Video Article

Whole-mount Clearing and Staining of *Arabidopsis* Flower Organs and Siliques

Afif Hedhly[1], Hannes Vogler[1], Christof Eichenberger[1], Ueli Grossniklaus[1]

[1]Department of Plant and Microbial Biology, Zurich-Basel Plant Science Center, University of Zurich

Correspondence to: Afif Hedhly at afif.hedhli@uzh.ch

URL: https://www.jove.com/video/56441
DOI: doi:10.3791/56441

Keywords: Developmental Biology, Issue 134, Herr's clearing, Hoyer's clearing, SDS clearing, Lugol's iodine staining, DAPI staining, aniline blue, ovule development, pollen development

Date Published: 4/13/2018

Citation: Hedhly, A., Vogler, H., Eichenberger, C., Grossniklaus, U. Whole-mount Clearing and Staining of *Arabidopsis* Flower Organs and Siliques. *J. Vis. Exp.* (134), e56441, doi:10.3791/56441 (2018).

Abstract

Due to its formidable tools for molecular genetic studies, *Arabidopsis thaliana* is one of the most prominent model species in plant biology and, especially, in plant reproductive biology. However, plant morphological, anatomical, and ultrastructural analyses traditionally involve time-consuming embedding and sectioning procedures for bright field, scanning, and electron microscopy. Recent progress in confocal fluorescence microscopy, state-of-the-art 3-D computer-aided microscopic analyses, and the continuous refinement of molecular techniques to be used on minimally processed whole-mount specimens, has led to an increased demand for developing efficient and minimal sample processing techniques. In this protocol, we describe techniques for properly dissecting Arabidopsis flowers and siliques, basic clearing techniques, and some staining procedures for whole-mount observations of reproductive structures.

Video Link

The video component of this article can be found at https://www.jove.com/video/56441/

Introduction

Flowers are among the most important defining organs of angiosperms. Flowering plants appeared some 90–130 million years ago[1], and diversified so fast that their rapid appearance was described as an "abominable mystery" by Charles Darwin[2]. The interests of plant researchers in flower development are diverse. Some research has focused on understanding the evolutionary origin of the flower as a whole, or the evolution of specific anatomical, structural, and functional properties of flowers[3,4,5,6]. The high variation in floral form and structure, as well as the modes of sexual and asexual reproduction relying on them, make the flower a highly complex structure. This has led to diverse efforts to characterize the anatomy and structural features of floral organs, using light and electron microscopical techniques that could be combined with genetic and molecular investigations[7]. Furthermore, as the source of fruits and seeds, flowers are of paramount importance for human and animal nutrition. Therefore, the characterization of flower and fruit development has many implications for applied research, including food security for an ever-increasing human population and ecological conservation strategies under a changing environment[8,9,10].

Flower development in *Arabidopsis* starts with flower induction and the transformation of the vegetative meristem to an inflorescence (group of flowers) meristem. Flower primordia are initiated laterally on the flank of the inflorescence meristem[11]. The floral organ primordia form progressively in concentric whorls from the outside to the center of the flower, and eventually develop into sepals, petals, stamens, and carpels[7]. These floral organs fulfill distinct nutritive, protective, and functional (*e.g.*, pollinator attraction) roles in different plant species, with the sexual organs sustaining the development of male and female gametophytes, respectively[12,13]. The gametophytes, in turn, each differentiate a pair of male (sperm) and female gametes (egg and central cell), which unite upon double fertilization to form the next generation, the zygote, and the primary endosperm, a terminal tissue supporting the development of the embryo[14,15]. Fruit and seed development support the growth, maturation, and preservation of the embryo and, eventually, its dispersal. Extensive research has been performed to characterize flower and embryo development in diverse plant species, especially in the model species *Arabidopsis*[7,16,17].

Early microscopic analyses of flower development were based on time-consuming sample processing and observation techniques, such as paraffin or resin embedding and sectioning, combined with light or electron microscopy. These traditional microscopic techniques were often used in combination with molecular genetic investigations, such as microscopical analyses of mutants, the localization of RNA by *in situ* hybridization, or the immuno-detection of proteins. Recent progress in wide-field and confocal fluorescence microscopy, in state-of-the-art 3-D computer-aided image analyses, and the continuous refinement of molecular methods that can be used on minimally processed whole-mount specimens, has led to a need for efficient and minimal sample processing techniques that are preferentially amenable to quantitative analyses. In recent years, significant progress has been made in developing clearing techniques on whole-mount animal specimen. They render the sample transparent either by using aqueous urea- or sugar-based reagents (*e.g.*, SCALE, SeeDB, CUBIC)[18,19,20], or by selectively removing lipids (using the detergent SDS) after embedding samples in stable hydrogels; the removal of lipids can be achieved either by passive diffusion (*e.g.*, modified

CLARITY protocol[21], PACT-PARS-RIMS[22]) or actively by electrophoresis (original CLARITY protocol[23] and ACT-PRESTO[24]). Encouraged by this fast progress, some derived techniques are also emerging for use in plants.

In this methods paper focused on the model *Arabidopsis*, we describe the procedure for the proper dissection of flower buds, flowers, and young siliques, and the clearing of whole-mount samples for diverse staining and observation procedures using classical or a recent SDS-based clearing method. Examples for starch, callose, and chromatin staining are given. Although these procedures may need further improvements and adaptations when used with other species, we hope they will set the stage for further research on these simple but critical methods that are the starting point of many research projects.

Protocol

1. Flower and Silique Fixation

1. Harvest flowers and siliques from plants synchronized at the opening of the first flower.
   NOTE: Under the experimental conditions used here, plants start flowering approximately 21 days after transplantation from Murashige and Skoog (MS) plates to soil. Seeds are stratified for 3–4 days at 4 °C and germinated/grown on MS plates at 22 °C/16 h light and 18 °C/8 h dark for 8 to 10 days, before transplanting the seedlings on nutrient-rich soil in pots kept under the same conditions (**Figure 1**). The number of replicates depends on the specific research objective, but a minimum of 5 inflorescences (from 5 individual plants) for each treatment is recommended. If flowers are the experimental unit, a minimum of 10 flowers per replicate is recommended.
2. Cut inflorescences or flowers (**Figure 1E-H**) using small scissors (*e.g.*, nail scissors) and place them immediately in a microcentrifuge tube (for single inflorescence/flower fixation) or a conical tube (for collective fixations) containing freshly made Carnoy's, methanol/acetic acid, or FPA50 fixatives (depending on the application, see below) on ice.
   NOTE: Samples should be completely submerged in the fixative.
3. Leave the tissue within the fixative for 4 h to overnight at 4 °C.
   NOTE: Vacuum infiltration can be used for speeding up the penetration of the fixative into the tissue, but this may be detrimental to preserving the structure of the tissue. The authors do not implement vacuum infiltration.
4. Remove the fixative, add enough 70% ethanol to cover the samples, and return them to 4 °C for at least 24 h; samples can stay indefinitely in this solution. After removing ethanol, proceed immediately to the next step; either dissection, or SDS clearing.

2. Dissection under the Stereomicroscope

1. Put freshly made 70% ethanol in a watch maker's glass placed on a small Petri dish for support.
2. **Place the inflorescence/flower/silique on this freshly made fixative and dissect under the stereomicroscope using forceps and a syringe with a needle.**
   1. Use a watch maker's glass or similar device that allows the samples to be totally covered with ethanol (recommended) for the dissection of inflorescences, and for tearing apart floral organs of mature flowers (sepals, petals, and stamen can be dissected at this stage) to avoid the risk of samples drying out.

3. **Dissect siliques and small flower buds on a slide as described below.**
   1. Put the watch maker's glass with pre-processed samples aside, and use a normal slide for the final dissection.
   2. Move the sample of interest to the slide and add 10 µL of fresh 70% ethanol. Work swiftly on the final dissection and add 10 µL of ethanol, if necessary, to keep the sample moist without excessive liquid.
   3. For releasing pollen grains from stamens, see step 5.1. For dissecting carpels and ovules, follow the procedure described in **Figure 2**. This procedure applies to all stages of development of the carpel, including the stage of development of green siliques.
      NOTE: Do not add ethanol if there is sufficient ethanol carry-over from moving the samples; dissecting very small samples is much easier in a minimal amount of liquid. Always keep only the organs of interest (sepals, petals, stamens, carpels, ovules, seeds, or pollen grains) on the slide. This procedure will ensure a uniform thickness between the slide and coverslip, corresponding to the thickness of the organ under examination, resulting in a higher homogeneity, and thus efficiency for microscopic examinations (higher number of target specimens in the same focal plane).

4. Use a small piece of blotting paper to absorb and remove as much ethanol as possible. Quickly proceed to the next step (section 3, 4, or 6) before the sample dries out.

3. Chloral Hydrate-Based Clearing and Combined Clearing-staining

NOTE: Best results for chloral hydrate-based clearing are obtained with FPA50 fixed material.

1. Place 20 µL of clearing solution (chloral hydrate/glycerol, modified Hoyer's medium, Herr's 4½, or Herr's IKI-4½ solutions) on the specimen-bearing slide. Using a pair of syringes with hypodermal needles, position the specimens as needed by reducing the space between them, and flipping those where the organ of interest faces downward. Remove any remaining air bubble using the needle.
2. Lower a coverslip sideways and gently place it, pressing very gently, and wait until the clearing solution fills the space in between. Add minimal clearing solution, if needed, to fill the entire space under the coverslip.
3. Place the slide on a slide holder and leave it under the fume hood. Proceed to microscopic observations after at least 4 h and during the following 4–5 days.

4. Combined Alexander Staining and Herr's 4½ Clearing of Anthers

NOTE: The original Alexander protocol is based on releasing pollen grains on the slide before staining. An efficient and more informative, modified Alexander staining and clearing procedure is the staining of mature pollen grains within mature but non-dehiscent anthers.

1. Choose freshly harvested flower buds that are about to open with non-dehiscent anthers.
   NOTE: Do not take younger flower buds because Alexander staining is intended for mature pollen grains and does not efficiently stain immature pollen grains. A minimum of 5 flowers per treatment harvested from different plants and inflorescences is recommended.
2. Prepare a 96-well plate with enough Alexander's solution to submerge a flower bud. Expose the anthers by removing the sepals and petals and immerse them in Alexander's solution. Keep the samples under the fume hood for 1–3 h.
3. Replace Alexander's solution with Herr's 4½ solution and place the multi-well plate back under the fume hood for an overnight incubation.
4. Using forceps, gently move the cleared flower buds onto a new slide. Since some carry-over of Alexander's solution may occur, use a blotting paper to remove as much clearing solution as possible.
5. Dissect the stamens and remove all other tissues. Follow the steps in section 3 using Herr's 4½ solution.

5. Removing the Exine from Pollen Grains

NOTE: We recommend using Carnoy's fixative for DAPI staining.

1. Follow the fixation and dissection procedures described in sections 1 and 2. By now, one should have one to many stamens on the slide within a minimum amount of 70% ethanol.
2. Use a blotting paper to remove excess ethanol, and add 5–10 µL of DAPI solution. Using a couple of syringes with needles, release the pollen grains within that small amount of solution (*e.g.*, use one syringe to immobilize the stamen and the other to release the pollen grains). Add another 5 µL of DAPI if the solution dries out.
3. Removing all debris of the stamen is a critical step, such that only pollen grains are left between the slide and the coverslip.
4. Place a coverslip on the specimen-bearing slide by lowering it sideways and add the minimal amount of DAPI solution required to fill the space. Place an index finger on the coverslip, and without squashing too much make a quick, firm, and short sliding movement.
   NOTE: To gauge the necessary force and amplitude of the sliding movement, this step should be practiced several times, checking the results each time under the microscope.
5. Add DAPI solution if needed and place the slide in a humid box in the dark at 4 °C for 15 min to 1 h. Proceed to observe samples under wide-field fluorescence or confocal microscopy within 24 h. Try to combine this procedure with SDS clearing to check whether further improvements can be obtained.

6. Sodium Dodecyl Sulfate (SDS) Clearing

NOTE: Depending on the floral organ to be analyzed, and on the researcher's skills to dissect very soft and small specimens, the SDS treatment can be carried out either before (for experienced researchers) or after the dissection step (for less experienced researchers) on methanol-acetic acid fixed material.

1. Use a watch maker's glass, a concave slide, or a microcentrifuge tube to incubate dissected or non-dissected samples in 1% SDS and 0.2 N NaOH solution overnight at room temperature.
2. Handle with care because the sample is very soft and has a transparent appearance. Rinse several times with water.
   NOTE: Remnants of the SDS solution might lead to precipitation when adding the staining solution, *e.g.*, aniline blue.
3. For non-dissected samples, follow the dissection procedure described in section 2, using water instead of 70% ethanol. After dissecting the desired tissue, remove any remnants and debris, and any excess water with a blotting paper, then add 20–40 µL of staining solution, and proceed with step 6.5.
4. For dissected samples, carefully move the sample from the washing solution and place it onto a clean slide, and add 20–40 µL of staining solution.
5. Gently place a coverslip on the slide by lowering it sideways. Take care not to squash the preparation. If the tissue is too soft, place any thin separator in between the slide and the coverslip at its corners (*e.g.*, tape or a broken coverslip), leaving a chamber-like space between slide and coverslip, such that the coverslip doesn't crush the specimen.
6. Place all the slides within a humid box, cover it with aluminum foil, and place it at 4 °C for at least 1 h. Proceed to observe the specimen using widefield fluorescence or confocal microscopy within 24 h.

Representative Results

Arabidopsis belongs to the Brassicacea family, bearing inflorescences with bisexual flowers arranged in a corymb (**Figure 1**). Each flower has four sepals, four petals, six stamens (four long and two short), and a syncarpous gynoecium consisting of two congenitally fused carpels (**Figure 1F-H**) arranged in four concentric whorls[25,26]. *Arabidopsis* has tenuinucellate, anatropous ovules arranged in parietal placentation in two rows (12-20 ovules in each row) within each of the two locules of the ovary (a total of 45–80 ovules) (unpublished data, and references[27,28,29,30]). In order to undertake robust comparative analyses of flower and embryo development within an individual plant or between different plants (as replicates or treatments), it is advisable to use the very first open flower as a reference (**Figure 1B**). At this stage, the inflorescence (**Figure 1E**) usually has between 20 and 30 flower buds spanning all stages of flower development, and at roughly similar stages in other individuals (unpublished data). More flower meristems (forming between 5 and 20 flowers) will be initiated by the inflorescence meristem after the first flower opened. The first two flowers might show fertility defects and, hence, should not be included in the analysis.

Clearing *Arabidopsis* flowers and floral organs using chloral hydrate-based clearing solutions[31,32], including Herr's 4½ clearing solution[33], is commonly executed for differential interference contrast (DIC) microscopic analyses of flower, silique, and embryo development (**Figure**

3A-J). The 4½ solution is specifically recommended for analyzing stamen development, and for organs requiring stronger clearing capacity. Some chloral hydrate-based solutions are used for the double purpose of clearing and staining simultaneously; this is the case for the original Alexander's[34] and Herr's IKI-4½[35,36] solutions. Alexander staining is widely used in Arabidopsis to evaluate pollen abortion. A modified and more informative Alexander staining procedure[37] is to stain whole anthers containing mature pollen grains. This procedure requires a posterior clearing of the anthers using Herr's 4½ solution (**Figure 3K-L**). Other staining techniques used in different plant species might provide a better estimation of pollen viability (*e.g.*, the fluorochromatic reaction using fluorescein diacetate, staining for callose, starch, DNA, and RNA, and enzymatic tests using redox dyes)[38,39,40]. However, most of these techniques evaluate a specific aspect of the pollen grain that might not necessarily correlate with pollen viability and, importantly, pollen fertility. An evaluation of pollen germination ability, and its ability to effect fertilization and set seeds might be more accurate[38-40]. A much less used clearing-staining procedure is Herr's IKI-4½, which combines clearing and starch-staining solutions. Besides staining for starch (**Figure 4**), this procedure can be used for general clearing purposes whenever higher contrast is needed (*e.g.*, **Figure 3A, E**).

A combination of auramine and calcofluor is used in many plant species[41] to simultaneously stain the pollen exine and intine (**Figure 5A**). Most of these and other chemical dyes do not stain a single cellular component and, hence, should be used in combination with more direct labeling techniques such as GUS or fluorescently-tagged marker lines, antibodies, or *in situ* hybridization. They might also show multiple excitation and emission spectra. DAPI, in contrast, is more specific, and is usually used to stain chromatin and chromosome spreads[42] during meiosis, and to follow pollen development[43], apart from its manifold applications as a counterstain for the nucleus. Here, we show how the staining intensity can be increased to obtain a more detailed view of the chromatin of sperm and vegetative nuclei by mechanically removing the exine (**Figure 5B-C**). Although this technique is very helpful for those interested in performing detailed analyses of chromatin, chromosomes, and nuclear structure, the mechanical removal of the exine might affect the organization of the pollen grain, such that results should be interpreted with care.

Most of these fluorescent dyes are used without prior clearing of the tissue; but in some cases, they are compatible with a posterior chloral hydrate-based clearing on the slide[44]. SDS clearing has recently been used as clearing reagent in animal and plant research and has been shown to be compatible with different staining procedures for widefield fluorescence and confocal microscopy (mPS-PI[45] in plants, and Clarity[23], PACT[22] or ACT-PRESTO[24] in animals). Using an SDS-NaOH clearing prior to aniline blue staining (**Figure 5D-H**), we managed to attain higher accessibility to internal tissues as well as a better staining for callose within floral tissues (**Figure 5I-L**). Similar results were obtained for calcofluor (data not shown).

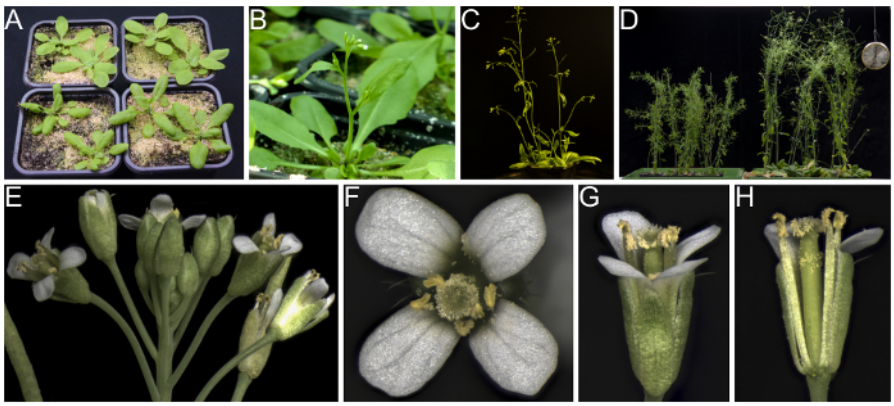

Figure 1: Photographs of *Arabidopsis* plants and flowers. (**A-D**) Arabidopsis plants at different stages of the life cycle: rosette before bolting (**A**), at first flower opening (**B**), with primary and a few secondary inflorescences (**C**), and at the end of flowering (**D**). (**E**) The typical corymb of an Arabidopsis inflorescence with flowers at different developmental stages, maturing acropetally. (**F-H**) An *Arabidopsis* flower at anthesis, illustrating self-pollination (stamen touching the stigma and releasing pollen grains). **Figures E-H** are focus stacks of 36, 53, 42, and 32 pictures, respectively, assembled using a software tool (*e.g.*, Helicon Focus). **F-H** represent the same flower where one sepal, two petals, and a short stamen were removed in **H**. Please click here to view a larger version of this figure.

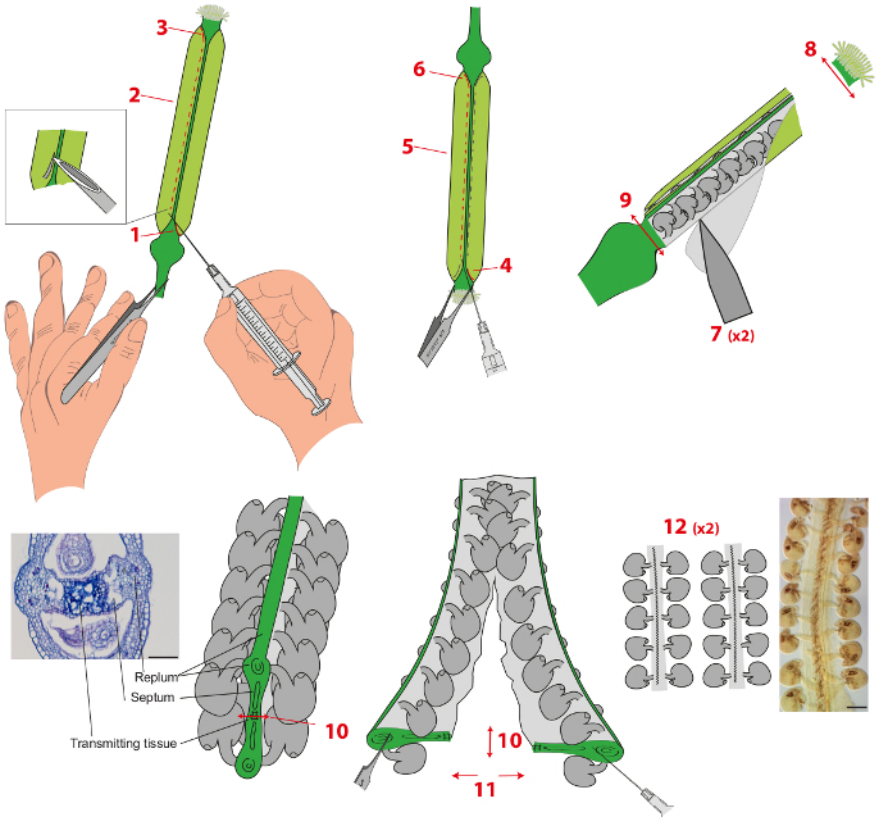

Figure 2: How to dissect a gynoecium and ovules. (**1-3**) Place the gynoecium on a slide with a minimal amount of 70% ethanol and position hands as depicted in the drawing. Make cuts 1-3 following the directions indicated by the red arrows. Use the needle with the opening facing upward and away from the ovules for cuts 2-3. (**4-6**) Rotate the carpel 180 degrees as shown, and make cuts 4-6 like the first three cuts. (**7**) Removing the valves: use the needle with the opening facing downward, place it below the ovules, and slide the needle quickly to the right along the valve margin. For bigger carpels (anthesis and onward), or with some dissection expertise, one can substitute this step by repeating steps 2 and 5 on the other side of the carpel. (**8-9**) Remove the stigma-style and the peduncle with sharp cuts using the needle with its opening sideways and away from the ovules. (**10**) Make a sharp small cut at the transmitting tissue level at one of the two extremes. (**11**) Use the forceps and the needle to tear apart the two halves. (**12**) Using the forceps and the needle, gently flip one row of ovules to obtain two parallel rows. Alternatively, place the two rows vertically with the replum lying above and push gently downward along the replum to spread the two ovule rows on either side. Two flattened but still attached rows of ovules after clearing and staining with Herr's IKI-4½ solution are shown as an illustration of the result of the dissection. Scale bars = 80 µm Please click here to view a larger version of this figure.

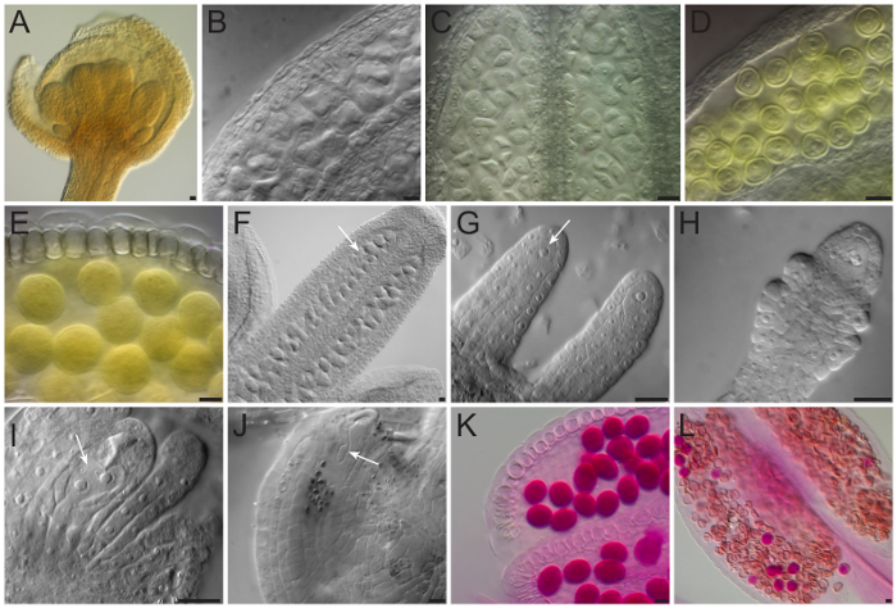

Figure 3: Chloral hydrate-based clearing with or without staining during flower development. (**A**) A young flower bud. (**B-E**) Stamen development with microspore mother cells in cytokinesis (**B**), tetrads of microspores (**C**), pollen mitosis I (**D**), and tricellular pollen grains (**E**). (**F-J**) Gynoecium containing ovules with megaspore mother cells (arrow) prior to meiosis (**F** and **G**) and during meiotic prophase I (**H**), with a binuclear embryo sac (arrow) (**I**), and at fertilization (**J**). Note the trace of a pollen tube (arrow) within the micropylar region of the ovule in **J**. (**K-L**) Modified Alexander's staining of anthers with full (**K**) or reduced pollen viability due to heat stress (**L**). Note the empty cytoplasm and irregular shapes of aborted pollen grains within the anther locules in *L. Arabidopsis* wild-type Col-0 accession. Clearing or staining: Herr's 4½ for **B-D** and **F-J**, Herr's IKI-4½ for **A** and **E**, and modified Alexander staining for **K-L**. Scale bars = 10 µm. Please click here to view a larger version of this figure.

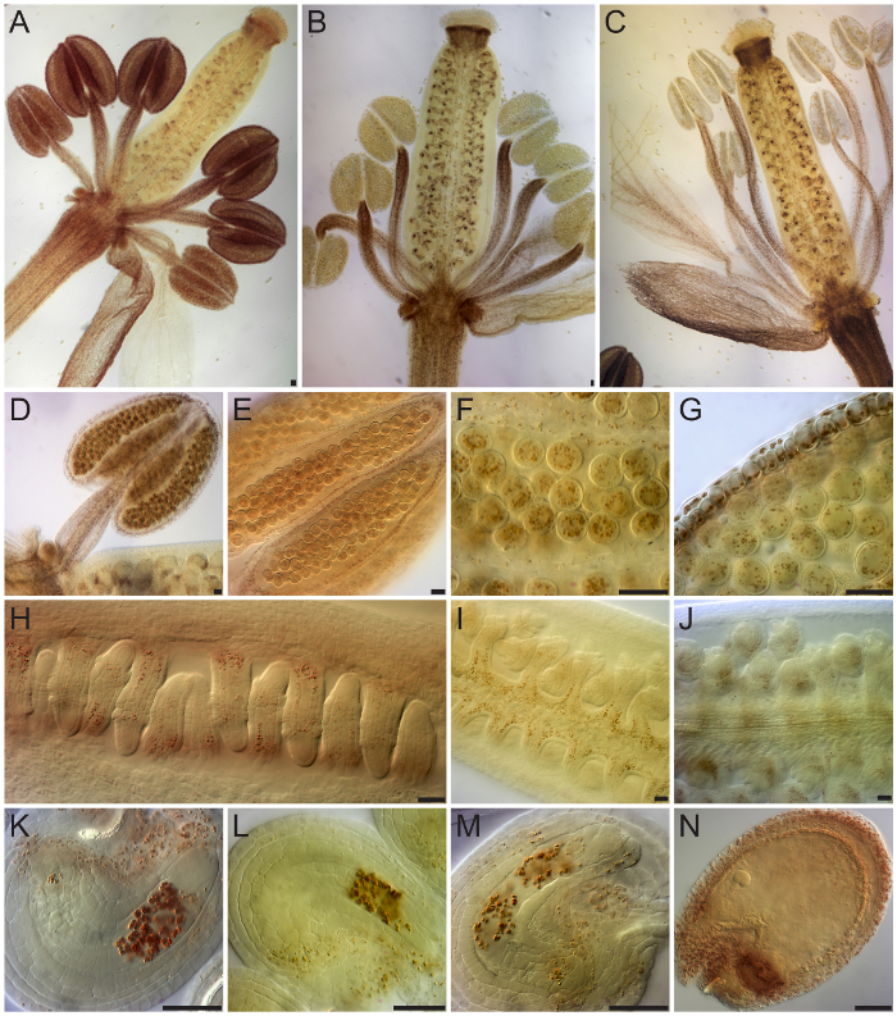

Figure 4: Starch dynamics during flower and early seed development. Representative results illustrating the dynamic starch turnover during flower development. (**A-C**) The third wave of starch amylogenesis/amylolysis during the last stages of stamen development as recently described[39]. (**D-G**) Representative results of the unique starch wave in the pollen grain with a peak of amylogenesis at the bicellular stage. (**H-N**) Representative results of starch dynamics in ovules at the megaspore mother cell stage (**H**), in developing female gametophytes (**I-K**), and during early seed development (just after fertilization in **L**, binucleate endosperm in **M**, and octant embryo stage in **N**). Arabidopsis wild-type Col-0 accession. Scale bars = 20 µm. Please click here to view a larger version of this figure.

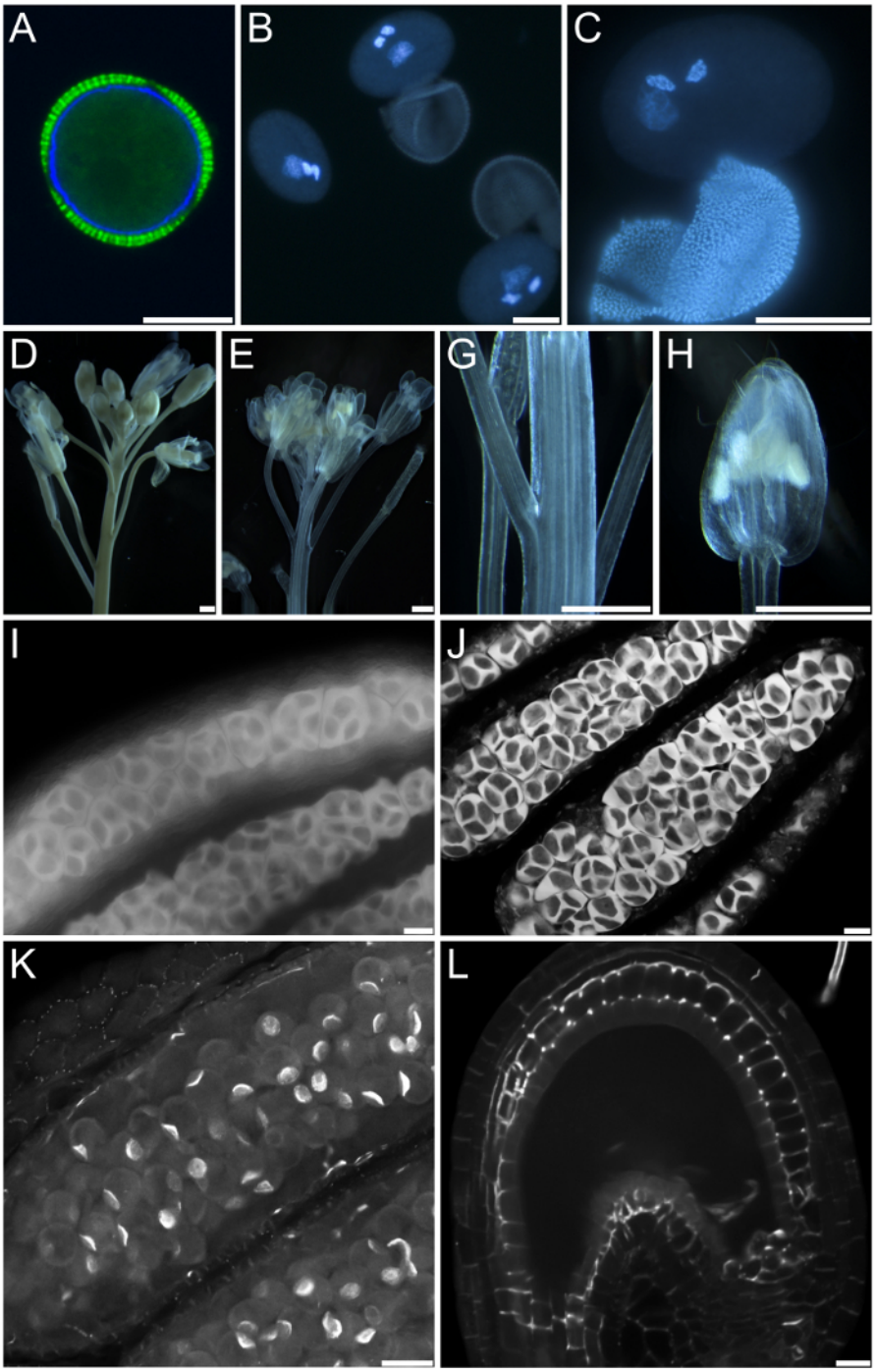

Figure 5: Some classical staining procedures for fluorescence microscopy and the effect of SDS-clearing. (**A**) Calcofluor-Auramine staining during pollen maturation where the intine is stained blue (calcofluor) and the exine intensely green (Auramine). (**B-C**) Mechanical removal of the exine and DAPI staining of tricellular pollen grains. (**D-H**) Representative pictures of an inflorescence before (**D**, as recovered from fixative) and after SDS clearing (**E-H**). Note the transparency of the tissue that allows the observation of the vasculature within the inflorescence stem (**G**) and the flower bud peduncle (**H**). (**I-J**) Aniline blue staining for callose in microspores at the tetrad stage within the stamen. Compare the clarity and the intensity of the staining between the non-SDS cleared anther (**I**) and the SDS-cleared anther (**J**). (**K**) An SDS-cleared and aniline blue stained anther with microspores during pollen mitosis I. Note the synchrony of many pollen grains at this stage in these two locules. (**L**) An SDS-cleared and aniline blue stained ovule after fertilization. Note callose staining in the remains of the pollen tube and in the seed coat. *Arabidopsis* wild-type Col-0 accession. Scale bar = 20 µm (A-C, I-L); 1 mm (D-H). Please click here to view a larger version of this figure.

Discussion

The existence of many flower buds within a single inflorescence of *Arabidopsis*, spanning all flower developmental stages, offers a unique opportunity for studies aimed at characterizing an effect of a treatment or a developmental feature simultaneously across the different stages of flower development. A good reference point between different individual plants is the opening of the first flower of the main inflorescence. Plants are treated in such a way that flowering is synchronized as much as possible (*e.g.*, 3–4 days stratification at 4 °C maximizes synchronous seed germination and hence more even flowering), and only plants that open their first flower on the same day are taken as a batch to distribute between treatments and replicates; batches ready on successive days can be used to replicate the experiment. Following this procedure, more robust comparative analyses, controlling for environmental and developmental variations within and between plants, can be performed.

Combining Lugol's stain with Herr's clearing solution allowed us not only to improve the contrast in cleared samples compared to classical clearing solutions, but also to characterize starch dynamics during flower and embryo development, as recently reported for *Arabidopsis*[46]. Using this simple procedure, we were able to unravel the existence of a new wave of starch amylogenesis-amylolysis during anther development, and to correlate starch dynamics with global changes in the expression of genes encoding proteins involved in sugar and starch metabolism. This analysis showed that GPT6 is the translocator of sugar-phosphate from the cytosol to amyloplasts for starch synthesis. The detailed results on starch dynamics in various tissues opens the possibility of characterizing other important genes in starch metabolism, and to address unresolved questions concerning less studied but important stages of plant development. Iodine-based starch staining is not quantitative, and should be complemented with other methods[46].

Research looking for improved clearing procedures that are compatible with fluorescence microscopy has intensified over the last years, especially in the animal field. Although the plant research field is lagging behind, and clearing largely relies on traditional clearing solutions for DIC analysis (*e.g.*, chloral hydrate-based solutions, benzyl benzoate, dibutyl phthalate, and methyl salicylate)[31,33,47,48], encouraged by the fast progress made in the animal field, some similar techniques are emerging using urea- (*e.g.*, ClearSee[49] and others[50]), and 2,2'-thiodiethanol-based clearing procedures[51,52]. Here, we show that the use of the SDS detergent in combination with NaOH renders the tissue transparent and improves some classical fluorescence staining procedures, such as aniline blue for callose and calcofluor for cellulose. Based on these promising results, similar progress in the SDS-based selective removal of lipids can be expected in the plant field in the near future. Since plant cells have rigid cell walls that are absent in animal cells, the prior embedding in hydrogels might not be necessary, as is the case for animal tissues. Other fluorescent dyes and fluorophores used in genetic marker lines might be assayed using this clearing procedure.

Disclosures

The authors have nothing to disclose.

Acknowledgements

This work was supported by the University of Zurich, an IEF Marie Curie Grant (grant no. TransEpigen-254797 to A.H.), an Advanced Grant of the European Research Council (grant no. MEDEA-250358 to U.G.), and a Research and Technology Development project (grant MecanX to U.G.) from SystemsX.ch, the Swiss Initiative in Systems Biology.

References

1. Crane, P.R., Friis, E.M., Pedersen, K.R. The origin and early diversification of angiosperms. *Nature.* **374** (6517), 27-33, (1995).
2. Friedman, W.E. The meaning of Darwin's 'abominable mystery'. *Am J Bot.* **96** (1), 5-21, (2009).
3. Sun, G., Dilcher, D.L., Zheng, S., Zhou, Z. In search of the first flower: A Jurassic angiosperm, *Archaefructus*, from Northeast China. *Science.* **282** (5394), 1692-1695, (1998).
4. Mulcahy, D.L. The rise of the angiosperms: a genecological factor. *Science.* **206** (4414), 20-23, (1979).
5. Friedman, W.E., Moore, R.C., Purugganan, M.D. The evolution of plant development. *Am J Bot.* **91** (10), 1726-1741, (2004).
6. Endress, P.K. Angiosperm floral evolution: morphological developmental framework. In Advances in botanical research Volume 44: *Developmental genetics of the flower.* (eds Soltis, D., Soltis, P., Leebens-Mack, J.), pp 1-61. Academic Press, (2006).
7. Alvarez-Buylla, E.R. *et al.* Flower development. *Arabidopsis Book.* **8** e0127, (2010).
8. Beddington, J. Food security: contributions from science to a new and greener revolution. *Philos Trans R Soc Lond B Biol Sci.* **365** (1537), 61-71, (2010).
9. Godfray, H.C. *et al.* Food security: The challenge of feeding 9 billion people. *Science.* **327** (5967), 812-818, (2010).
10. Burkle, L.A., Alarcón, R. The future of plant-pollinator diversity: understanding interaction networks across time, space, and global change. *Am J Bot.* **98** (3), 528-538, (2011).
11. Meyerowitz, E.M. *et al.* A genetic and molecular model for flower development in *Arabidopsis thaliana. Dev Suppl.* **1** 157-167, (1991).
12. Hafidh, S., Fíla, J., Honys, D. Male gametophyte development and function in angiosperms: a general concept. *Plant Reprod.* **29** (1-2), 31-51, (2016).
13. Schmidt, A., Schmid, M.W., Grossniklaus, U. Plant germline formation: common concepts and developmental flexibility in sexual and asexual reproduction. *Development.* **142** (2), 229-241, (2015).
14. Sprunck, S., Dresselhaus, T. Three cell fusions during double fertilization. *Cell.* **161** (4), 708-709, (2015).
15. Dresselhaus, T., Sprunck, S., Wessel, G.M. Fertilization mechanisms in flowering plants. *Curr Biol.* **26** (3), R125-39, (2016).
16. Smyth, D.R., Bowman, J.L., Meyerowitz, E.M. Early flower development in Arabidopsis. *Plant Cell.* **2** (8), 755-767, (1990).
17. Roeder, A.H., Yanofsky, M.F. Fruit development in *Arabidopsis. Arabidopsis Book.* **4** e0075, (2006).

18. Hama, H. *et al.* Scale, a chemical approach for fluorescence imaging and reconstruction of transparent mouse brain. *Nat Neurosci.* **14** (11), 1481-1488, (2011).
19. Ke, M.-T., Fujimoto, S., Imai, T. SeeDB. a simple and morphology-preserving optical clearing agent for neuronal circuit reconstruction. *Nat Neurosci.* **16** (8), 1154-1161, (2013).
20. Susaki, E.A. *et al.* Whole-brain imaging with single-cell resolution using chemical cocktails and computational analysis. *Cell.* **157** (3), 726-739, (2014).
21. Tomer, R., Ye, L., Hsueh, B., Deisseroth, K. Advanced CLARITY for rapid and high-resolution imaging of intact tissues. *Nat Protoc.* **9** (7), 1682-1697 (2014).
22. Yang, B. *et al.* Single-cell phenotyping within transparent intact tissue through whole-body clearing. *Cell.* **158** (4), 945-958, (2014).
23. Chung, K., Deisseroth, K. CLARITY for mapping the nervous system. *Nat Methods.* **10** (6), 508-513, (2013).
24. Lee, E. *et al.* ACT-PRESTO: rapid and consistent tissue clearing and labeling method for 3-dimensional (3D) imaging. *Sci Rep.* **6**, 18631, (2016).
25. Endress, P.K. Evolution and floral diversity: the phylogenetic surroundings of *Arabidopsis* and *Antirrhinum. Int J Plant Sci.* **153** (3, Part 2), S106-S122, (1992).
26. Reyes-Olalde, J.I., Zuñiga-Mayo, V.M., Chávez Montes, R.A., Marsch-Martínez, N., de Folter, S. Inside the gynoecium: at the carpel margin. *Trends Plant Sci.* **18** (11), 644-655, (2013).
27. Hill, J.P., Lord, E.M. Floral development in *Arabidopsis thaliana*: a comparison of the wild type and the homeotic *pistillata* mutant. *Can J Bot.* **67** (10), 2922-2936, (1989).
28. Sessions, R.A., Zambryski, P.C. *Arabidopsis* gynoecium structure in the wild type and in *ettin* mutants. *Development.* **121** (5), 1519-1532, (1995).
29. Schneitz, K., Hülskamp, M., Pruitt, R.E. Wild-type ovule development in *Arabidopsis thaliana*: a light microscope study of cleared whole-mount tissue. *Plant J.* **7** (5), 731-749, (1995).
30. Sundberg, E., Ferrándiz, C. Gynoecium patterning in *Arabidopsis*: a basic plan behind a complex structure. In Annual Plant Reviews Volume 38: *Fruit Development and Seed Dispersal.* (ed Østergaard, L.), pp 35-69. Wiley-Blackwell, Oxford, UK, (2009).
31. Meinke, D.W. 10 seed development in *Arabidopsis thaliana. Cold Spring Harbor Monograph Archive.* **27** 253-295, (1994).
32. Weigel, D., Glazebrook, J. *Arabidopsis: a laboratory manual.* Cold Spring Harbour Laboratory Press, Cold Spring Harbour, N.Y., (2002).
33. Herr, J.M. A new clearing-squash technique for the study of ovule development in angiosperms. *Am J Bot.* **58** (8), 785-790, (1971).
34. Alexander, M.P. A versatile stain for pollen fungi, yeast and bacteria. *Stain Technol.* **55** (1), 13-18, (1980).
35. Herr, J.M. Applications of a new clearing technique for the investigation of vascular plant morphology. *J Elisha Mitchell Sci Soc Chapel Hill N C.* **88** (3), 137-143, (1972).
36. Herr, J.M. Clearing techniques for the study of vascular plant tissues in whole structures and thick sections. In *Tested studies for laboratory teaching.* Volume 5 (eds Goldman, C.A., Hauta, P.L., O'Donnell, M.A., Andrews, S.E., van der Heiden, R.), Toronto, Ontario, Canada, Proceedings of the Fifth Workshop/Conference of the Association for Biology Laboratory Education (ABLE), pp 63-84, (1993).
37. Colcombet, J., Boisson-Dernier, A., Ros-Palau, R., Vera, C.E., Schroeder, J.I. Arabidopsis somatic embryogenesis receptor kinases1 and 2 are essential for tapetum development and microspore maturation. *Plant Cell.* **17** (12), 3350-3361, (2005).
38. Heslop-Harrison, J., Heslop-Harrison, Y., Shivanna, K.R. The evaluation of pollen quality and a further appraisal of the fluorochromatic (FCR) test procedure. *Theor Appl Genet.* **67** (4), 367-379, (1984).
39. Stone, J.L., Thomson, J.D., Dent-Acosta, S.J. Assessment of pollen viability in handpollination experiments: A review. *Amer J Bot.* **82** (9), 1186-1197, (1995).
40. Dafni, A., Firmage, D. Pollen viability and longevity: practical, ecological and evolutionary implications. *Plant Syst Evol.* **222** (1-4), 113-132, (2000).
41. Lora, J., Testillano, P.S., Risueño, M.C., Hormaza, J.I., Herrero, M. Pollen development in *Annona cherimola* Mill. (Annonaceae). Implications for the evolution of aggregated pollen. *BMC Plant Biol.* **9** 129, (2009).
42. Ross, K.J., Fransz, P., Jones, G.H. A light microscopic atlas of meiosis in *Arabidopsis thaliana. Chromosome Res.* **4** (7), 507-516, (1996).
43. Park, S.K., Howden, R., Twell, D. The *Arabidopsis thaliana* gametophytic mutation *gemini pollen1* disrupts microspore polarity, division asymmetry and pollen cell fate. *Development.* **125** (19), 3789-3799, (1998).
44. Haseloff, J. Old botanical techniques for new microscopes. *BioTechniques.* **34** (6), 1174-1182, (2003).
45. Truernit, E. *et al.* High-resolution whole-mount imaging of three-dimensional tissue organization and gene expression enables the study of phloem development and structure in *Arabidopsis. Plant Cell.* **20** (6), 1494-1503, (2008).
46. Hedhly, A. *et al.* Starch turnover and metabolism during flower and early embryo development. *Plant Physiol.* **172** (4), 2388-2402, (2016).
47. Crane, C.F., Carman, J.G. Mechanisms of apomixis in *Elymus rectisetus* from eastern Australia and New Zealand. *Am J Bot.* **74** (4), 477-496, (1987).
48. Young, B.A., Sherwood, R.T., Bashaw, E.C. Cleared-pistil and thick-sectioning techniques for detecting aposporous apomixis in grasses. *Can J Bot.* **57** (15), 1668 1672, (1979).
49. Kurihara, D., Mizuta, Y., Sato, Y., Higashiyama, T. ClearSee: a rapid optical clearing reagent for whole-plant fluorescence imaging. *Development.* **142**, 4168-4179, (2015).
50. Warner, C.A. *et al.* An optical clearing technique for plant tissues allowing deep imaging and compatible with Fluorescence Microscopy. *Plant Phys.* **166**, 1684-1687, (2014).
51. Hasegawa, J., Sakamoto, Y., Nakagami, S., Aida, M., Sawa, S., Matsunaga, S. Three-dimensional imaging of plant organs using a simple and rapid transparency technique. *Plant Cell Physiol.* **57** (3), 462- 72, (2016).
52. Musielak, T.J., Slane, D., Liebig, C., Bayer, M. A Versatile Optical Clearing Protocol for Deep Tissue Imaging of Fluorescent Proteins in Arabidopsis thaliana. *PLoS ONE.* **11** (8), e0161107, (2016).

