compound microscope [3].
 - 2.5.1. Talent placing the tape sticky side down onto a clean microscope slide.
 - 2.5.2. Talent smoothing the tape onto the slide to avoid folds or overlaps.
 - 2.5.3. Talent places the slide under the microscope.

3. Leaf Clearing and Toluidine Blue O Staining Protocol for Microscopic Analysis

- 3.1. Transfer leaf pieces placed in the fixative solution into plastic vials and wash them three

times with distilled water [1]. Once the fixative solution is removed completely, immerse the samples in 1 molar sodium hydroxide solution [2].

3.1.1. Talent places the samples in plastic vials and adds water.

3.1.2. Talent removes water from the vials and adds the prepared sodium hydroxide solution.

3.2. During leaf clearing, observe the sodium hydroxide solution turning green [1]. Replace the sodium hydroxide solution once per day with a fresh solution [2] and continue this process for 3 to 4 days or until the samples turn pale yellow and the solution remains clear [2].

3.2.1. Shot of the sodium hydroxide solution turned green during leaf clearing.

3.2.2. Talent replacing the sodium hydroxide solution with fresh solution in the sample container.

3.2.3. Shot of the samples after sodium hydroxide clearing.

3.3. After removing the sodium hydroxide solution, cover the samples with 5 percent sodium hypochlorite bleach for 1 to 2 minutes [1]. Observe the samples turning white and translucent [2].

3.3.1. Talent covering the samples with bleach solution.

3.3.2. Shot of samples appearing white and translucent after bleach treatment.

3.4. Now, remove the previous solution and cover the samples with distilled water for 2 minutes [1]. Dehydrate the samples through a graded series of distilled water to ethanol of 75 to 25, 50 to 50, 25 to 75, and 100 percent ethanol [2].

3.4.1. Talent removing the bleach solution and adding distilled water to the samples.

3.4.2. Talent sequentially changing the solution in the samples with increasing ethanol concentrations.

3.5. Then, remove the ethanol [1], cover the samples with a 3 percent alcoholic solution of toluidine blue O, and leave overnight [2].

3.5.1. Talent removing ethanol from the samples.

3.5.2. Talent covering the samples with toluidine blue O staining solution.

3.6. Once the staining solution is removed, cover the samples with 100 percent ethanol for 2 minutes [1], then repeat using a graded series of distilled water to ethanol of 25 to 75, 50 to 50, 75 to 25, and finally distilled water [2]. Then, prepare a slide and examine under a compound light microscope at 400x magnification [3].

3.6.1. Talent covers the samples with 100 % ethanol.

3.6.2. Talent sequentially changing the solution in the samples with decreasing ethanol concentrations and final distilled water.

3.6.3. Talent places the prepared glass slide under the microscope.

3.7. Next, use Fiji ImageJ for image processing and analysis [1].

3.7.1. SCREEN: 69615_3.7.1.mp4: 00:08-00:12

3.8. Using the **Line tool**, draw a line over the scale bar [1]. Select **Analyze**, then choose Set Scale, and modify the known distance to match the length of the scale bar, and set the unit of length to micrometers [2].

3.8.1. SCREEN: 69615_3.8.mp4: 00:07-00:19, 00:53-01:00

3.8.2. SCREEN: 69615_3.8.mp4: 02:00-02:20

3.9. Go to **Analyze** and select **Set Measurements**. Ensure that the Area and Feret's diameter options are checked [1]. Using the Polygon Selection tool, outline the object of interest and press the M key to record the measurement [2].

3.9.1. SCREEN: 69615_3.9.1(until-0029).mp4: 00:06-00:21

3.9.2. SCREEN: 69615_3.9.2.mp4: 00:21-00:57

4. AI-Assisted Stomatal and Epidermal Segmentation Analysis

4.1. After preparing the images for machine learning, start StoManager1 (*Sto-Manager-One*) via the graphical user interface [1]. Choose the input directory containing leaf images [2].

4.1.1. SCREEN: 69615_4.1.mp4: 00:10-00:24

4.1.2. SCREEN: 69615_4.1.mp4: 00:29-00:38

4.2. For model selection, check the YOLOv8 (*Yolo-V-Eight*) segmentation extra-large model for segmentation-based detailed metrics, or leave it unchecked to use the YOLOv3 bounding box model with fewer metrics [1].

4.2.1. SCREEN: 69615_4.2.mp4: 00:25-00:30

4.3. Run segmentation by clicking **Start Process** to detect, segment, and measure stomata and whole stomata [1].

4.3.1. SCREEN: 69615_4.3.mp4: 00:14-00:34.

4.4. Collect the results saved to the specified output path [1]. Review the generated comma-separated value files containing per-object and summary metrics along with masks and result images [2].

4.4.1. SCREEN: 69615_4.4.mp4: 00:54-01:00

4.4.2. SCREEN: 69615_4.4.mp4: 01:44-02:08

4.5. Review outputs using Excel, R, or Python for further statistical analysis and visualization

[1].

4.5.1. SCREEN: 69615_4.5.mp4: 00:20-00:32.

Results

5. Results

- 5.1. Peeled samples provided sharper cell boundaries and clearer visualization of guard cell shape and pore aperture compared to cleared samples [1].
 - 5.1.1. LAB MEDIA: Figure 4. *Video editor: Show the panel labeled “Peeled” with clearly defined cell outlines*
- 5.2. In *Fraxinus excelsior*, average stomatal area and stomatal width were significantly higher in cleared samples than in peeled samples [1]. In *Taraxacum officinale*, stomatal area and stomatal width were not significantly different between cleared and peeled samples [2].
 - 5.2.1. LAB MEDIA: Figure 5A and B. *Video editor: Highlight the box for “Cleared” under F. excelsior showing higher stomatal area and width*
 - 5.2.2. LAB MEDIA: Figure 5A and B. *Video editor: Highlight both “Cleared” and “Peel” boxes under T. officinale showing similar stomatal area and stomatal width*
- 5.3. Stomatal density was significantly higher in peeled samples of *Fraxinus excelsior*, while no method effect was detected in *Taraxacum officinale* [1]. Epidermal cell area did not differ between preparation methods in either species [2].
 - 5.3.1. LAB MEDIA: Figure 5C. *Video editor: Highlight the box for “Peeled” under F. excelsior*
 - 5.3.2. LAB MEDIA: Figure 5D.
- 5.4. Epidermal cell density was slightly higher in peeled samples of *Fraxinus excelsior*, but the difference was not statistically significant [1].
 - 5.4.1. LAB MEDIA: Figure 5E. *Video editor: Highlight the “Peel” box under F. excelsior showing a slightly higher value*
- 5.5. The stomata-specific model detected stomata more reliably on peeled images compared to cleared images [1].
 - 5.5.1. LAB MEDIA: Figure 6 and 7. *Video editor: Show red detection boxes marking stomata*
- 5.6. In *Taraxacum officinale*, the model detected stomatal presence and density across preparation methods [1], but values differed significantly from manual measurements [2].
 - 5.6.1. LAB MEDIA: Figure 8. *Video editor: Highlight the T. officinale panel*
 - 5.6.2. LAB MEDIA: Table 2. *Video editor: Highlight the columns labelled “Man” and “AI”*
- 5.7. In *Fraxinus excelsior*, detection was limited to the abaxial surface and was inconsistent

in cleared samples with frequent false positives [1]

5.7.1. LAB MEDIA: Figure 8. *Video editor: Highlight the abaxial panel under F. excelsior*

5.8. Both tools produced numerous false positives, especially under low-contrast or blurred conditions where contours were indistinct [1].

5.8.1. LAB MEDIA: Figure 10.

1. Fraxinus excelsior
Pronunciation link: <https://www.howtopronounce.com/fraxinus-excelsior>
IPA: /'fræksɪnəs ɪk'selsɪ,ər/
Phonetic Spelling: FRAK·sɪh·nuhs ɪk·SEL·see·or
2. Taraxacum officinale
Pronunciation link: <https://www.howtopronounce.com/taraxacum-officinale>
IPA: /tə'ræksəkəm ə'fɪsə'næli/
Phonetic Spelling: tuh·RAK·suh·kuhm ə·fis·uh·NAL·ee
3. Non-extendible
Pronunciation link: No confirmed link found
IPA: /,nɑːn ɪk'stendəbəl/
Phonetic Spelling: naan·ɪk·STEN·duh·buhl
4. Nitrile
Pronunciation link: <https://www.merriam-webster.com/dictionary/nitrile>
IPA: /'naɪ,traɪl/
Phonetic Spelling: NYE·tryle
5. Epidermal
Pronunciation link: <https://www.merriam-webster.com/dictionary/epidermal>
IPA: /,ɛpə'dɜːməl/
Phonetic Spelling: ep·uh·DER·muhl
6. Toluidine
Pronunciation link: <https://www.merriam-webster.com/dictionary/toluidine>
IPA: /tə'luːə,diːn/
Phonetic Spelling: tuh·LOO·uh·deen
7. Fixative
Pronunciation link: <https://www.merriam-webster.com/dictionary/fixative>
IPA: /'fɪksətɪv/
Phonetic Spelling: FIK·suh·tiv

8. Molar
Pronunciation link: <https://www.merriam-webster.com/dictionary/molar>
IPA: /'moʊlər/
Phonetic Spelling: MOH·ler
9. Sodium hydroxide
Pronunciation link: <https://www.merriam-webster.com/dictionary/sodium%20hydroxide>
IPA: /'soʊdiəm haɪ'drɑːksaɪd/
Phonetic Spelling: SOH·dee·uhm hye·DRAAK·syde
10. Sodium hypochlorite
Pronunciation link: <https://www.merriam-webster.com/dictionary/sodium%20hypochlorite>
IPA: /'soʊdiəm ˌhaɪpə'klɔːraɪt/
Phonetic Spelling: SOH·dee·uhm hye·puh·KLOR·ite
11. Translucent
Pronunciation link: <https://www.merriam-webster.com/dictionary/translucent>
IPA: /trænz'luːsənt/
Phonetic Spelling: tranz·LOO·suhnt
12. Dehydrate
Pronunciation link: <https://www.merriam-webster.com/dictionary/dehydrate>
IPA: /di:'haɪ,dreɪt/
Phonetic Spelling: dee·HYE·drayt
13. Ethanol
Pronunciation link: <https://www.merriam-webster.com/dictionary/ethanol>
IPA: /'εθə,nɑːl/
Phonetic Spelling: ETH·uh·naal
14. Micrometers
Pronunciation link: <https://www.merriam-webster.com/dictionary/micrometer>
IPA: /maɪ'krɑːmɛtər/
Phonetic Spelling: my·KRAA·muh·ter
15. Feret's diameter
Pronunciation link: No confirmed link found
IPA: /fə'reɪz daɪ'æmɛtər/
Phonetic Spelling: fuh·RAYZ dye·AM·uh·ter
16. Polygon
Pronunciation link: <https://www.merriam-webster.com/dictionary/polygon>
IPA: /'pɑːli,gɑːn/
Phonetic Spelling: PAA·lee·gaan
17. Stomatal
Pronunciation link: <https://www.merriam-webster.com/dictionary/stomatal>
IPA: /'stoomətəl/
Phonetic Spelling: STOH·muh·tuhl
18. Epidermal
Pronunciation link: <https://www.merriam-webster.com/dictionary/epidermal>
IPA: /,εpə'dɜːməl/
Phonetic Spelling: ep·uh·DER·muhl

19. Segmentation
Pronunciation link: <https://www.merriam-webster.com/dictionary/segmentation>
IPA: /,sɛgmən'teɪʃən/
Phonetic Spelling: seg·muhn·TAY·shuhn
20. Graphical
Pronunciation link: <https://www.merriam-webster.com/dictionary/graphical>
IPA: /'græfɪkəl/
Phonetic Spelling: GRAF·ih·kuhl
21. Stomata
Pronunciation link: <https://www.merriam-webster.com/dictionary/stomata>
IPA: /'stoomətə/
Phonetic Spelling: STOH·muh·tuh
22. Visualization
Pronunciation link: <https://www.merriam-webster.com/dictionary/visualization>
IPA: /ˌvɪʒuələ'zeɪʃən/
Phonetic Spelling: vɪzh·oo·uh·luh·ZAY·shuhn
23. Aperture
Pronunciation link: <https://www.merriam-webster.com/dictionary/aperture>
IPA: /'æpərtʃər/
Phonetic Spelling: AP·er·cher
24. Abaxial
Pronunciation link: <https://www.merriam-webster.com/dictionary/abaxial>
IPA: /æb'æksɪəl/
Phonetic Spelling: ab·AK·see·uhl
25. Contours
Pronunciation link: <https://www.merriam-webster.com/dictionary/contour>
IPA: /'kɑ:n,tʊr/
Phonetic Spelling: KAHN·toor