Methods in Palynology

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Preparation of Recent and Fossil Material for LM, SEM, and TEM

Multiple methods and techniques should be used when investigating pollen grains in order to provide comprehensive and accurate information about pollen morphology and ultrastructure (see also "Misinterpretations in Palynology"). The preparation methods used depend on the material to be studied, if the pollen grains are to be obtained from recent flower material (herbarium sheets, newly collected) or from various sedimentary rocks, sediments or soils (fossil to subfossil pollen). Recent and fossil pollen grains are easily studied using both LM and SEM, but recent pollen grains are also more often studied using TEM.

For an accurate description of any taxonomic value, it is important to study pollen grains in both LM and SEM. The LM will provide, among others, information on the endoaperture that cannot be obtained using SEM. Likewise the SEM will provide detailed information on the sculpture of the pollen grain that is not visible under the low magnification provided by the LM. For example, terms with "micro-" (like microreticulate) or "nano-" (like nanoechinate) can only be observed using SEM (Fig. 1).

Annotation: The methods described in this section are the standard palynological techniques applied by the authors of this book and may differ in other working groups/labs around the world. All LM, SEM, and TEM micrographs in this book are produced following these standard protocols. Recipes for preparations are included at the end of this section.

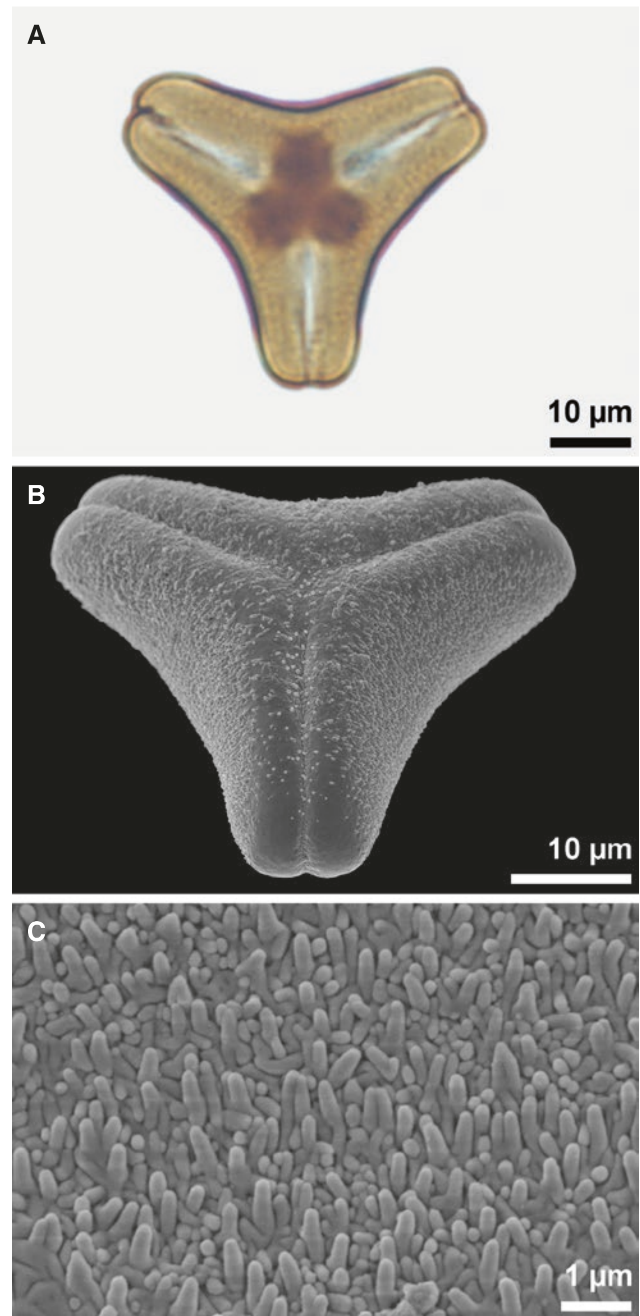


Fig. 1 LM vs. SEM. A-C. *Aetanthus coriaceus*, Loranthaceae. **A**. Pollen grain looks psilate or scabrate in LM. **B**. Sculpture elements become visible under SEM. **C**. The sculpture elements are nano- to microbaculate and only identifiable using high magnification

Light Microscopy

Pollen Hydration Status at Dispersal

To clarify the dehydration status of pollen grains at anthesis, pollen must be collected from newly opened anthers (Fig. 2). Fresh pollen grains are transferred immediately into a drop of pure glycerine and should be observed as soon as possible, as pollen grains expand in glycerine (within days or

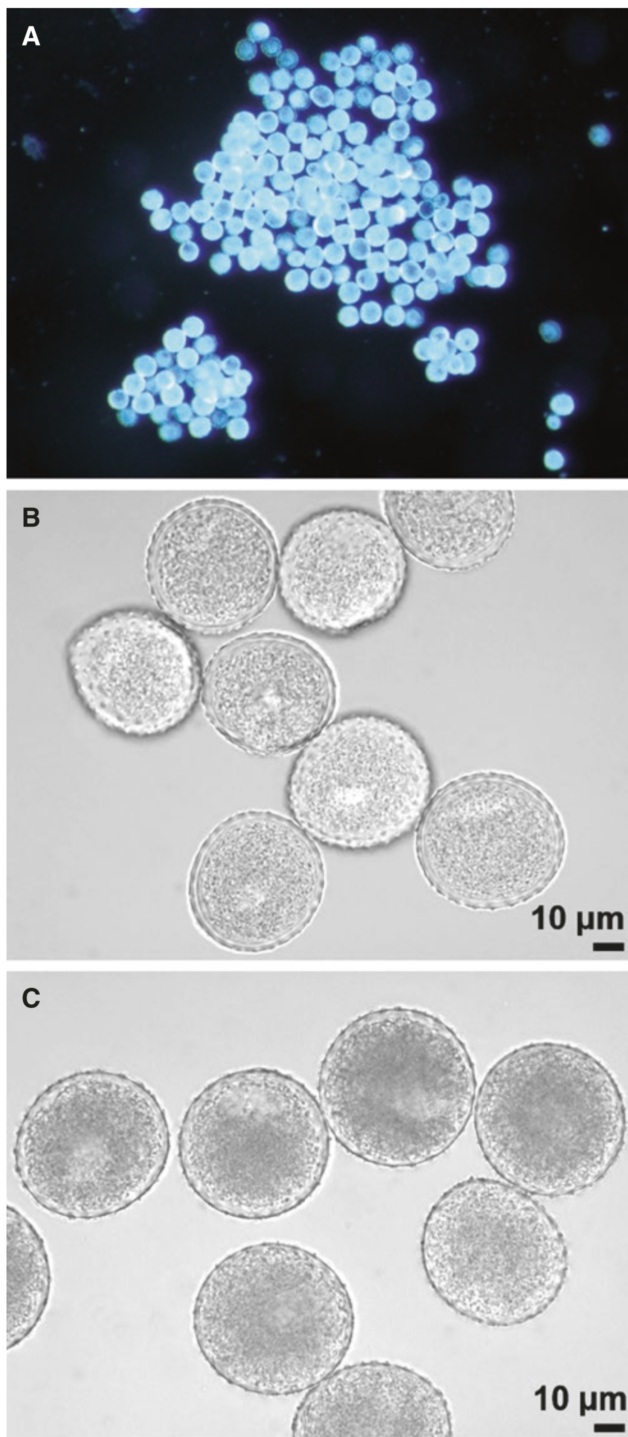


Fig. 2 Pollen hydration status at dispersal. **A-C**. *Alocasia* sp., Araceae. **A**. Pollen grains fully hydrated at anthesis, binocular microscope. **B**. Pollen in glycerine, LM. **C**. Pollen hydrated in water, LM

weeks). The water content of pollen grains at the time of dispersal varies and pollen can be fully hydrated, partially hydrated, or partially dehydrated (Heslop-Harrison 1979; Nepi et al. 2001; see also **harmomegathic effect** in "Pollen Morphology and Ultrastructure").

Pollen Hydrated in Water

Fresh or dry pollen grains are hydrated in a drop of water on a glass slide and observed in LM. This should be the first step before preparing pollen for SEM to get an impression about the quality of the collected material, to make sure that the material is not degenerated or contaminated by fungi (Fig. 2). Observations on pollen hydrated in water with the LM can reveal interesting aspects. One example is *Montrichardia* (Araceae), where a drop of water triggers a massive expansion of the thick intine resulting in an explosive opening of the pollen wall (Weber and Halbritter 2007).

Clarify the Pollen Polarity and Aperture Type

To clarify the pollen polarity and the aperture type, anthers with pollen tetrads must be collected before anthesis (usually found in flower buds). Pollen tetrads can be released from the anthers in a drop of water or in glycerine. Quite often different developmental stages can be found in one anther: microspores in early and late tetrad stages (with or without callose wall), but also young microspores (before first pollen mitosis) released from the tetrad as well as mature pollen grains (Fig. 3; see also Fig. 1 in "Pollen Development"). For the investigation it might be useful to stain the material, e.g. with toluidine blue or basic fuchsin (Siegel 1967).

Acetocarmine Staining: Detection of the Cellular Condition

For the detection of the cellular condition of pollen grains, fresh pollen are put into a drop of acetocarmine and warmed on a heating plate (up to

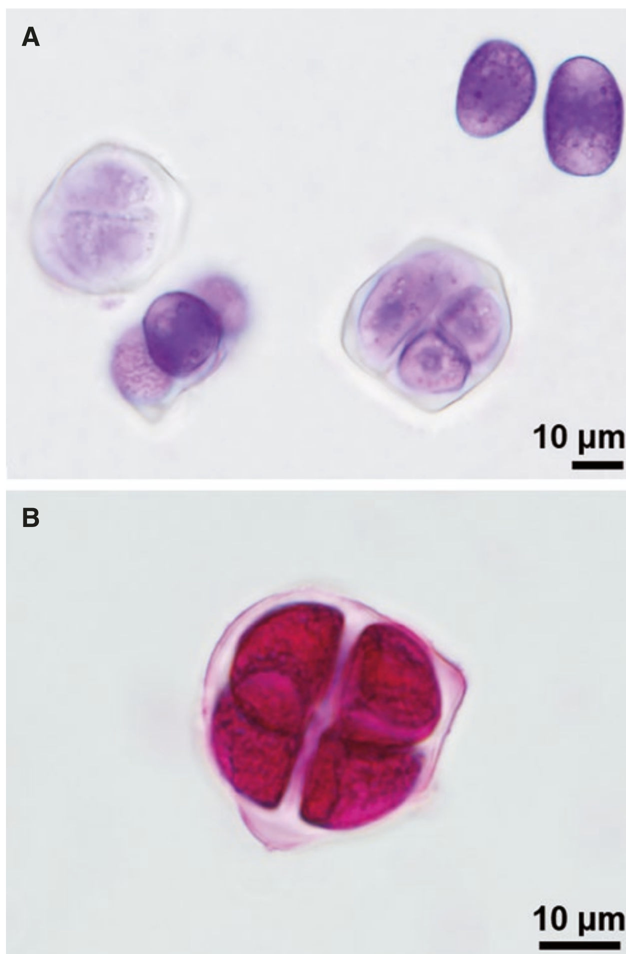


Fig. 3 Clarify the pollen polarity. **A-B**. *Calla palustris*, Araceae, tetrads in different stages as well as free microspores stained with toluidine blue (**A**) and basic fuchsin (**B**)

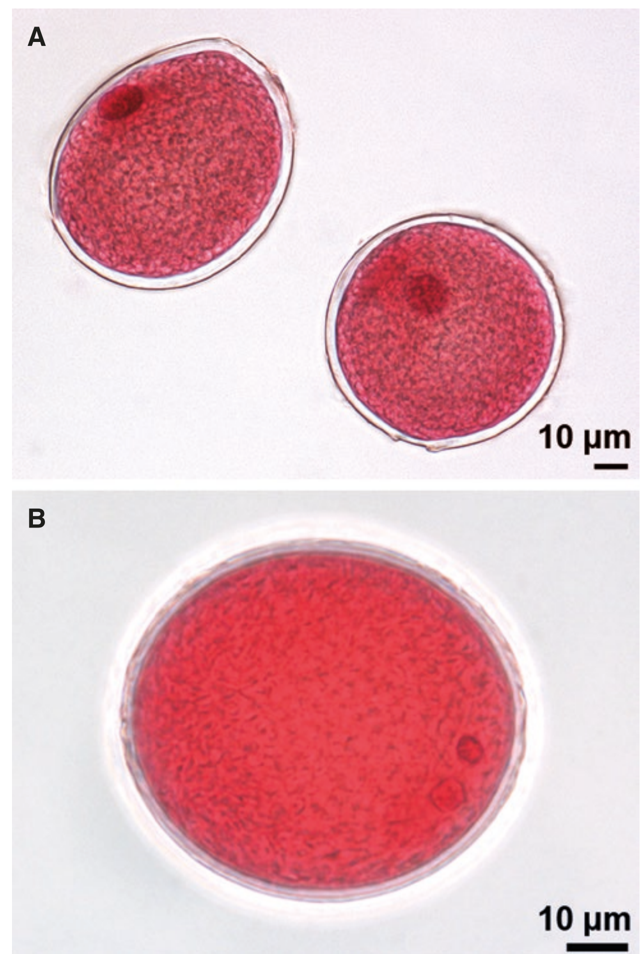


Fig. 4 Clarification of the cellular condition using aceto-carmine. **A.** Binucleate pollen of *Anchomanes welwitschii*, Araceae, generative nucleus stains intensive red. **B.** Trinucleate pollen of *Amorphophallus krausei*, Araceae, sperm nuclei stain intensive red

70 °C), for a few seconds to several minutes (species dependent), and observed under the LM (Gerlach 1984). The generative nucleus in binucleate pollen grains and the sperm nuclei in trinucleate pollen stain intensively red with aceto-carmine (Fig. 4). The generative nucleus usually stains less intensive.

Potassium Iodine: Detection of Starch

For the detection of starch as reserves in the cytoplasm, pollen grains are stained with aqueous potassium iodine (Gerlach 1984). Fresh or dry pollen grains are transferred into a drop of staining solution on a glass slide. Starch present in pollen grains will stain dark brown to black (Fig. 5).

Acetolysis: Visualizing Pollen Ornamentation and Aperture Number in Recent and Fossil Pollen

Acetolysis (Erdtman 1960) is a standard palynological preparation technique and an indispensable method for illustrating pollen grains with the LM. Untreated or stained pollen grains will hide much of the important information for the description of a pollen grain. The acetolysis treatment should remove the cellular content and the intine, but can also destroy the aperture membrane. Moreover, it cleans pollen surfaces and colors pollen grains brown, which makes it easier to observe all details of the pollen wall.

The normal preparation procedure is a combination of two steps, chlorination and acetolyzation

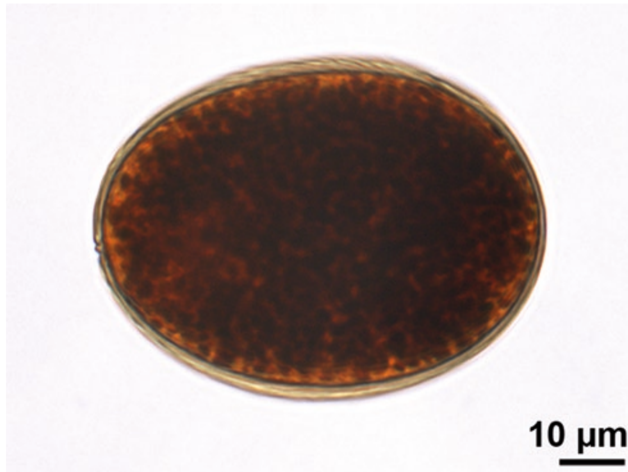


Fig. 5 Detection of starch using potassium iodine. *Amorphophallus interruptus,* Araceae, starch (in amyloplasts) stained with potassium iodine

(Fig. 6). For **chlorination**, the sample is transferred to a test tube and covered with a layer (1.5 cm) of glacial acetic acid and a layer (ca. 3 cm) of a freshly prepared solution of saturated sodium chlorate. After adding 3 or 4 drops of concentrated HCl, the mixture is stirred with a glass rod, heated in a bath of boiling water for 3 min, centrifuged, and the liquid fraction decanted. The residue is carefully rinsed to eliminate any remaining chemicals and then finally washed in concentrated acetic acid or acetic anhydride to remove the water. For the **acetolyzation**, the sample is put into a mixture of 9 parts acetic anhydride and 1 part concentrated sulfuric acid and heated to 100 °C (at least 80 °C) for approximately 4 min (up to 10 min). The samples are ideally acetolyzed in an ultrasonic bath to avoid boiling retardation and to reduce water condensation. After the mixture has been centrifuged and the liquid fraction decanted, the residue is washed in acetic acid and 3 times with water. After washing, test tubes are turned upside down and the content dried. Glycerine is then added to the sample. For fossil pollen material both steps (chlorination and acetolyzation) are usually applied.

When **preparing recent material** (Fig. 7) it is routine to apply only the second step (acetolyzation). Traditionally, the term "acetolysis" is also used even when pollen grains have been acetolyzed only and not previously chlorinated. For acetolysis of recent pollen fresh or air dried pollen/anthers are transferred into test tubes and can be acetolyzed directly. For the analysis of soil, dust, honey, or any other samples, the material has to be washed in a beaker with about 200 ml distilled water (and

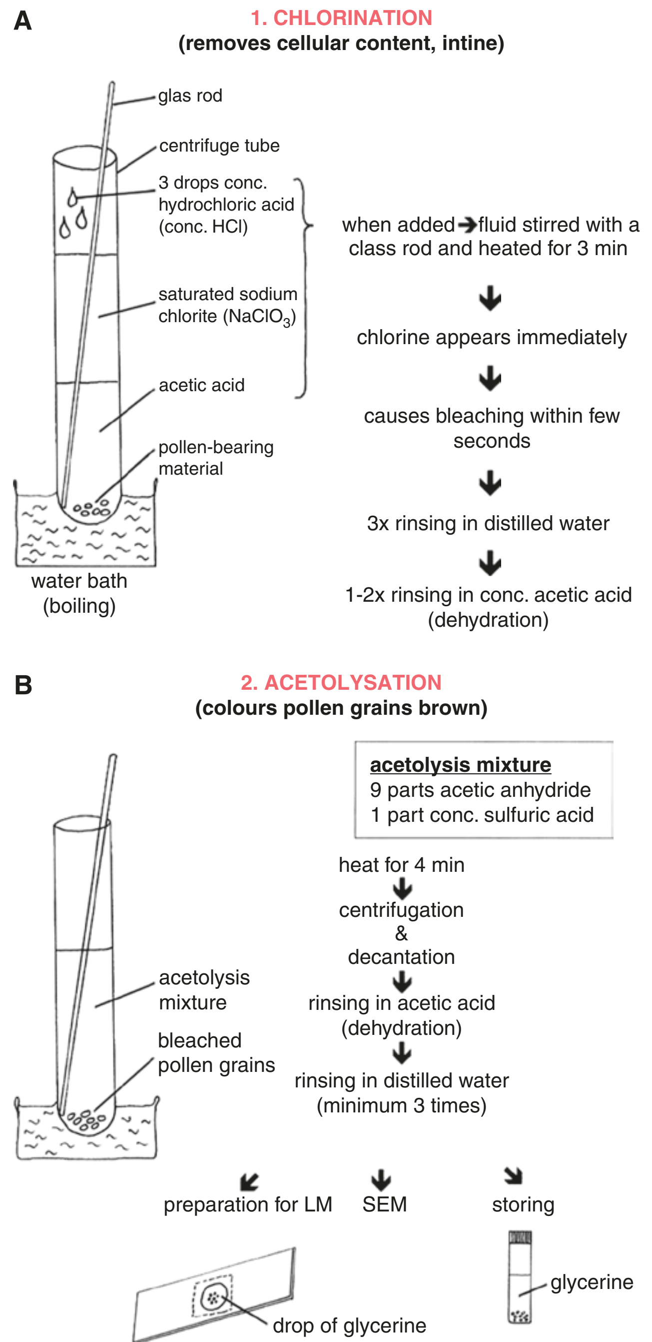


Fig. 6 Acetolysis treatment. Chlorination (**A**) and acetolyzation (**B**), the two steps of acetolysis

detergent, e.g., Tween) and can be sieved to remove bigger parts (leaves, branches) from the sample. In order to prevent pollen loss, it is important to use sieves with big mashes (E-D-quick sieve "260 µm"). The material is then concentrated in test tubes by centrifuging at 3000 rpm and the water decanted. The residue is washed in concentrated acetic acid to remove the remaining water and

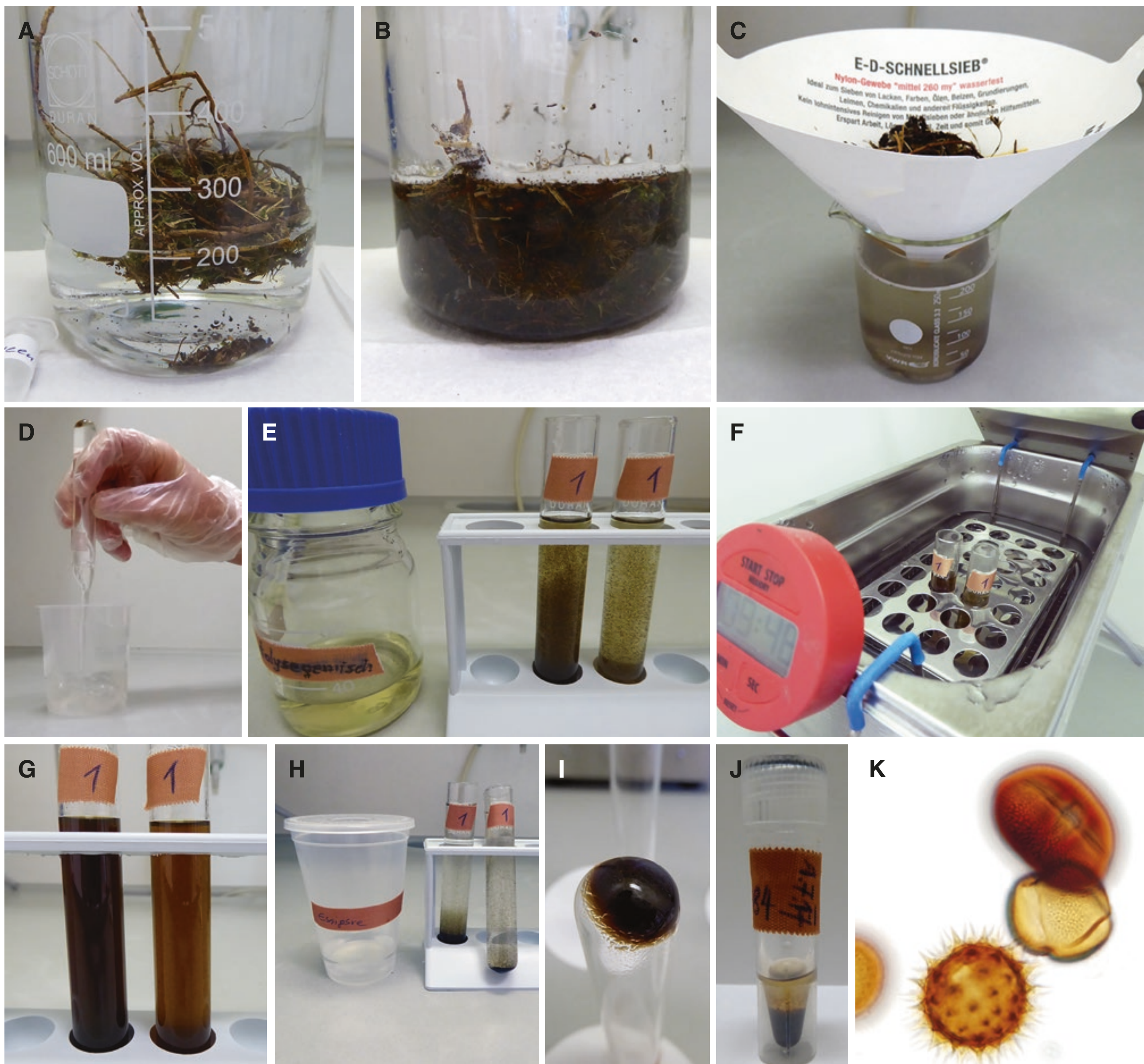


Fig. 7 Acetolyzation treatment of recent material. A. Washing the sample in a beaker. **B**. Washing with a detergent "Tween". **C**. Sieving the sample. **D**. Decanting water from the test tube after centrifuging; the organic fraction remains at the bottom. **E**. Fresh acetolysis mixture is added to the sample in the test tube. **F**. Samples are heated in an ultrasonic bath. **G**. During acetolyzation the solution turns brown. **H**. Residue washed in acetic acid followed by water. **I**. Drying of the acetolyzed sample. **J**. Acetolyzed material in glycerine stored in cryo tubes. **K**. acetolyzed pollen from honey in LM

subsequently acetolyzed (see description "acetolyzation" above). For light microscopy one part of the acetolyzed material is transferred into glycerine. For scanning electron microscopy, acetolyzed pollen is transferred into a drop of anhydrous ethanol on a SEM stub and sputter coated with gold (see also below "Preparation of fossil material").

Annotation: After rehydration or washing of the material (pollen/anthers) use acetic acid before and after the use of the acetolysis mixture, as it reacts intensively with water. Fresh acetolysis mixture is light yellow colored and highly reactive. Over time the mixture obtains a dark brown color and becomes less reactive.

Heavy Liquid Separation

Samples (recent and fossil) that still contain a very high mineral content after acetolysis should be treated with heavy liquid (e.g., zinc bromide solution; e.g., Eyring 1996, Traverse 2007; Fig. 8). Add ca. 2 cm of zinc bromide solution into the centrifuge tube and mix with the organic residue. Distilled

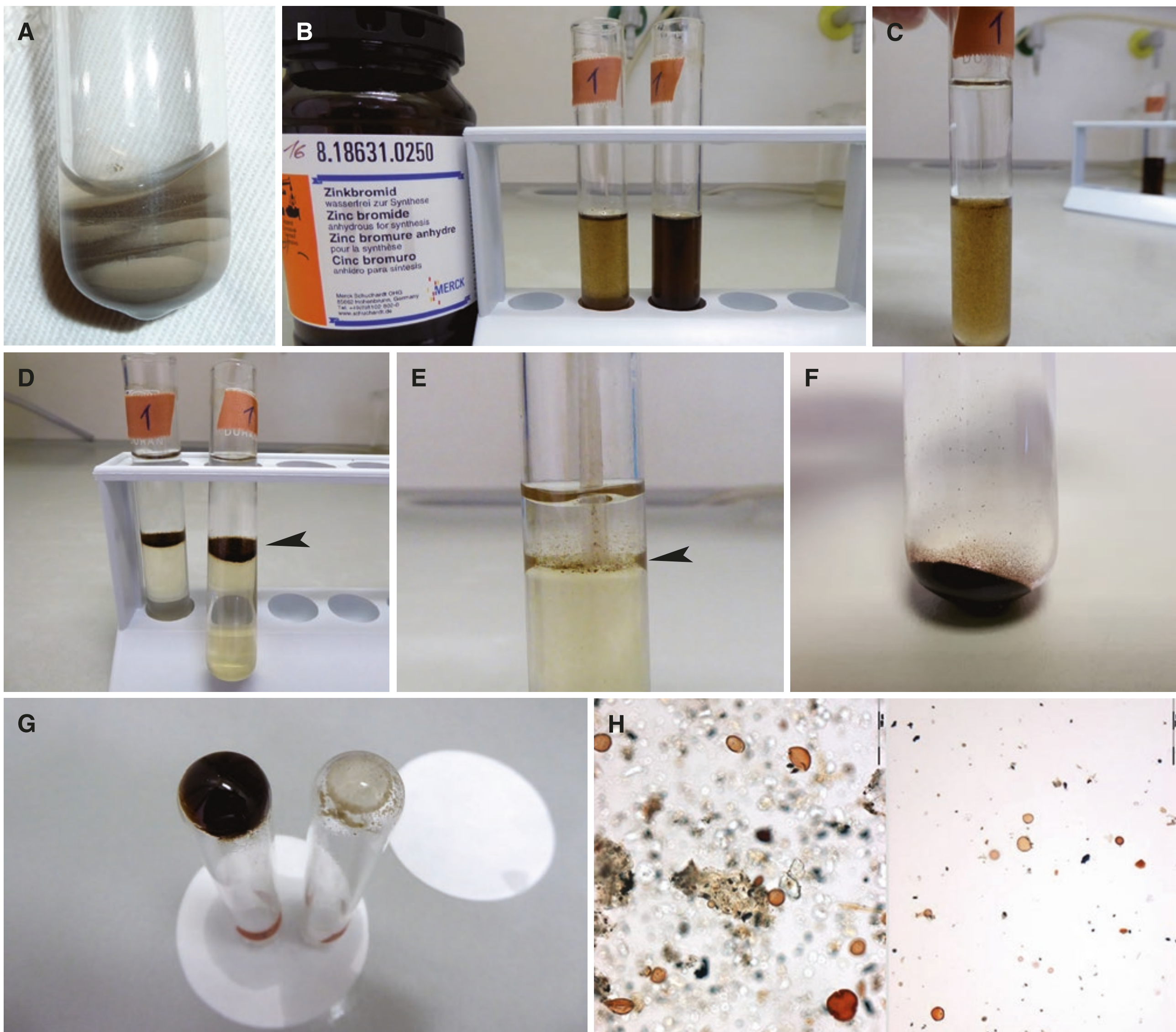


Fig. 8 Heavy liquid separation. **A**. Sample with high mineral content (light grey layers) after acetolysis. **B**. Mixing the sample with heavy liquid. **C**. Distilled water added without intermixing the liquids. **D**. Organic fraction (arrowhead) floating on the heavy liquid. **E**. Organic fraction (arrowhead) pipetted to a new test tube. **F**. Washing the organic fraction with water. **G**. Drying the acetolyzed sample (left) and the mineral fraction (right). **H**. Sample untreated (left) and treated with heavy liquid separation (right)

water is then carefully poured into the test tube (ca. 2 cm) and make sure that the two liquids do not intermix. After centrifuging for about 5–8 min at 3000 rpm the organic material is floating on the heavy liquid and below the distilled water. The organic material can then be transferred with a pipette into a new test tube for further washing. The inorganic parts remain at the bottom of the solution.

Acetolysis the Fast Way

A fast and easy way to prepare recent pollen grains for LM and SEM is to have a small glass bottle with a readymade acetolysis fluid (nine to one mix of 99% acetic anhydride and 95–97% sulfuric acid) at hand. Place a drop(s) of the acetolysis fluid on a glass slide. Remove anthers from the flowers and place them into the fluid on the glass slide (Fig. 9). To soften up the material let it lay in the liquid for some time and break the anther/flower material by squeezing and pressing it with the tip of a teasing needle. The slides are then heated over a candle flame for a short time to soften up the anthers, release the pollen grains from the anthers, dissolve extra organic material on pollen grain surfaces, "rehydrate" pollen grains and release their cell contents, and finally, to stain the pollen grains for LM photography. Make sure not to hold the slide over the flame for too long since it will make the pollen grains too dark. Best is to

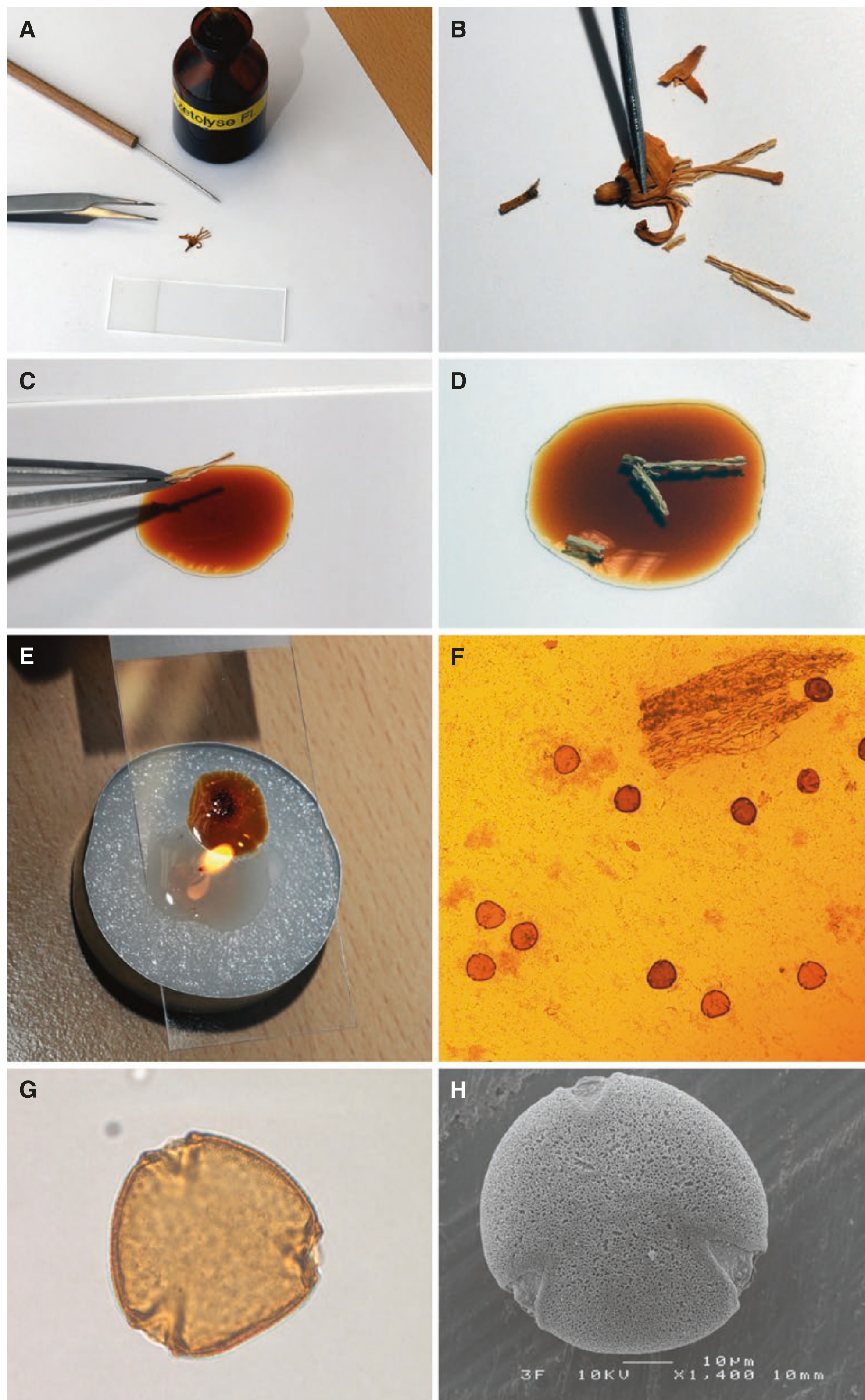


Fig. 9 Acetolysis the fast way. **A**. Flower and tools needed for preparation. **B**. Brake or cut off anthers. **C**. Transfer anthers into acetolysis fluid on glass slide. **D**. To soften up the material it can lay in the fluid for some time. **E**. Carefully heat the slides over a candle light. **F**. Readymade pollen grains in the acetolysis fluid. **G**. Transfer pollen grains to fresh drops of glycerine on new glass slides and photograph in LM. **H**. Same grain photographed in SEM using the "single-grain method"

heat the slides shortly and then use the teasing needle to break down the anther material. This should be repeated until the pollen have gained the required color. Using a micromanipulator (see below) selected pollen grains are then transferred into fresh drops of glycerine on new glass slides and photographed under LM. Some pollen grains can also be transferred to SEM stubs using the technique of the "single-grain method" described below, sputter coated with gold and photographed under the SEM.

Scanning Electron Microscopy: Preparation of Recent Pollen

SEM techniques cannot substitute LM, but they can provide a great deal more information, especially about ornamentation. Samples prepared for SEM should ideally reflect the fully hydrated condition of a living pollen grain. In addition, all types of pollen coatings must be removed from the pollen surface, not to obscure details of the pollen wall.

For scanning electron microscopy dehydration and drying techniques are of great importance. The principle of critical point drying (CPD) is to avoid any damaging to the pollen due to surface tension forces occurring during transition from the liquid to the vapor phase. Due to the slow penetration time of DMP, large samples (e.g., large anthers, whole parts of flowers) should be dehydrated in a series of alcohol (70–85–96%, each about 20 min) and acetone or dehydrated in 70% ethanol (3 days) and formaldehyde dimethyl acetal (FDA, 1 day or overnight).

The DMP Direct Method: Dimethoxypropane

With the DMP direct method (Halbritter 1998) important details of hydrated pollen grains, which may be lost by conventional methods (alcohol), are well preserved without shrinkage, distortion, or dissolution (Fig. 10). The best results are obtained using acidified dimethoxypropane (DMP) for dehydration. Anthers should be collected at anthesis. Take whole or parts of anthers, or loose pollen grains and put them into a pouch made of filter paper. For analyzing pollen in hydrated condition, moisture the filter pouch with a droplet of water and wait for a few seconds before transferring them into acidified 2,2-dimethoxypropane. After 20–30 min (or up to 24 h) in DMP samples are transferred into pure acetone for a few minutes and critical-point dried in CO_2 using acetone as the intermediate fluid. The CPD-pollen samples are then mounted on stubs using double-sided adhesive tape, sputter coated with gold and observed with an SEM. CPD samples can be stored, e.g., in a sealed plastic box to protect them from humidity.

This method can be used for fresh material as well as for herbarium samples (after rehydration in water). The chemical dehydration of unfixed plant material with DMP is a simple and fast method and can be applied to small samples only.

Unless stated otherwise, the pollen grains shown in this book are prepared using the DMP direct method by Halbritter (1998).

Transmission Electron Microscopy: Pollen Wall Stratification and Ultrastructure

For TEM studies of recent and fossil pollen, more than one protocol for fixation and staining may be needed.

Fixation and Embedding

Fixation of samples for TEM studies (Hayat 2000) is a time-consuming process that starts with fixation on the first day (Fig. 11), followed by dehydration and infiltration on the second and third day and ends with embedding on the fourth day (Fig. 12). For pre-fixation, the samples (closed anthers or pollen suspension) are placed in phosphate buffered glutaraldehyde (3%). In case of large specimens (flower/anther), the relevant parts of the sample are prepared/cut within the fixation solution under a binocular microscope (placed at the fume hood to prevent toxic substances from inhalation). Samples must be free of gaseous/air-bubbles. Transfer samples into Eppendorf tubes and make holes into the lid. Place the tubes into the vacuum desiccator and evacuate from air for 10–30 min. For pre-fixation the evacuated samples are then placed for 6 h in a specimen rotator (at room temperature). After rinsing in buffer and distilled water, samples are post-fixed in 2% osmium tetroxide plus 0.8% phosphate-buffered potassium ferrocyanide (2:1) for 8–12 h at 6 °C (for osmium storage see also Fig. 28). On the second day osmium tetroxide is removed and samples are washed in distilled water (3 times for 5 min each) followed by dehydration in 2,2-dimethoxypropane (3 times, for 10 min each) and finally by pure acetone (2 times for 15 min each). The infiltration process starts by adding a few drops of the embedding media (1:2) to the samples

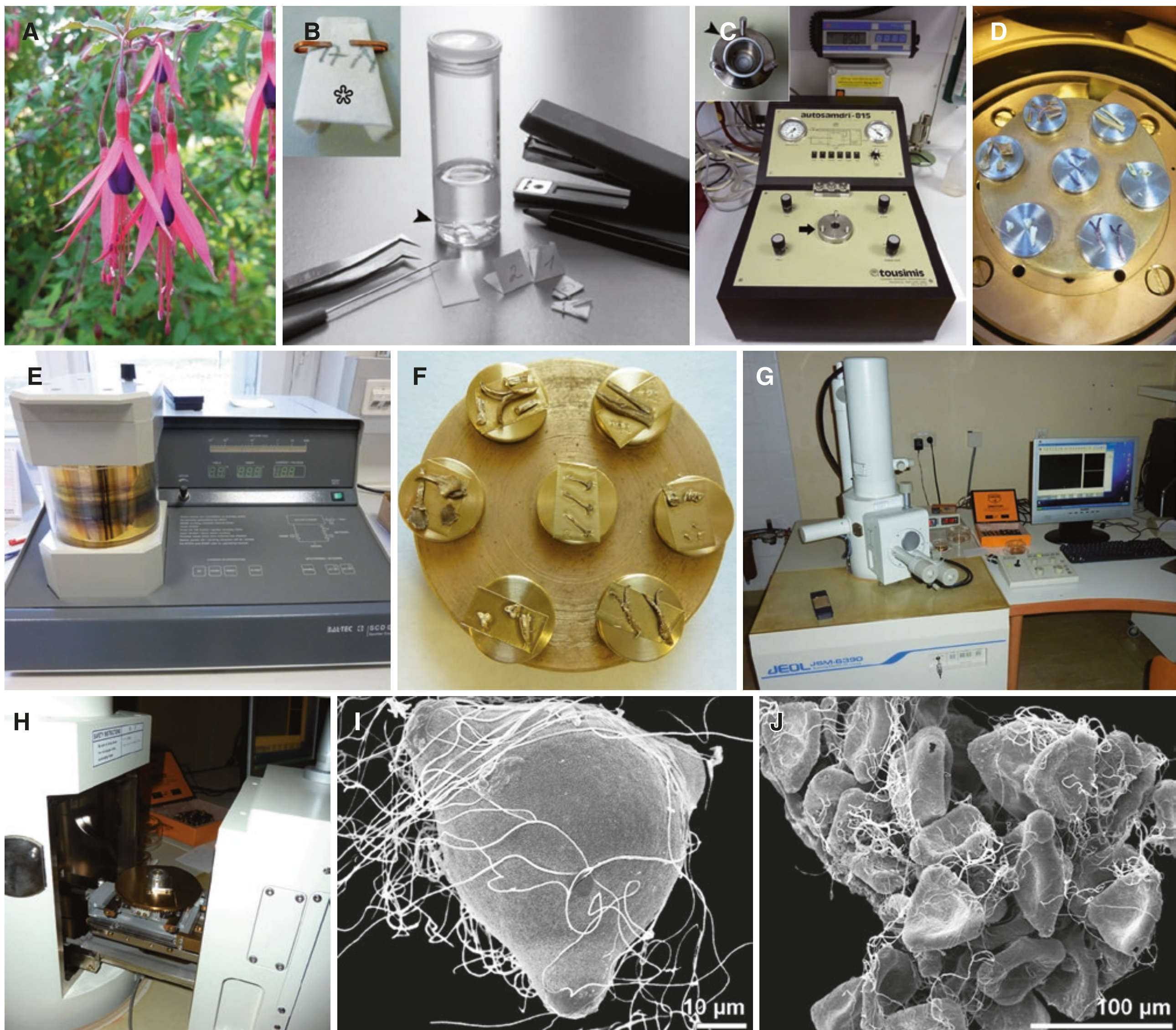


Fig. 10 The DMP direct method. A. Pollen collected at anthesis, *Fuchsia magellanica*, Onagraceae. **B**. Filter pouches for pollen preparation; moisture filter pouches (pollen samples) with a droplet of water (asterisk) before dehydration in DMP (arrowhead). **C**. Critical point dryer (CPD) with closed chamber and upper view on open chamber (arrowhead). **D**. CPD-pollen samples mounted on stubs using double-sided adhesive tape. **E**. Sputter coater. **F**. Samples sputter-coated with gold. **G**. SEM. **H**. Open chamber. **I**. Pollen in hydrated condition, SEM. **J**. Pollen in dry condition, SEM

and swirl the mixture. Repeat the procedure in 6–7 h, then let samples infiltrate overnight. This process has to be repeated on the third day. On the fourth day, acetone has to be removed before embedding the material: extract half of the acetone-resin-mixture with a pipette and wait for 2–3 h until the remaining acetone evaporates. After the fixation process the material should be stained intensive black (due to osmium), if not start from the beginning with new material.

The fixed material can now be transferred into embedding forms filled with fresh **embedding media** (Agar low-viscosity resin, see section "Recipes for TEM"). Polymerization takes place in an oven for about 12 h at 70 °C. After polymerization the specimen blocks can be stored in small plastic bags and are ready for ultrathin sectioning.

Annotation: For fixation of pollen, the material must be centrifuged after each step and the fixation mixture/water/DMP must be extracted with a pipette.

Ultramicrotomy

A lot of equipment and preliminary steps are involved in the ultramicrotomy process: **preparation of formvar film-coated grids, section-manipulators**

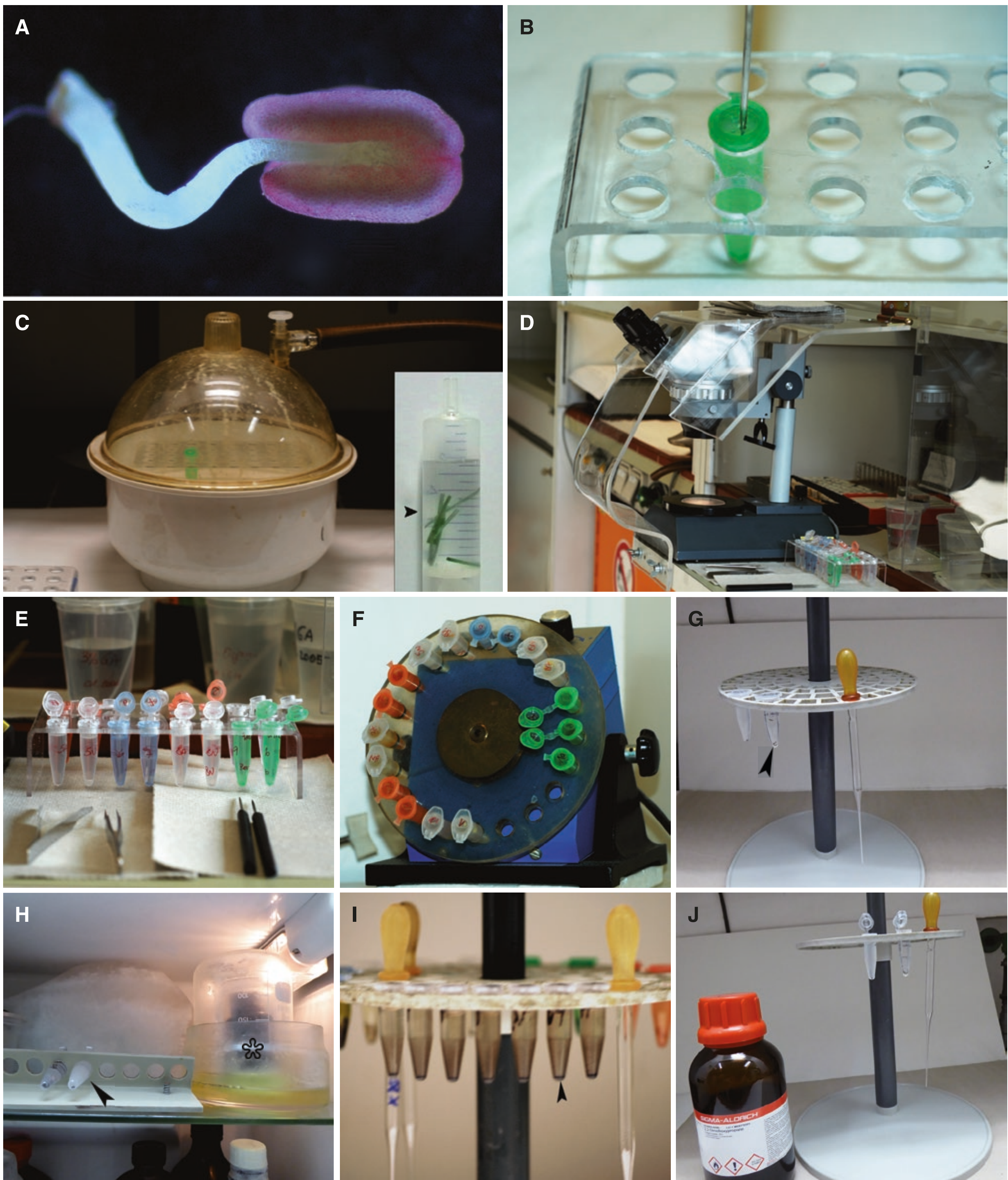


Fig. 11 Fixation and embedding day 1–2. **A**. Closed anther for pre-fixation. **B**. Material in Eppendorf tube with fixation solution, make holes in lid before evaporation. **C**. Evacuation in vacuum desiccator (left) or manually in a syringe (right). **D**. Preparation/cutting of samples within the fixation solution under a binocular microscope (placed at the fume hood). **E**. Transfer of selected parts of the sample into small Eppendorf tubes with fixation solution (3% GA). **F**. Samples in specimen rotator. **G**. Post-fixation; arrowhead indicates sample with osmium solution. **H**. Post-fixation of samples (arrowhead) for 8–12 h at 6 °C (fridge in a fume hood) in Eppendorf tubes; Note: osmium solution stored in fridge (asterisk). **I**. Samples after 8–12 h: material blackened due to osmium (arrowhead). **J**. After removal of osmium, samples are dehydrated, followed by pure acetone

Fig. 12 Fixation and embedding day 2–4. A. Infiltration starts by repeatedly adding few drops of embedding media. **B**. Embedding solution (Agar low-viscosity resin) mixed using a magnetic stirrer. **C**. Final embedding into adequate embedding forms under binocular microscope. **D**. Polymerization at 70 °C in a thermostat oven (arrow). **E**. Examples of various embedding forms. **F**. Polymerized samples. **G**. Specimen blocks stored in small plastic bags

and preparation of loops, specimen block trimming, semi-thin sectioning, making of glass knives, diamond knives, and **ultra-thin sectioning**. Another indispensable equipment for ultramicrotomy are tweezers with an ultra fine pointed, curved, and angled precision tip.

Formvar Film-Coated Grids

Coated grids are made with a formvar solution (see "Recipes for TEM"; Fig. 13). New and cleaned glass slides are dipped with a special self-made "filming machine" into the formvar solution (minimize evaporation of the chloroform). The extraction speed of the slide influences the thickness of the formvar film: a thin film is produced by a slow, steady movement. After 1–2 min remove the glass slide steadily from the solution and dry for 2–3 min. The film can then be transferred onto a clean water surface (use distilled water in a clean staining cuvette). To loosen the film, cut the film with a scalpel along the edges of the slide and blow moist air (with a straw from your mouth) onto the film. In the same instance, dip the slide into the water at an angle of 45° to remove the film from the glass slide. When the film is floating on the water surface, don't pull out the slide, but let it slowly set into the cuvette. The quality of the film is indicated by the color: a thin film is grey to silver, whereas gold is too thick. Grids cleaned with chloroform are placed using fine pointed tweezers onto the film. To know which side of the grid is coated, always put one side (either

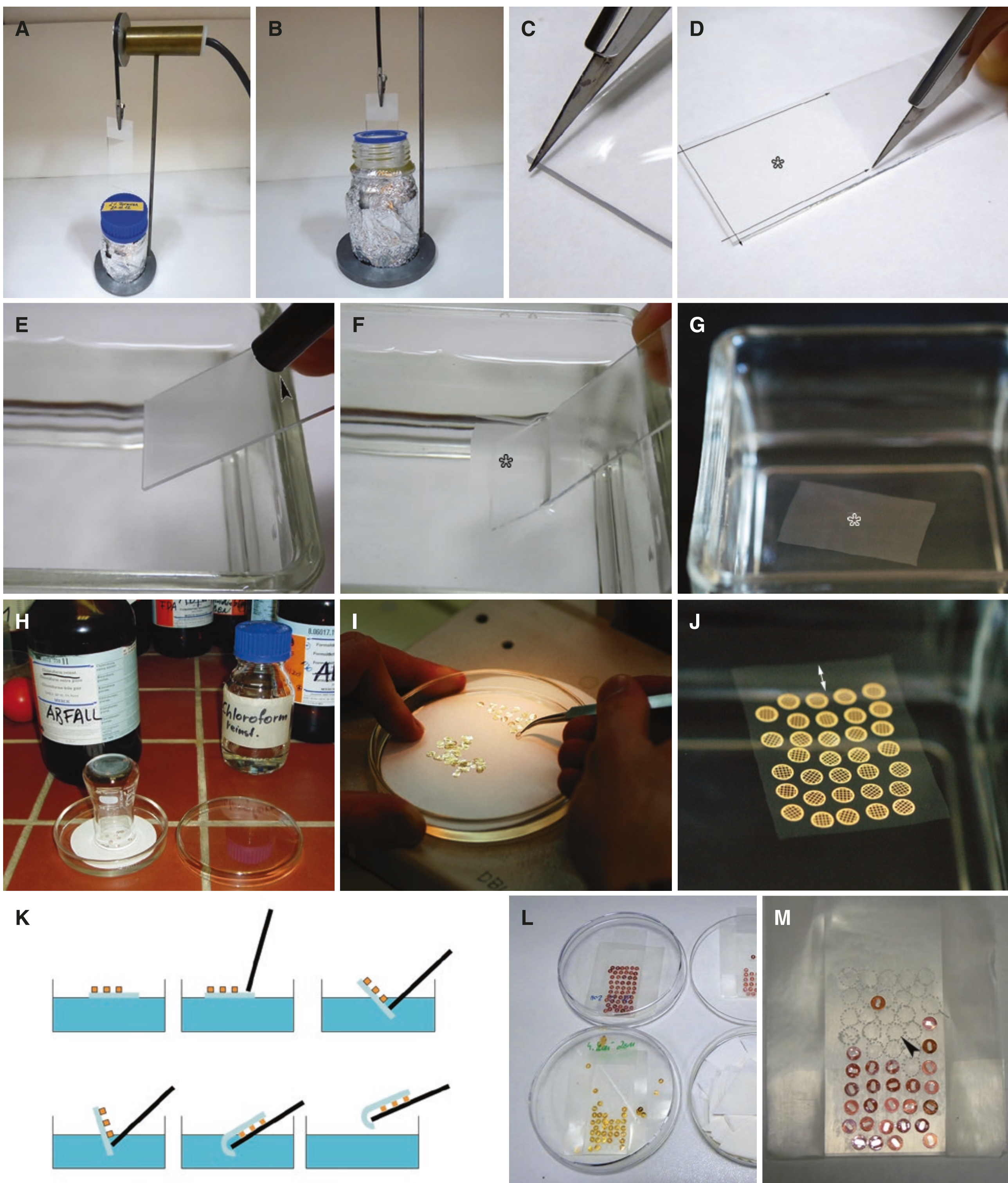


Fig. 13 Making formvar film-coated grids. **A**. Filming machine with holder for glass slide; filming solution should be protected from light. **B**. Glass slide dipped into formvar solution (under fume hood). **C-D**. Film cut along edges (arrowhead); arrows indicate cutting line on film (asterisk). **E**. Moisture film before dipping the slide under water; arrowhead indicates straw. **F**. Dipping the slide into the water at an angle of 45°; film partly floating on water (asterisk). **G**. Thin film floating on water surface (silver colored). **H**. Clean grids with chloroform. **I**. Shiny or dull side of the grid is visible under binocular. **J**. Grids on thin floating formvar film, arrow indicates space left for film extraction. **K**. Extraction of coated grids from the water surface using a parafilm-coated glass slide. **L**. Coated grids dried and stored in petri dish. **M**. Parafilm-coated slide with formvar coated grids, perforations (arrowhead) outline removed grids

shiny or dull side) of the grid down on the film. Make sure to leave enough space between grids and along one short margin to extract the film from the water surface. Use a parafilm-coated glass slide to extract the filmed grids: place the slide on free space of the film and dip with quick and steady motion at about 45° angle into the water and then pull out the slide again (Fig. 13K). Place the slide on a filter paper in a petri dish and let it dry. Formvar film-coated grids should be stored protected from light and dust-free (e.g., in the petri dish). To isolate the grids, use a needle to make perforations around the grids and remove them carefully with a forceps. Before ultra-thin sectioning, check the formvar film-coated grids for defects (e.g., holes, dust) under binocular microscope and place them with the filmed side up on a filter paper (see also Fig. 20 "Section pick up").

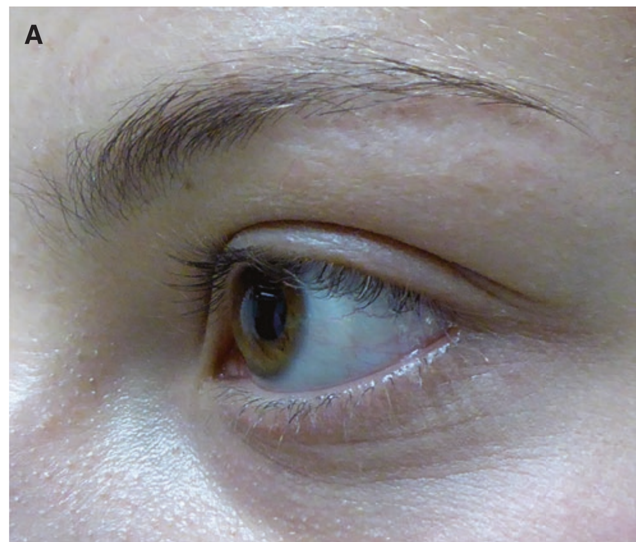
Section Manipulators (Eyelash or Other Adequate Type of Hair)

To separate and move semi-thin and ultra-thin sections floating on the water surface an **eyelash manipulator** is used. Usually a human eyelash (untreated) is fixed with glue or wax on a short glass pipette or wooden stick. The eyelashes should be cleaned with alcohol each time used and stored dust-free (e.g., covered with the back end of a bigger plastic pipette) (Fig. 14).

Loops

Loops are used to transfer ultra-thin sections onto formvar-coated grids (see Fig. 20 "Section pick up"). A loop should take up a droplet of water accurately and should fit exactly onto the grid. Therefore, two types of loops are produced (1) **circular loops**, that fit onto mesh-grids and (2) **oval** loops, used for slot-grids (Fig. 15).

Loops are made with wires from conventional electric cables (wires should not be too thick or thin). For making a circular loop a small piece of wire can be twisted around a circular object with appropriate diameter (e.g., screw driver). To produce an oval loop make a smaller circle and press it from two sides with a plier into an oval shape (fitting the grid slot). More ideally wrap the wire around a self-made model form fitting the grid size/slot. The wire of the loop is finally flattened with a hammer and the twisted (non-flattened) appendices fixed with glue or wax, e.g., on a short glass pipette. The loop should be cleaned before use with alcohol and stored free of dust.



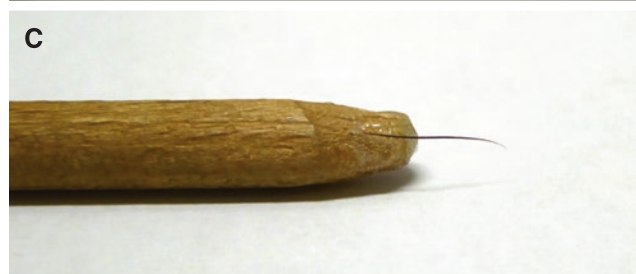


Fig. 14 Making a section manipulator. **A**. Human eyelash. **B**. Technical equipment for making a section manipulator; arrowheads indicate eyelashes. **C**. eyelash fixed with glue on wooden stick

Specimen Block Trimming

Criteria for block trimming are: (1) a small sample size, (2) the location of the sample should be in the center of the block-face (trapezoid) and surrounded by resin, (3) the straightness of the block-face edges (parallel edges).

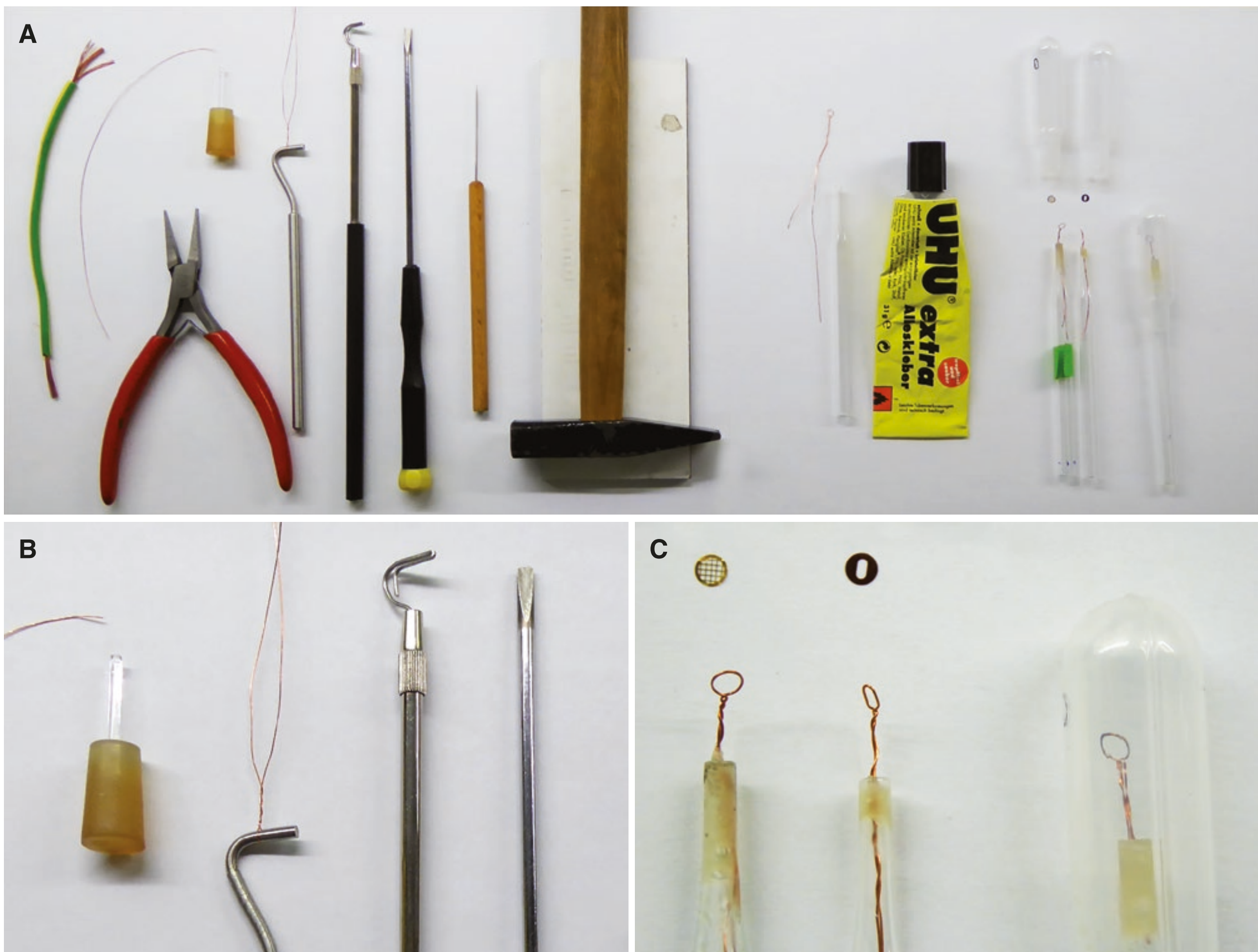


Fig. 15 Making loops. **A**. Technical equipment for making loops. **B**. Wire twisted around a circular object (model form) for mesh grids. **C**. Loops fixed with wax on glass pipettes (left), for storage loop covered with the back end of a disposable plastic pipette (right)

A specimen block must be trimmed (cut) to get small sections with a block-face of 4 mm by 4 mm in size (Fig. 16 O). A small block-face ensures good sectioning performance. Trimming is conducted with razor blades (for each block use a new razor blade). The block is fixed in a specimen holder and trimmed under a binocular microscope. The specimen block is trimmed into a pyramid with a trapezoid-shaped block-face. The tip of the pyramid should be cut away until you reach the appropriate level within the sample. A glass knife is used for initial cuts. If the specimen is rather big, the block-face can be larger for semi-thin sectioning (max. 4 mm^2) to ensure that the area of interest is preserved. Such a large block must be trimmed further to reach the final required block-face for ultra-thin sectioning.

Glass Knives

Glass knives are generally used for semi-thin sectioning and are replaced by diamond knives for ultra-thin sectioning. Glass knives are produced with a "knife-maker" (Fig. 17). Specially produced glass strips (e.g., 6.4 × 25 mm) are first cut into squares. The squares are then cut diagonally into two triangles, each with a knife edge (Fig. 17 E). The breaking line (stress line) indicates the quality of the knives. The left side of the glass knife is sharper and can also be used for ultra-thin sectioning, whereas the right side is used for semi-thin sectioning only. "**Glass knife boats**" (disposable plastic forms) are attached and sealed with hot melted dental wax (hot plate and ethanol burner) to the glass knife (see also Fig. 18). Glass knives should be stored dust-free and safe in a "glass knife box."

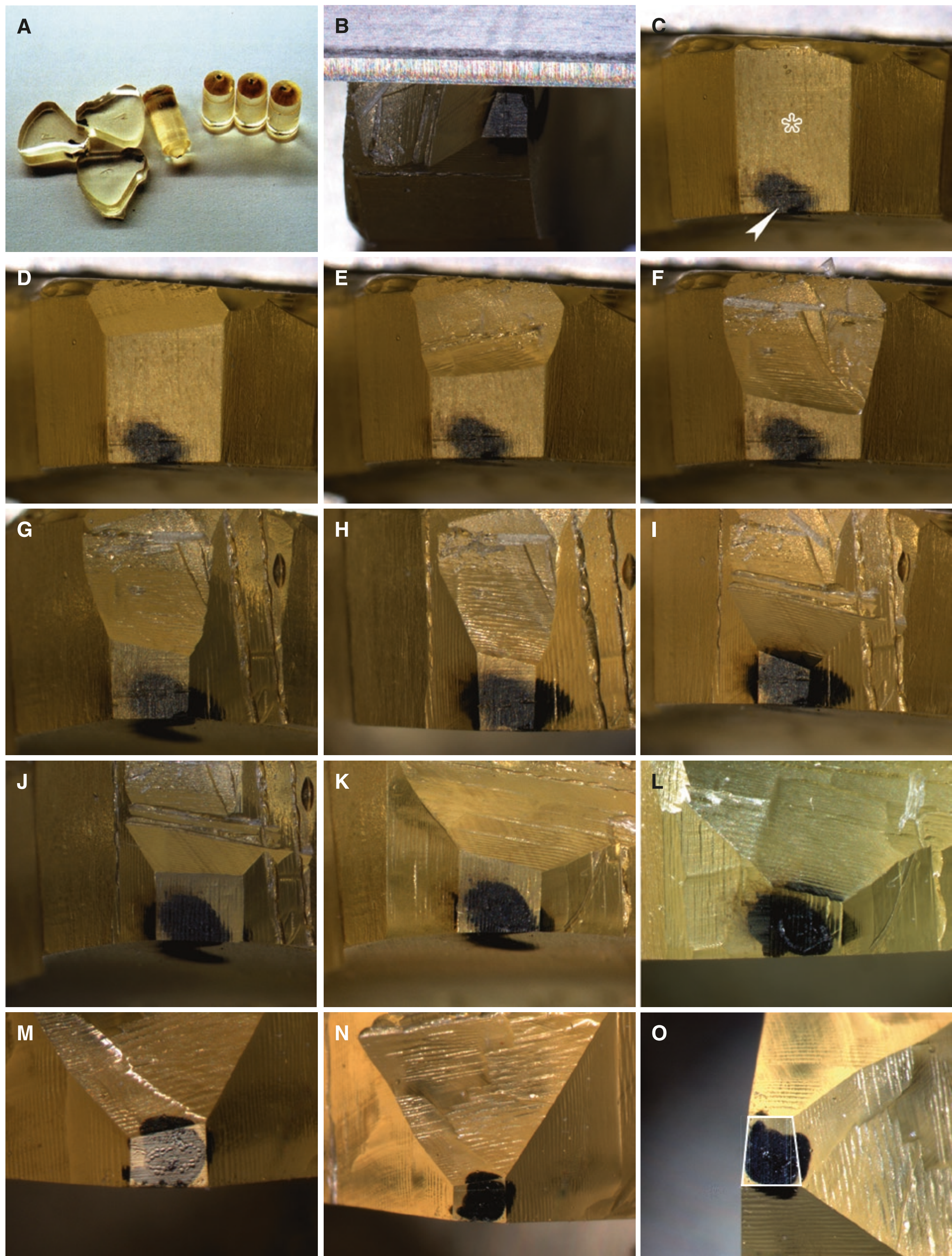


Fig. 16 Specimen block trimming. **A**. Specimen blocks of various shapes. **B**. Trimming is conducted with razor blades. **C**. Untrimmed specimen block with view on block-face (asterisk), arrowhead indicates position of specimen inside block. **D-N**. Blocks are trimmed into a pyramid with a +/– trapezoid shaped block-face and parallel edges. **O**. Final block-face with trapezoid form (white trapeze)

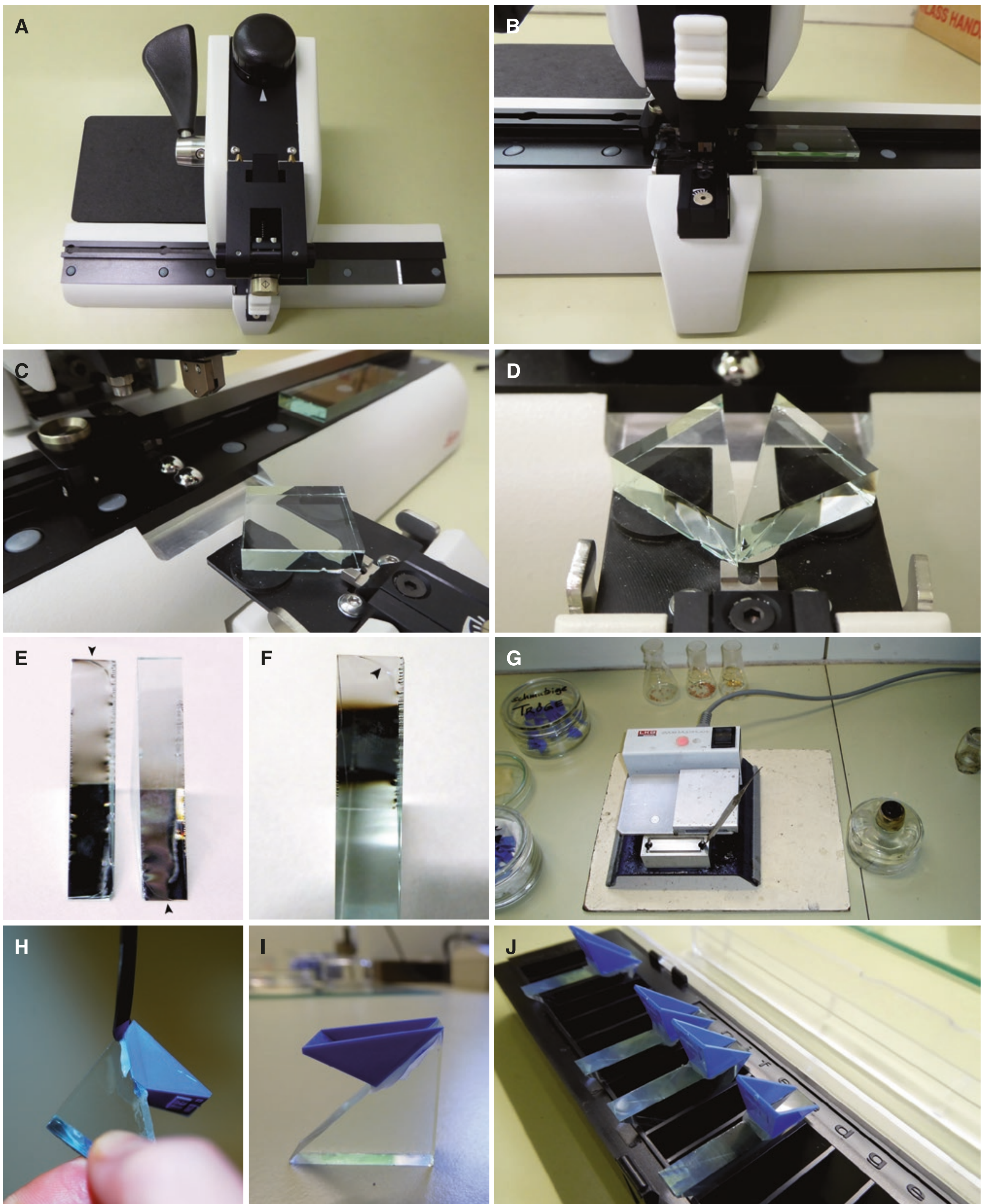


Fig. 17 Making glass knives. **A**. Knife maker. **B**. Glass stripes cut into squares. **C**. Squares are cut into two triangles. **D**. Two triangles (glass knives). **E.** Each triangle has a knife edge (arrowhead). **F.** Detail of triangle with knife edge, arrowhead indicating breaking (stress) line. **G.** Hot plate and ethanol burner for melting dental wax. **H.** Glass knife boats attached and sealed with hot wax using a spatula. **I.** Readymade glass knife. **J.** Knives stored in glass knife box

Semi-Thin Sectioning

Before selecting an area of the specimen block for ultra-thin sectioning, semi-thin sections are cut with an ultramicrotome, using a glass knife (Fig. 18). The settings for semi-thin sectioning are: section thickness between 0.5 and 2 µm (interference color purple to blue) and cutting speed 2 mm per second. Semi-thick sections are transferred with a loop into a drop of water on a glass slide. For a fast drying process put the slide on a hot plate (approx. 70 °C). While the water evaporates the sections will stretch. The dried sections are stained with toluidine blue on the glass slide, which can be sped up by placing the slide for max. 5 s on the hot plate. Carefully wash the slide with water and dry the glass slide in a filter paper block. The stained semi-thin sections are controlled with the LM to determine the quality of the fixation and to ensure that the appropriate area of the specimen is in the correct position for ultra-thin sectioning.

Ultra-Thin Sectioning

Ultra-thin sections between 60 and 90 nm (interference color silver to pale gold) are cut using an ultramicrotome (Fig. 19). **Diamond knives** are more suitable for cutting plant material, as e.g., crystals in cells destroy the cutting edge of glass knives, generating scratches within the sections or even splitting the sections.

The knife is placed in the knife holder and the knife boat filled with distilled water. The knife should be clean, free of dust and moistened with water. The specimen block has to be placed in the specimen arm in the upper position. Then the block has to be positioned parallel to the knife-edge by rotational or lateral adjustments of block as well as the knife. By moving the block up and down in front of the knife a slit of reflected light helps to adjust the block to the knife. A narrow slit of light indicates that the block is close to the knife and a constant thickness of the slit, along the whole block-face, indicates that the block face and the knife-edge are parallel. This is the ideal position for sectioning. The settings for ultra-thin sectioning are: section thickness between 60 and 90 nm and cutting speed 1 mm per second. The section settings can be adjusted while cutting until pale gold to silver sections are produced. Sections are floating on the water surface and can be manipulated with an eye-lash. Before the ultra-thin sections can be transferred to grids, sections must be stretched to remove compressions due to cutting. For stretching a solvent (e.g., xylol, chloroform, acetone vapor) or a hot pen can be used. For the vapor method use a thin, wedge-shaped piece of filter paper moistened with a drop of solvent, hold it closely above the sections while moving it back and forth.

Section Pick-Up

The stretched sections are picked up from the water surface with a loop (Fig. 20). Depending on the size of the sections between 3 and 10 sections can be picked up at once. Center the loop above the selected sections, dip it on to the water surface, lift the sections up within a droplet of water and transfer onto a grid under a binocular microscope. Center the loop above the grid and lower it onto the grid surface. Lift up the loop and the attached grid. The water is removed slowly with a filter paper touching the first twist by the loop (Fig. 20 D). Transfer the grid with a forceps into a grid-box (sections should face the same side). Make a section protocol. Store the grid box away from light.

Staining Methods

The application of different TEM staining techniques for one and the same sample is very important and highly recommended to avoid misinterpretations of the pollen wall structure. Therefore, sections of pollen grains are routinely stained using the several different staining methods (Figs. 21 and 22). Most staining solutions are harmful or even toxic and therefore applied under fume hood.

Annotation: In electron microscopy there is no grey-scale terminology from white to black. Use "electron dense" for black or darkly colored structures and "electron translucent" for white to light grey colored.

Uranyl Acetate-Lead Citrate Staining: U + Pb

Uranyl acetate-lead citrate staining is a conventional staining method (Hayat 2000; Figs. 21 and 22). Ultra-thin sections are usually collected on copper grids. Sections are stained in uranyl acetate solution (Leica Ultrastain-1) for 45 min followed by lead citrate staining (Leica Ultrastain-2) for 1–5 min at room temperature. Use of sodium hydroxide pellets for lead citrate staining prevents crystalline precipitation by absorbing moisture and carbon dioxide from the air. Sections are thoroughly washed in distilled water after each staining step (3 times for 5 min in a row of water drops).

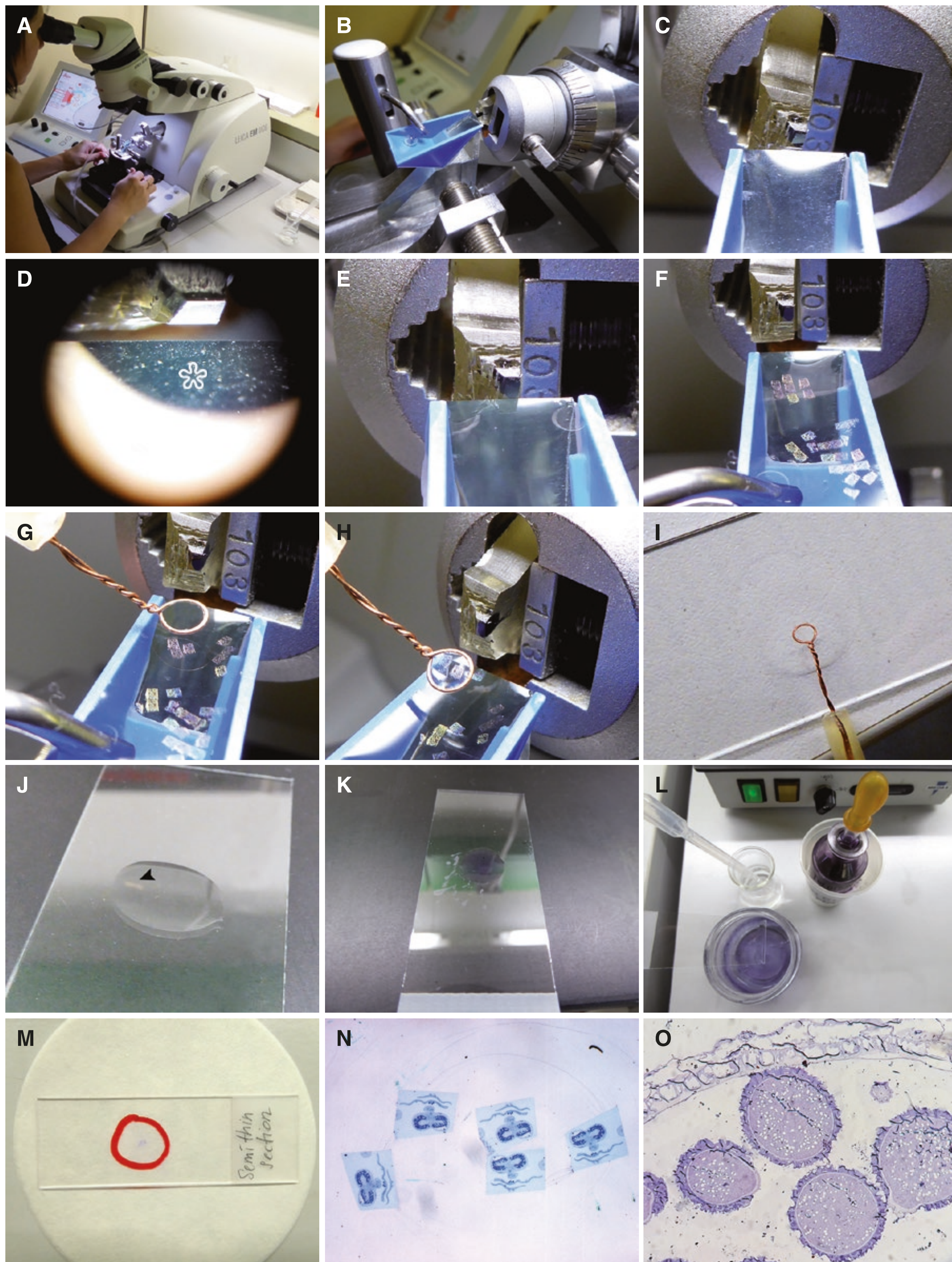


Fig. 18 Semi-thin sectioning. **A**. Ultramicrotome. **B.** Glass knife positioned in the knife holder and knife boat filled with distilled water; specimen block fixed within the specimen holder. **C**. Block adjustment parallel to knife-edge by use of reflecting light. **D**. Knife should be clean, free of dust and moistened with water, asterisk indicates slightly lowered water level at knife edge for sectioning, but still moistened. **E.** Block must be close enough to knife (until slit of light almost disappears) to start sectioning. **F.** Semi-thin sections between 0.5 and 2 µm (interference color purple to blue) floating on water. **G-H**. Section pick-up with a loop (see "Section pick-up"). **I**. Transfer of sections in a drop of water on a glass slide. **J**. Slide on a hot plate (arrowhead indicates semi-thin sections). **K.** Staining sections with toluidine blue on hot plate. **L**. Rinsing the stained sections with water. **M.** Stained semi-thin sections ready for LM. **N**. Toluidine blue sections seen under LM. **O**. Final quality check before ultra-thin sectioning

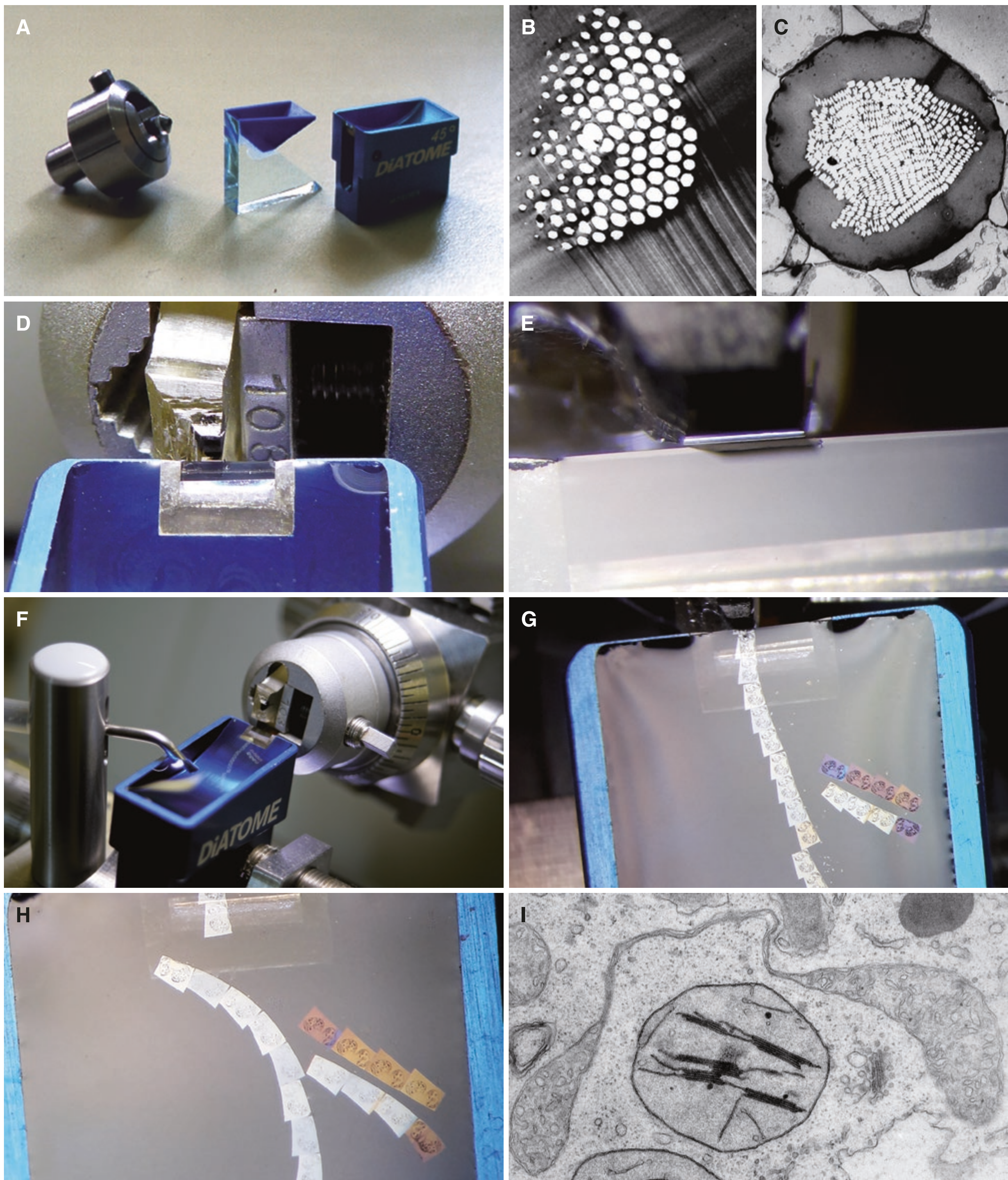


Fig. 19 Ultra-thin sectioning. **A**. Specimen block holder with trimmed block, glass and diamond knife. **B**. Crystals in plant cells cut with glass knife, note scratches. **C**. Crystals in plant cells cut with diamond knife. **D-E**. Block adjusted parallel to knife-edge by use of reflecting light. **F-G**. Sections between 60 and 90°nm (interference color silver to pale gold). **H**. Stretched sections, note the change in size and thickness (for color change compare to picture **G**). **I**. Ultrastructure of a plant cell showing high quality fixation of several organelles in TEM

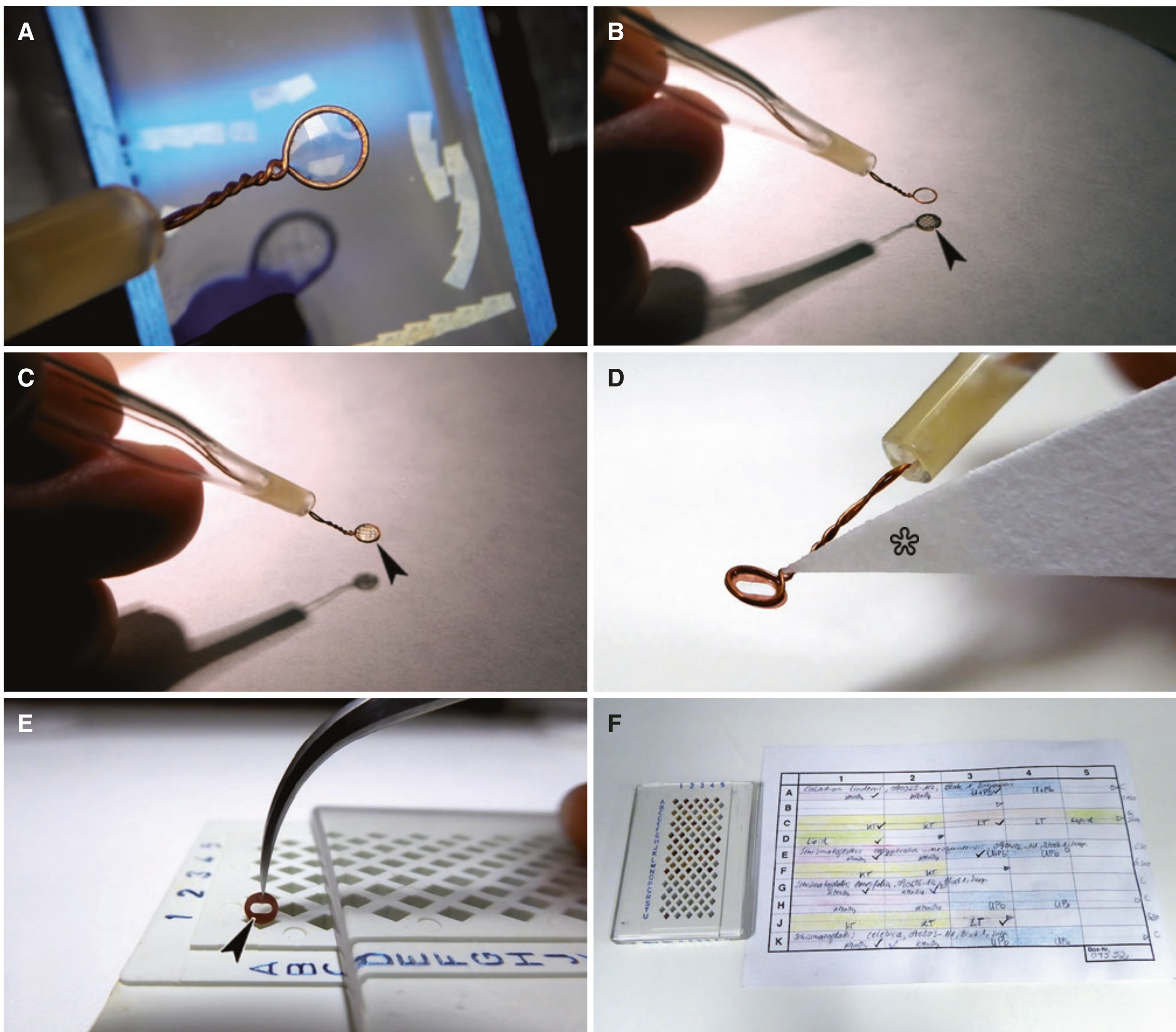


Fig. 20 Section pick-up. **A**. Loop for section pick-up. **B**. Loop centered above grid (arrowhead). **C**. Grid attached to loop (arrowhead). **D**. Water removed from grid, asterisk indicates wet filter paper. **E**. Dry grid placed into grid-box, sections on the left side (arrowhead). **F**. Grid-box and section protocol with color code used for different staining methods

The Lipid Test for the Detection of Unsaturated Lipids: TCH + SP

The endexine can be differentiated from the ektexine and the intine by thiocarbohydrazide-silver proteinate (TCH+SP) staining in osmium-fixed material. The endexine stains electron dense after the lipid test, indicating lipidic compounds (Fig. 22 B).

Ultra-thin sections on gold grids are treated with 0.2% TCH for 8–15 h and 1% SP for 30 min and thoroughly washed in water (3 times for 5 min in a row of water drops) (Rowley and Dahl 1977; Weber 1992).

Thiéry-Test: PA + TCH + SP

The Thiéry-test is used for the detection of neutral polysaccharides in osmium-free material (Thiéry 1967). Ultra-thin sections from osmium-free material are placed on gold grids and treated with 1% periodic acid (PA) for 45 min, 0.2% thiocarbohydrazide (TCH) for 8–15 h, and 1% silver proteinate (SP) for 30 min (Thiéry 1967). The polysaccharide intine and starch grains in amyloplasts stain electron dense (Fig. 22 C). For control samples leave out the thiocarbohydrazide step. If osmium fixed material is

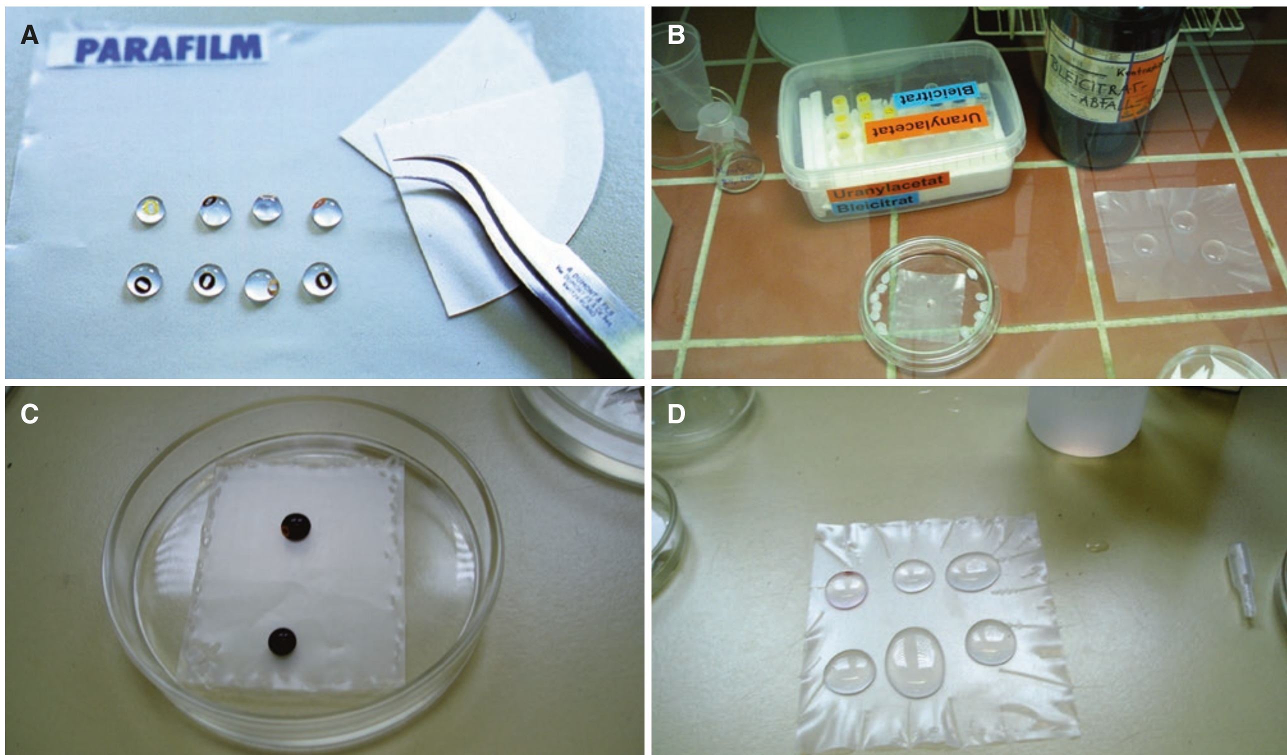


Fig. 21 Staining methods for ultra-thin sections. **A**. Ultra-thin sections on copper or gold grids stained in a small drop of uranyl acetate on parafilm. **B**. Small drops of lead citrate on parafilm and sodium hydroxide pellets in a closed petri dish. **C**. Small drops of potassium permanganate on parafilm. **D**. Row of large water drops for washing placed on parafilm

used for the Thiéry-test, the staining time for 1% periodic acid has to be prolonged up to 60 min (instead of 30 min), to remove the osmium tetroxide from the material.

Modified Thiéry-Test: PA + TCH + SP (short)

The modified (short) Thiéry-test (Weber and Frosch 1995) is especially effective after fixation of specimens with osmium and potassium ferrocyanide and is a good method for general enhancement of contrast in the cytoplasm and the pollen wall (Fig. 22 D). Ultra-thin sections are collected on gold grids and stained with 1% periodic acid (PA) for 10 min, 0.2% thiocarbohydrazide (TCH) for 15 min, and 1% silver proteinate (SP) for 10 min (at room temperature). After all steps the sections are thoroughly washed in distilled water (3 times for 5 min in a row of water drops), and following the TCH first washed in 3% acetic acid.

Potassium Permanganate: $KMnO_4$

Potassium permanganate staining is a simple method for the detection of the endexine. Using uranyl acetate and lead citrate, ektexine and endexine may differ in their electron opaqueness in that the endexine is higher in electron density than the ektexine, or vice versa. When the endexine is thin and less compact or discontinuous, the differentiation of the two layers may be insufficient. Typical for the endexine is its increasing thickness close to the aperture. Potassium permanganate stains the endexine electron dense, producing a distinct contrast (Weber and Ulrich 2010; Fig. 22 E). Ultra-thin sections from osmified material on copper grids are treated with 1% aqueous potassium permanganate solution for 7 min and thoroughly washed in water (3 times for 5 min in a row of water drops).

Preparation of Fossil Pollen

There are numerous methods currently used to extract organic material, including fossil and subfossil pollen, from all different types of sediments (rocks) and soils. These methods have been summarized in detail by, e.g., Erdtman (1943), Brown (1960), Fægri and Iversen (1989), Moore et al. (1991), Wood et al. (1996), and Traverse (2007). Most of these preparation methods involve sieving of some sort and the final production of palynomorphs enclosed in

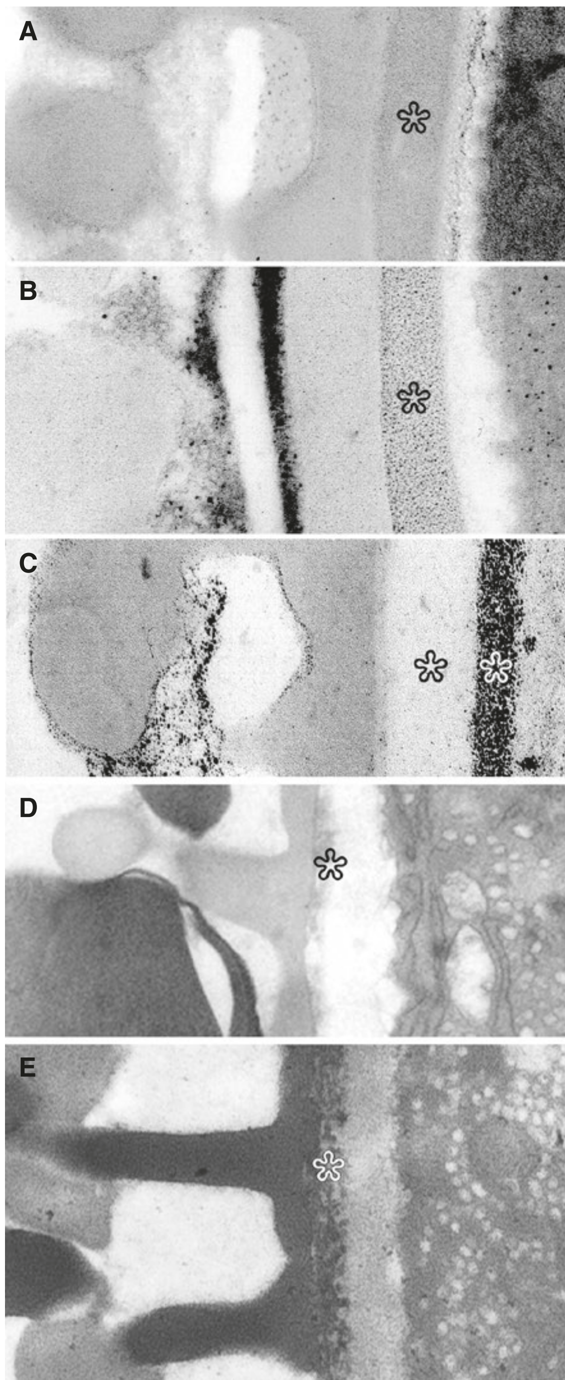


Fig. 22 Stained pollen walls and behavior of endexine (cross-section, TEM). **A-C**. *Apium nodiflorum*, Apiaceae. **A**. Uranyl acetate + lead citrate (U + Pb), compact-continuous endexine (asterisk). **B**. Lipid test (TCH+SP). compact-continuous endexine (asterisk) stains electron dense. **C**. Thiéry-Test (PA + TCH + SP), compact-continuous endexine (asterisk) stains electron translucent, intine electron dense (white asterisk). **D-E**. *Mentha aquatica*, Lamiaceae. **D**. Modified Thiéry-Test (PA + TCH + SP), thin compact-continuous endexine (asterisk) only slightly visible. **E**. Potassium permanganate ($KMnO_4$), thin compact-continuous endexine (asterisk) electron dense

glycerine gelatine on sealed glass-slides. Majority of paleopalynological studies then focus on counting the quantity of each pollen type observed on the slides (often between 300 and 600 grains), with an unfortunate minor emphasis on pollen morphology and ultrastructure. The following preparation procedure has been used by the paleopalynology team at the University of Vienna for over 30 years and is suitable for most sedimentary rocks with minor variations. During preparation the solution is not sieved at any stage, so not to lose any small or exceptionally large palynomorphs, and the final solution is stored in glycerine suspension in small sample tubes so the palynomorphs can be studied using the so-called "single grain method." This method has been evolved to able researchers to obtain pollen characters from single fossil grains using both LM and SEM and sometimes TEM.

Preparation Method: From Rock to Palynomorphs

Sedimentary rock samples (20–50 g) are washed and dried and hand ground in a mortar with a pestle (Fig. 23). Using a glass beaker the resulting powder is boiled in ≥200 ml of concentrated hydrochloride acid (HCl) for 5–10 min; this should remove all carbonates. Let the solution stand and when the residue has settled, decant most of the HCl liquid. Transfer the remainder of the solution into a copper pan or pot and add ≥150 ml of hydrofluoric acid (HF) and boil for approx. 10 min while stirring with a copper stick or spoon (or let stand in cold HF for 3–5 days, stir regularly, use acid-resistant plastic containers and tools); this should remove all the silicates. The solution is then poured slowly into a 4 L plastic beaker filled with water. After settling, the liquid is decanted and the remainder solution poured into glass beakers along with ≥200 ml of HCl and boiled again for 5–10 min; this prevents the formation of fluorite crystals. After cooling and settling decant most of the HCl and pour the remainder of the solution into two separate test tubes (glass centrifuge type). Wash the solutions 4 times with water and centrifuge and decant the liquid following each wash. Fill one large glass tube with cold water and add 1–2 teaspoons of sodium chlorate (pure crystalline powder; $NaClO_3$). Shake this large tube and when there are crystals that cannot be dissolved in the water the solution is ready. Pour ca 1 ml of acetic acid glacial (100%, CH_3COOH) and 3–4 ml of the sodium chlorate solution into the two original test tubes, then add five drops of HCl. Place the tubes in boiling water for at least 5 min and

Fig. 23 From rock to palynomorphs. **A**. Different types of sedimentary rocks: reddish, yellowish, and white-greyish samples usually contain few and/or badly preserved palynomorphs (back row), brown, dark-grey to blackish samples often contain well preserved pollen (front row). **B**. Sedimentary rock sample (ca 30 g) hand grounded in a mortar with a pestle. **C**. Sample boiling in ≥200 ml of HCl. **D**. Sample boiled in a copper pan with ≥150 ml of HF. **E**. The HF solution is poured slowly into a large plastic beaker filled with water. **F**. Organic material settled on the bottom of the beaker. **G**. Acetolysis, test tube in boiling water, note the stirring glass stick. **H**. Acetolyzed sample before decanting of water following the final wash

have a stirring glass stick in it at all times. The color of the sample solutions should change from dark blackish to brown or reddish. Centrifuge the test tubes and decant the liquid. Wash the residue 3–4 times with water and one last time with acetic acid glacial. Prepare a new solution in a clean and dry measuring glass-tube with 9 parts acetic anhydride (99%, $(CH_3CO)_2O$) and one part sulfuric acid (95–97%, H_2SO_4). Make sure to produce at least 10 ml of this solution for each original (fossil) test tube you process. Pour ca 10 ml of the new solution into each test tube. Direct tube away from your face and make sure no water comes into contact with the solution. Place the tubes again into the boiling water bath for at least 5 min. Then centrifuge and decant the liquid (again avoid contact with water). First wash the remaining residue once with acetic acid glacial, centrifuge and descant liquid, and then wash them 3–4 times with water. The remaining organic material in the test tube is finally mixed with glycerine and transferred, using pipettes, into small closable plastic test tubes. Test tubes are labelled accordingly.

The Single-Grain Method

A combined method for the investigation of fossil pollen grains was initiated by Daghlian (1982), suggesting that the same individual fossil grain should/could be observed in LM, SEM, and even TEM. This idea of how to properly investigate fossil pollen grains in a taxonomically valid way was taken further by Zetter (1989) who evolved a relatively easy method to investigate the same single fossil grain using the so-called "single-grain method," also described in Ferguson et al. (2007). To apply this method the following equipment and tools are necessary: samples prepared in the way described above, narrow glass-pipettes (see below, Fig. 24), teasing needle with an attached human nasal hair (see below, Fig. 25), an erect image compound microscope with a photographing unit, 10 and/or 20× objective lens with a minimum 10 mm working distance, glass slides, ethanol absolute, SEM stubs, sputter coater, and a functional scanning electron microscope.

Making Glass-Pipettes

It is important to have enough cheap and dispensable glass-pipettes to transfer parts of the sample from the storage tubes onto the glass slides for primary LM investigations. These pipettes are also used to make very small drops of ethanol on the surface

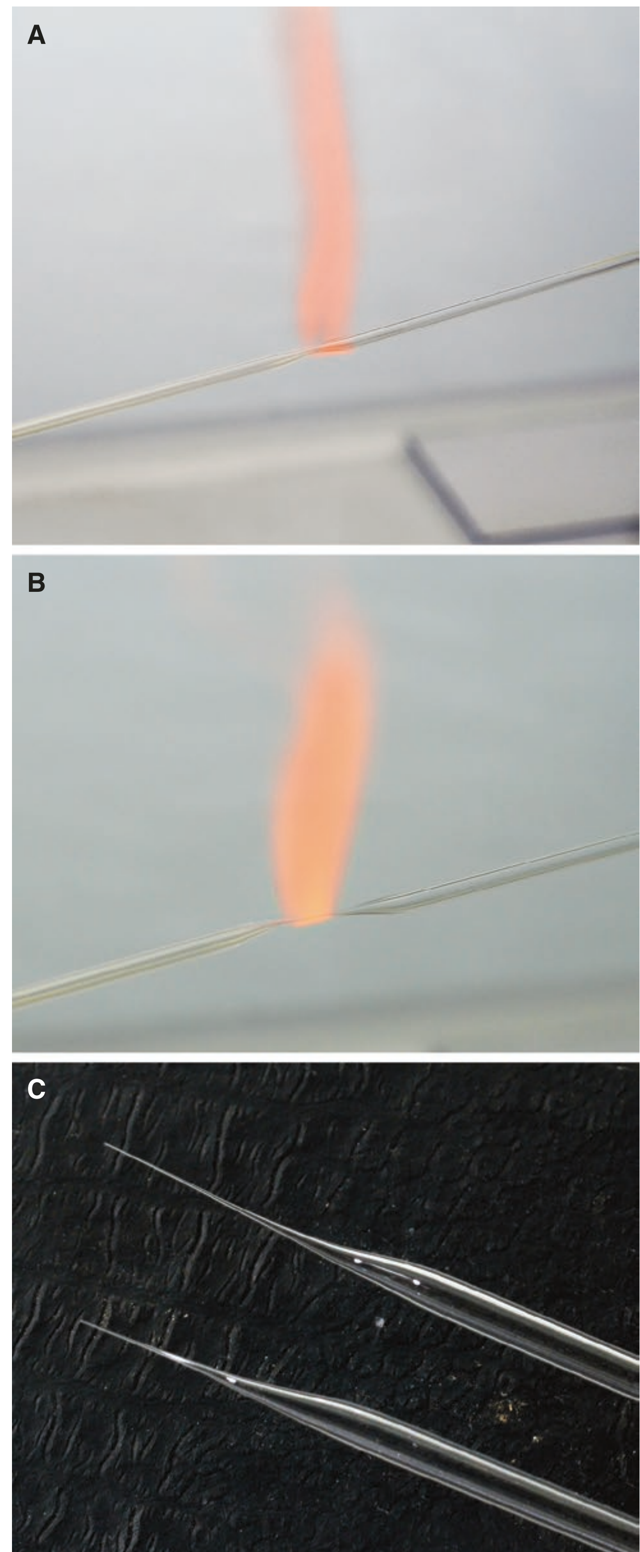


Fig. 24 Making glass-pipettes. **A**. Glass pipe held in burning gas flame starting to melt. **B**. Melting glass pulled very slowly and gently apart. **C**. Two freshly made pipettes ready for use

of the SEM stubs when transferring pollen from glycerine drops using the micromanipulator (see below

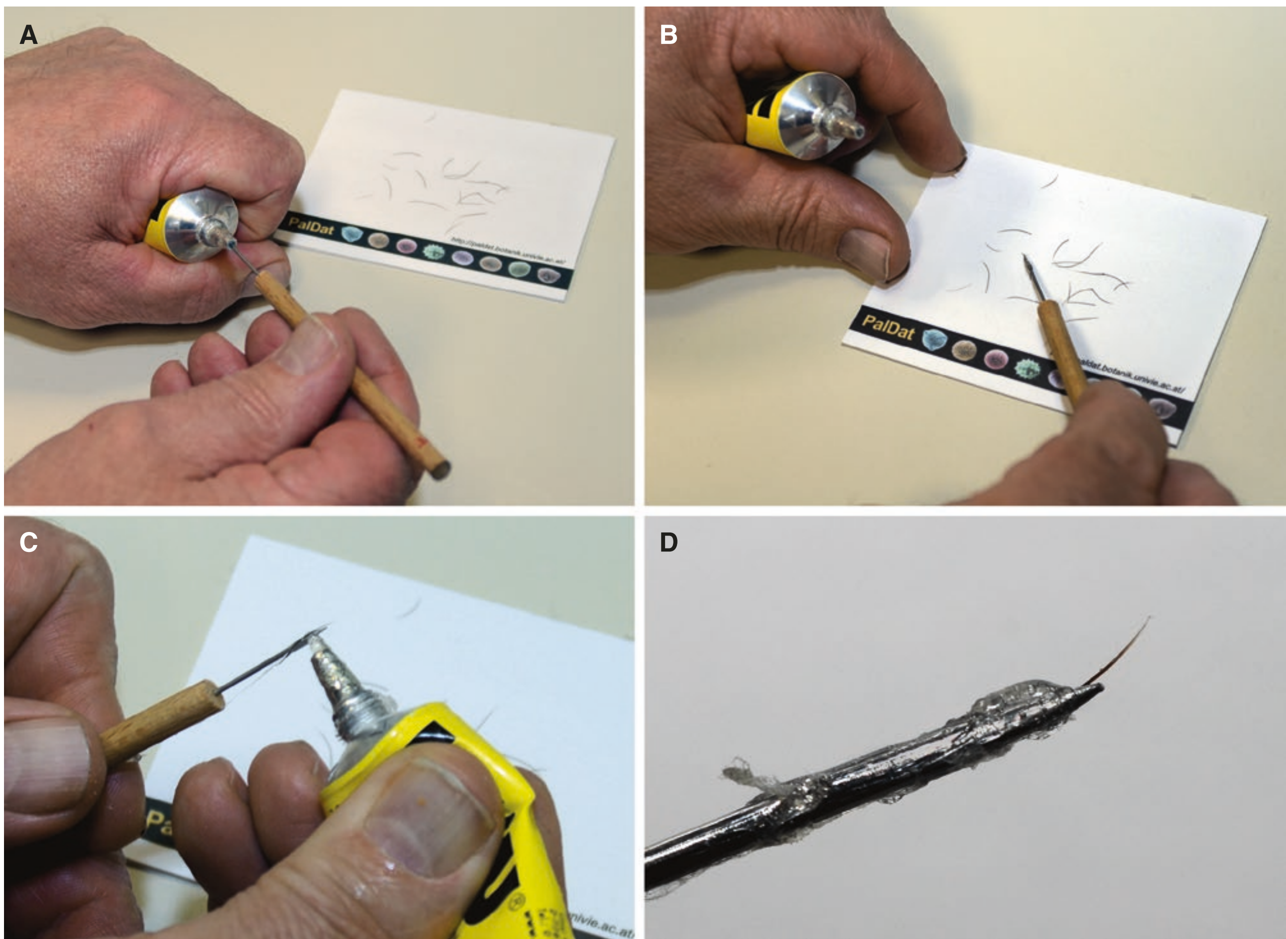


Fig. 25 Producing a micromanipulator. **A**. Needle pushed ca 1 cm into glue tube and turn in circles. **B**. Needle pressed onto a nasal hair. **C**. Extra glue added around the proximal part of the hair. **D**. Readymade micromanipulator

"Producing a Micromanipulator"). One possibility is to make your own pipettes (Fig. 24) by cutting down 4 mm wide glass pipes (cylinders) into ca 30 cm long units. The middle part of these is then held in/over a burning gas flame. While the glass starts to heat and melt you pull it apart from each end. The pipes will quickly give away in the middle as the glass melts. When pulled apart the glass will form two very long and narrow cones until they finally detach and one holds a perfect pipette in each hand.

Producing a Micromanipulator

The easiest way to make a really good and functional micromanipulator, that can be used to push around and pick out single pollen grains, is to attach a human nasal hair to a teasing needle (Fig. 25). Collect fresh nasal hairs from your professor or senior scientist (avoid the grey and white hairs) and lay them on a sheet of paper. Take a teasing needle and push it ca 1 cm into a glue tube while squeezing gently and turning the needle in circles. Pull out the needle and press onto one of the hairs already laying on the sheet of paper. Make sure that the distal end of the hair is facing the same way as the distal end of the needle and that it extends a few mm longer than the needle. When the hair is attached to the glue, add a little extra glue to cover the proximal part of the hair. Place the needle across the small opening of the glue tube, then press the tube gently for additional glue and at the same time turn the needle in circles while moving it back and forward.

Applying the Single-Grain Method

Use one of the self-made glass-pipettes to stir the sample and blow air through it to mix up the particles real good. Then suck up a tiny portion of the sample using the pipette and transfer onto a glass slide. When the tip of the pipette touches the glass slide drag the pipette along the middle section of the slide (left to right) to produce a long and relatively

narrow glycerine strip. Using an erect image compound microscope (meaning when something is moved under the objective lens from left to right it is also seen moving in that same direction when observed through the eyepiece) place the glass slide under the special working distance 10× or 20× objective lens and move the distal end of the micromanipulator in-between the glass slide and the lens and then gently press the tip of the micromanipulator (the nasal hair) into the glycerine (Fig. 26 A-B). Using the micromanipulator grains of particular interest are brushed or pushed to the edge of the glycerine, then out of the glycerine until they are attached to the nasal hair and can be picked up and transferred to another glass slide (Fig. 26 C-H). Have a fresh drop of glycerine ready on a new glass slide. Dip the tip of the hair with the attached pollen into the glycerine drop and the pollen will automatically detach from the hair and rest in the glycerine. Because no cover slip is used this pollen can now be turned around with help from the micromanipulator and photographed in polar and equatorial views as well under different foci (high-, low focus, optical section), documenting important features such as sculpture, apertures, and thickenings or thinnings of the pollen wall (Fig. 27 A-D). After this, the pollen grain is transferred to a SEM stub to which a drop of absolute ethanol has been added to remove all traces of the glycerine from the surface of the pollen grains (Fig. 27 E-G). For this, the best way is to position the light microscope close beside a binocular stereoscope. Place a single SEM stub under the stereoscope and have a small container with fresh ethanol at your side as well as one of the glass-pipettes mentioned above. First pick out a pollen grain with the micromanipulator from the glycerine drop and slowly move over to the stereoscope. Dip the tip of the pipette into the ethanol container and it will automatically suck up a small portion of the ethanol. Press the tip of the pipette on the surface of the SEM stub to leave a tiny drop of ethanol. Then gently press the tip of the nasal hair with the attached pollen into the drop of ethanol and the pollen will be detached from the hair, float a bit in the drop and finally rest on the stub surface when the ethanol evaporates. Try to make the ethanol drops small and close to the center of the SEM stub. Up to 10 different types of grains can be placed on a single stub and additional ethanol drops can be added to clean the glycerine thoroughly off the pollen grains. The stub is then sputter coated with gold and the pollen photographed using a SEM (overviews and close-ups). Pollen of particular interests can be turned. Add a drop of ethanol to the sputtered sample and flip the grain over using the micromanipulator before the ethanol evaporates (under the stereoscope). Re-sputter the sample and photograph it again using the SEM. This applies especially to any kind of heteropolar pollen/spores or tetrads of some sort.

Recipes

Recipes for Light Microscopy (LM)

Acetocarmine (Staining)

30 g acetocarmine + 2 L 45% acetic acid, 4 h boiled and filtered.

Potassium Iodine (Lugol's Iodine, Detection of Starch)

2 g potassium iodine + 1 g iodine + 100 ml distilled water

Toluidine Blue (Staining)

0.1 g Toluidine blue + 100 ml 2.5% sodium carbonate ($NaCO_3$); durable at +4 °C

Chlorination Mixture

Acetic acid (CH_3COOH) + saturated sodium chlorate ($NaClO_3$)* + 3–5 drops hydrochloric acid (conc. HCl)

*Saturated sodium chlorate solution: about 10 g of $NaClO_3$ in 10 ml distilled water (25 °C); the solution is saturated when crystals are still present.

Annotation: solubility of sodium chlorate is depending on the temperature of water.

Acetolysis Mixture

Acetolysis mixture: 9 parts acetic anhydride (99%) are mixed with 1 part concentrated sulfuric acid (96%).

Zinc Bromide Solution (Heavy Liquid Separation for Samples with a High Mineral Content)

250 g zinc bromide (Merck 8.18631.0250) + 25 ml 10% HCl*, mix until all zinc bromide is solved (takes some time!), then add 100 ml distilled water.

*10% HCl-Lösung: 27 ml H_2O + 10 ml HCl (37%) = 37 ml 10% HCl

Recipes for Scanning Electron Microscopy (SEM)

Dimethoxypropane (Dehydration)

30 ml 2,2-dimethoxypropane (DMP) + 1 drop 0.2 n hydrochloric acid (HCl)

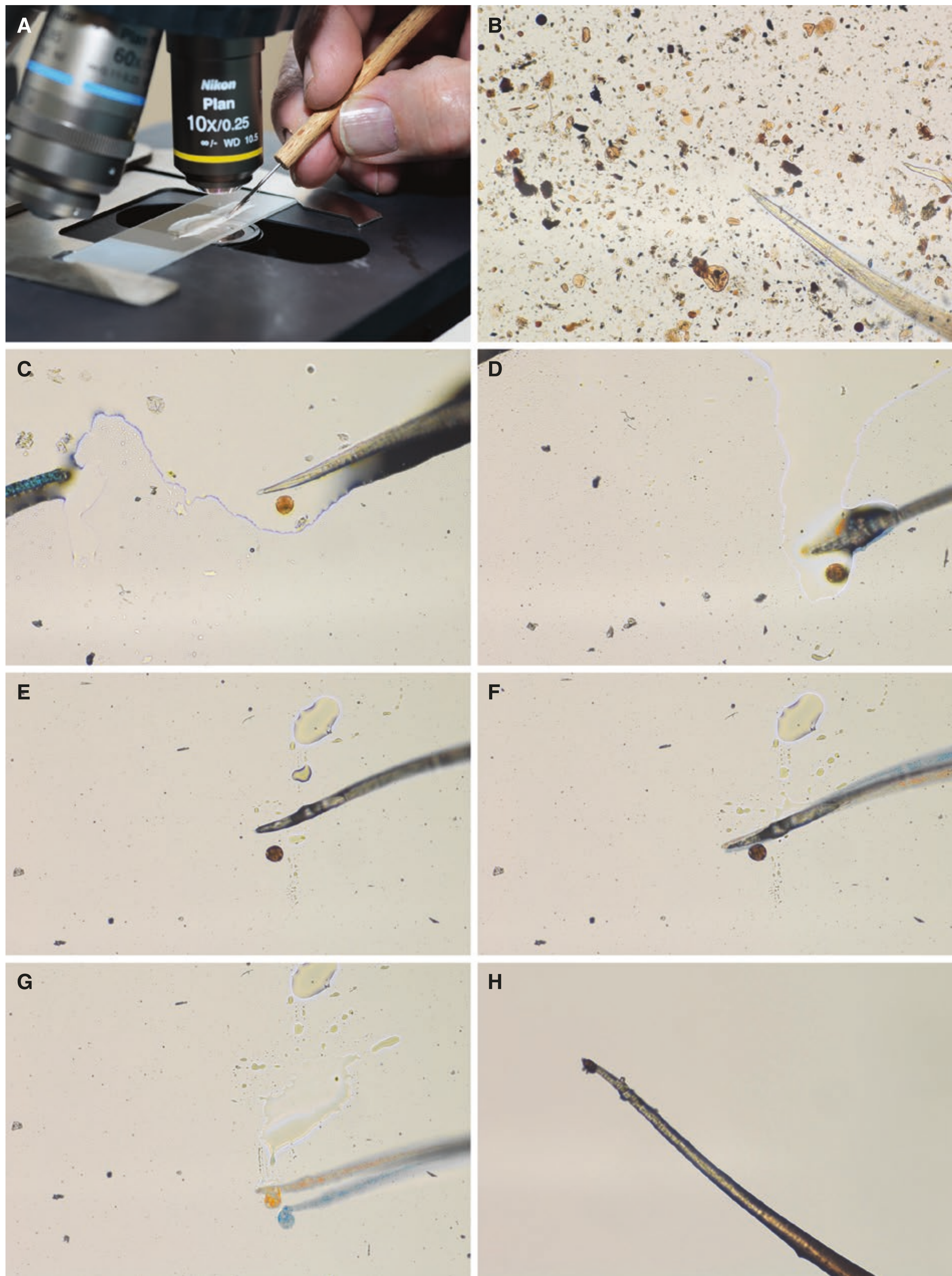


Fig. 26 Applying the single-grain method — Part 1. **A**. Sample on a glass slide under LM, working distance from sample to objective approx. 1 cm. **B**. Organic-rich sample and the tip of a nasal hair seen through the LM. **C**. Fossil pollen grain being brushed/pushed towards the margin of the glycerine. **D**. Fossil pollen grain pushed further away from the glycerine. **E**. Grain out of glycerine and ready to be picked up by the nasal hair. **F**. Pollen pushed a bit further. **G**. In a pushing or brushing motion the pollen is picked up from the glass slide. **H**. Single fossil pollen grain attached to tip of nasal hair

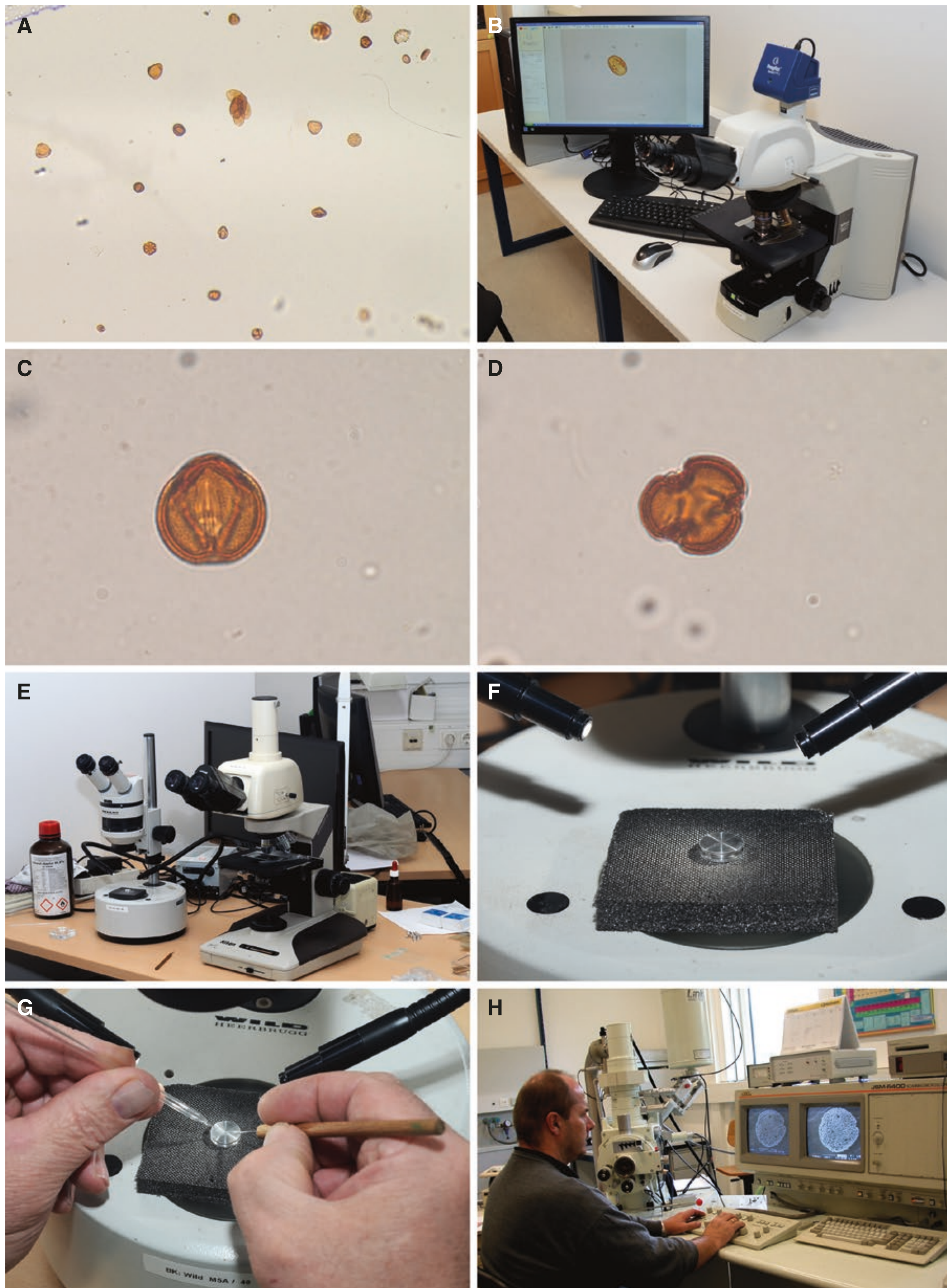


Fig. 27 Applying the single-grain method — Part 2. **A**. Selected well-preserved pollen grains in a fresh drop of glycerine. **B**. Light microscope equipped with a photographic unit to document pollen grains and their diagnostic features. **C**. Pollen turned and photographed in equatorial view. **D**. Same pollen grain turned and photographed in polar view. **E**. Arrangement of the light microscope and stereomicroscope along with a bottle of ethanol and other tools used when transporting pollen grains from glass slides over to SEM stubs. **F**. Cleaned SEM stub under a stereomicroscope waiting for fossil pollen grains. **G**. How to hold the pipette with the ethanol (left) and the micromanipulator (right) when transferring fossil pollen grains onto SEM stubs. **H**. Photographing fossil pollen using SEM

Recipes for Transmission Electron Microscopy (TEM)

3% Glutaraldehyde (Fixation)

100 ml glutaraldehyde: 12 ml glutaraldehyde (GA, 25%) + 88 ml phosphate buffer (pH 7.2).

1 % Osmium Tetroxide (Fixation)

0.1 g osmium tetroxide (OsO_4) + 10 ml distilled water.

Osmium can be acquired in crystalline form within glass ampullae. The osmium crystals usually adhere inside the ampulla and can be loosened by dipping the ampulla in liquid nitrogen (in a styrofoam box). The ampulla can then be opened and the osmium crystals transferred into distilled water in a vial. Close the vial and seal it with parafilm. For faster dissolution, place the vial in an ultrasonic bath. Mix the osmium solution and pipette it into a vapor-tight bottle. Store it at 6 °C.

*Annotation: osmium is volatile and toxic, use in fume hood only; for storage, use oil with high percentage of unsaturated fatty acids (e.g. corn oil) to bind volatiles of osmium tetroxide (*Fig. 28*).*

Phosphate Buffer pH 7.2 (Fixation)

1 phosphate buffer saline tablet (phosphate buffer saline tablets, $Na_2HPO_4 \cdot 2H_2O$, sodium hydrogen phosphate) + 200 ml distilled water (dispense tablet in ultrasonic bath).

0.8% Potassium Ferrocyanide (Accelerator for Osmium)

0.1 g potassium hexacyanoferrate (II) ($K_4Fe(CN)_6 \cdot 3H_2O$) + 12.5 ml distilled water (dispense in ultrasonic bath).

Annotation: the fresh solution is uncolored and becomes yellow after a few days.

Agar Low Viscosity Resin Kit (Embedding)

LV-resin (Agar Scientific): 48 g LV Resin + 8 g hardener VH1 + 44 g hardener VH2 + 2.5 ml accelerator.

Annotation: Mix the embedding solution in a disposable plastic beaker by using a magnetic stirrer. The first two components must be mixed first before adding the remaining ingredients, then mix well again. The mixture can be used immediately for infiltration and then for embedding. Embedding solution can be stored in a freezer.

Potassium Iodine (Staining)

3 g potassium iodide + 7 g iodine + 100 ml ethanol (92%).

Fig. 28 Osmium storage. **A**. Osmium solution stored at 6 °C (fridge placed in a fume hood). **B-D**. Osmium solution in a sealed bottle and stored in a plastic container, plastic container placed in glass vessel containing oil. **C**. Arrowheads showing osmium contamination from volatiles. **D**. Second glass vessel placed over the osmium containers, osmium vapor is bound to the oil and cannot escape into the atmosphere

1% Potassium Permanganate (Fixation and Staining)

1% potassium permanganate: 1 g potassium permanganate in 100 ml distilled water

1% Periodic Acid (Staining)

1 g periodic acid (PA, Firma Fluka) + 100 ml distilled water

0.2% Thiocarbohydrazide (Staining)

0.2 g thiocarbohydrazide (TCH, by *Serva*) + 100 ml 20% acetic acid (20 ml 100 % CH_3COOH + 80 ml distilled water)

1% Silver Proteinate (Staining)

0.25 g silver proteinate (SP, by *Merck*) + 25 ml distilled water

Uranyl Acetate (Staining)

Prefabricated solution: "Ultrostain 1" by Leica

Lead Citrate (Staining)

Prefabricated solution: "Ultrostain 2" by Leica; used with potassium hydroxide pellets

Formvar Filming Solution (Film-Coated Grids)

2 g formvar (15/45 E) + 100 ml chloroform (pure); mix with a magnetic stirrer

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