JOHN E. SASS

Botanical Microtechnique

Botanical Microtechnique

JOHN E. SASS
Professor of Botany
Iowa State College

THIRD EDITION

The Iowa State College Press, Ames, Iowa

Second Edition, 1951
Third Edition, 1958

Library of Congress Catalog Card Number: 58-13416

Preface

Permanent slides for microscopic study are indispensable in the teaching of a basic course in botany and also in specialized advanced courses. In some advanced courses, the students prepare many of the slides used in the course, but in elementary courses the slides are furnished. In the latter case, the slides either are purchased from commercial sources or made in the departmental laboratory. Biological supply houses make excellent slides of the subjects commonly used in elementary teaching, but the quality is likely to be variable. Jobbing houses that purchase slides from constantly changing sources also may furnish disappointing slides at times.

The relative merits of making slides and of purchasing them must be decided on the basis of local conditions. Uncertainties in the commercial supply and the need for specialized or unlisted items necessitates the preparation of slides in the biological departments of schools. This service work often is performed by a skilled professional technician with more or less supervision by the departmental staff. In other departments a member of the teaching staff, usually a morphologist, assumes this responsibility, with the aid of student assistants.

Some research organizations maintain a technician for the preparation of research slides. There are many types of investigation in which it is possible for the technician to prepare and place the finished slides before the investigator, who then carries out the study and interpretation of the material. However, in many investigations, some or all steps in the preparation require an intimate knowledge of the history, structure, and orientation of the material and the aims of the study. The use of a technician who allegedly merely "turns the crank" is then less valid, and the so-called technician may in fact be a research collaborator. The investigator in any field of plant science is urged to utilize microtechnique as a tool, but to do so critically and intelligently and in proper fairness to the workers who contribute their skill, patience, and understanding to the furtherance of the research. It cannot be too strongly emphasized that in order to

have a proper appreciation of the possibilities and limitations of present-day techniques, and to utilize the services of commercial or institutional technicians to best advantage, every teacher and investigator in the biological sciences should be familiar with at least the elements of microtechnique. We can do no better than to quote the late Dr. Charles J. Chamberlain, the dean of American microscopists: "The student who has not had sufficient experience to make a first-class preparation for microscopic study cannot safely interpret slides made by others. He is in the same class with the one who claims he sees it but can't draw it; while the real trouble is not in his hand, but in his head."

The term *histology* is very commonly misused to imply histological methods or technique. Histology means the study of the structure and development of tissues, and does not refer to the preparation of slides. A good textbook of histology need not contain a word about sectioning and staining of tissues. A person who takes an afternoon off and learns to whittle some fair freehand sections is neither a histologist nor a technician.

Botanical microtechnique may be defined in terms of its functions, which fall into the following overlapping categories:

1. the preparation of plant tissues for microscopic study.
2. the skillful use of the microscope and related equipment for the critical study and interpretation of the material.
3. the recording and illustrating of the results by means of the graphic arts.

In some schools microtechnique is taught as part of the work in some branch of morphology, such as anatomy or cytology. That system has marked advantages. The student who has collected and processed his own plant materials, and made his slides, can visualize the orientation of the sections in the plant and interpret the relationship of parts to the whole plant. A disadvantage of the system is that specialized courses in morphology are likely to utilize a limited number of methods — for instance, the smear method in cytogenetics. The student may acquire remarkable skill in making preparations of one type and have no experience with other useful methods. He may develop great skill in making smear preparations of pollen mother cells, but one cannot smear a kernel of corn or a pine stem. He may even acquire disdain for methods which versatile and experienced workers regard as indispendsable.

The maintenance of a separate course in microtechnique makes possible the presentation of the fundamentals of useful standard

methods, which experience has shown to be the backbone of research and which have long served the routine needs in teaching. A course should be organized to give a systematic, graded series of exercises, each exercise pointing to some definite objective and yielding superior preparations of a given type. Student interest can be maintained by working with plants that are of interest to the student or the institution, and with plants that are characteristic of the region. It is not desirable to permit the student to interject his research problem into the course. The integrity of the teaching functions of the course could be compromised by using it as a device to do the research of the student, the teacher, or a colleague.

This manual has evolved over a period of years in connection with the teaching of a college course in botanical microtechnique. The author is not a technician, he is a teacher and researcher in plant science. It is not his function to train technicians, but to contribute to the training of future teachers and researchers in plant science.

Since this is primarily a training manual rather than a reference work, use is made of a graded series of assignments, beginning with subjects in which orientation is easily visualized, few sectioning difficulties are encountered, and a simple stain is used. Subsequent assignments require greater skill in the processing, sectioning, and differential staining of cell and tissue components. A few carefully selected processing and staining methods are presented in detail. Emphasis is placed on gaining an understanding of the aim of the undertaking and the function of every operation, rather than on memorizing and mechanically following a written outline or numbered jars. After mastering the fundamentals, the worker can readily delve into the literature of specialized fields by consulting the brief bibliography. The advanced worker will use the excellent texts by Johansen (1950) and Gray (1954), and in particular the comprehensive bibliography in the latter.

The drawings in this book were made by Miss Ruth McDonald. Grateful acknowledgment is made for a grant from the Graduate College for the preparation of the first edition. The author's colleagues and students have given much valuable criticism, advice, and encouragement.

JOHN E. SASS

IOWA STATE COLLEGE
August, 1958

Contents

Part I
General Principles and Methods

1. Introduction

The study of the microscopic details of the structure of plants usually requires some preparation of the material to facilitate observation. Unicellular, filamentous, or other minute plants require comparatively little preparation. The material may simply be mounted on a slide in a drop of water and thus studied, even under considerable magnification. Larger plants, or parts of plants, must be dissected or cut into thin slices in order to expose inner regions and to permit light to penetrate through the object. Some materials have enough natural coloration to be visible even when finely divided or sectioned. Highly transparent or colorless structures, on the other hand, must be made visible by the use of stains. Preparations that are to receive considerable handling over a period of time should have some degree of permanence. The desirable properties of microscopic preparations are, therefore, adequate thinness, coloration or refractile visibility, and permanence.

The processes used in the preparation of plant materials for microscopic study can be roughly classified in the following categories:

1. Unicellular, filamentous, and thin thalloid forms can be processed *in toto* – without sectioning – and mounted as "whole mounts" to make temporary or permanent slides.

2. Some succulent tissues can be crushed or smeared into a thin layer on a slide or cover glass. The preparation is then stained and treated to make temporary or permanent slides.

3. The more complex and massive tissues are usually sliced into very thin slices, freehand or with a microtome. Materials that are not sufficiently rigid to be cut without support are embedded in a supporting matrix before sectioning. The sections are stained and mounted to make temporary or permanent slides.

The method used for the perparation of a given subject depends on the character of the material, the use that is to be made of the

slides, and such facilities as equipment, reagents, and time. The experienced worker does not overstress the merits and applicability of some one method. For example, important advances in smear methods and related processes for the study of nuclear and chromosome details have replaced to some extent embedding and sectioning. The whole-mount method is recognized to be entirely satisfactory for many algae, fern prothalli, and similar subjects. However, microtome sections of embedded material must be made if we wish to study the undisturbed cellular organization of a tissue, the development and arrangement of organs, or the structural relationship between a fungus or insect parasite and the tissues of its host. The much-maligned celloidin method must be used to keep intact a badly decayed, fungus-infected piece of oak railroad tie for an examination of the mycelium in the wood. In order to avoid undue emphasis on any particular method, we should recognize that each of the well-established methods has its proper sphere, in which it is the most effective and economical method of performing a given task.

The sequence in which processes are arranged in this book takes cognizance of the fact that the paraffin method furnishes by far the largest number of slides produced for teaching and research. Certain operations, such as the killing of protoplasm and the preservation of fixation images, are essentially similar for smears, sectioning, and whole-mount methods. The preliminary processing of material is much the same in the several embedding and sectioning methods. In view of these facts, the paraffin method is presented with unbroken continuity of its operations.

2. Collecting and Subdividing Plant Materials

The preservation of structural details of cells and tissues is influenced by the condition of the plant at the time of collecting and by the subsequent preparation for killing (fixation). For the study of normal structure, select healthy, representative plants. Remove the plant or the desired part with the least possible injury to the sample. If the material is to be killed at once, follow the procedure outlined in Chap. 3. If the material cannot be killed promptly, it should be stored and transported in such manner that bruising, desiccation, molding, and other injuries are minimized. Do not use material that has been obviously damaged in storage or shipment. The unsatisfactory slides obtained from such material are likely to be interpreted by uncritical observers as the result of poor technique. Dried herbarium specimens can be softened and sectioned to make slides in which it is possible to determine the gross features of vascular arrangement or carpellary organization (Hyland, 1941). However, such material is not suitable for detailed microscopic study.

The following general directions are introduced at this point for the use of readers who have selected subjects on which to work. The reader who seeks suggestions concerning suitable and tested subjects should turn to Part II and use the recommendations made therein in conjunction with the present chapter.

LEAVES

Remove a leaf or leaflet by cutting the petiole, without squeezing or pulling the petiole. The vascular bundles in the petioles of some plants become dislodged easily. For transportation or brief storage, place the leaves between sheets of wet toweling paper and keep in a closed container such as a tin can or a Mason jar. If the leaves appear to be wilted on arrival in the laboratory, freshen them in a moist chamber or in water before processing.

STEMS

Leafy stems can be kept fresh for several days by standing them in a container of water, preferably in a refrigerator. If such storage is not practicable, cut the stems into the longest pieces that will fit into the available closed container without folding or crushing. Wrap the pieces promptly in wet paper and store in a cool place. Dormant woody twigs, large limbs, and disks cut from logs can be kept for weeks in a refrigerator without appreciable injury.

ROOTS

Do not collect roots or other underground organs by pulling up the plant. The delicate cortex is easily damaged, in fact, the woody stele may be pulled out of the cortex, leaving the cortex in the ground. To collect roots without damaging them, dig up the plant, soak the mass of soil in water until thoroughly softened. Wash the soil away carefully, cut off the desired roots and brush them gently with a camel's hair brush to remove as much soil as possible. Wrap the pieces and store as in the case of stems.

FLORAL ORGANS

Remove entire flowers or flower clusters and wrap in wet paper. Store in a closed container in a cool place. Large buds like those of lily can be kept in a Mason jar of water until you are ready to dissect and preserve them. Fruits may be collected and stored in a similar manner.

LIVERWORTS AND MOSSES

Remove groups or mats of the material with a generous quantity of the substratum. Store in a moist chamber until the plants are turgid. Saturate the substratum in order to permit the removal of complete plants without excessive damage. Dissect out the desired parts under a binocular and transfer to the preserving fluid promptly.

ALGAE

Collect in a quantity of the water in which the plants are growing, and keep in a cool place in subdued light. Many filamentous forms disintegrate rapidly in the laboratory, and even in the greenhouse unless the temperature and light can be carefully controlled. It is best to kill algae promptly after collecting.

FLESHY FUNGI

The larger fleshy fungi can be transported and stored, wrapped

loosely in waxed paper. Sporulation continues and may indeed be promoted in this manner. However, since molding and disintegration take place during prolonged storage, material should be processed promptly. Small fungi should be wrapped in moist paper, enclosed in waxed paper, and processed as soon as possible.

PATHOLOGICAL MATERIAL

Particular care should be exercised to insure that the condition of the host tissues is not altered by handling, in order that abnormal structure may be properly interpreted as an histological symptom of the disease. Prevent wilting of the material, or revive it in a moist chamber, but avoid the development of bacteria, molds, or other secondary organisms. For a pathological investigation, always collect normal, disease-free tissues of age comparable with the diseased samples. It is imperative to work out the best technique for preserving the "normal" condition of the host before attempting an authoritative interpretation of slides of pathological material.

The foregoing general remarks will serve as a basis from which the worker can develop effective methods and habits of collecting and handling material in accordance with facilities and circumstances. Hold rigidly to the view that the finished slide should represent the original structure of the plant, whether that structure is presumably normal or pathological or is the result of experimental treatment.

The handling of materials that are to be used for bulk specimens or whole mounts is described in Chap. 10. The preparation of permanent slides from microtome sections consists essentially of the following processes:

1. Selecting desired plants or parts of plants and, if necessary, subdividing into suitable pieces.
2. The killing and preservation of the contents of cells and the preservation of cellular structures in a condition approximating that in the living plant.
3. Embedding in a matrix if necessary, in order to support the tissues for sectioning. See page 91 for the sectioning of unembedded tissues.
4. Sectioning of the tissues into very thin slices.
5. Staining the slices and covering with a cemented cover glass to make a permanent slide.

Subdividing Material for Processing

Some preliminary remarks concerning the action of reagents in the preservation of cells and tissues will aid in understanding the following description of this process. The reagents used for killing

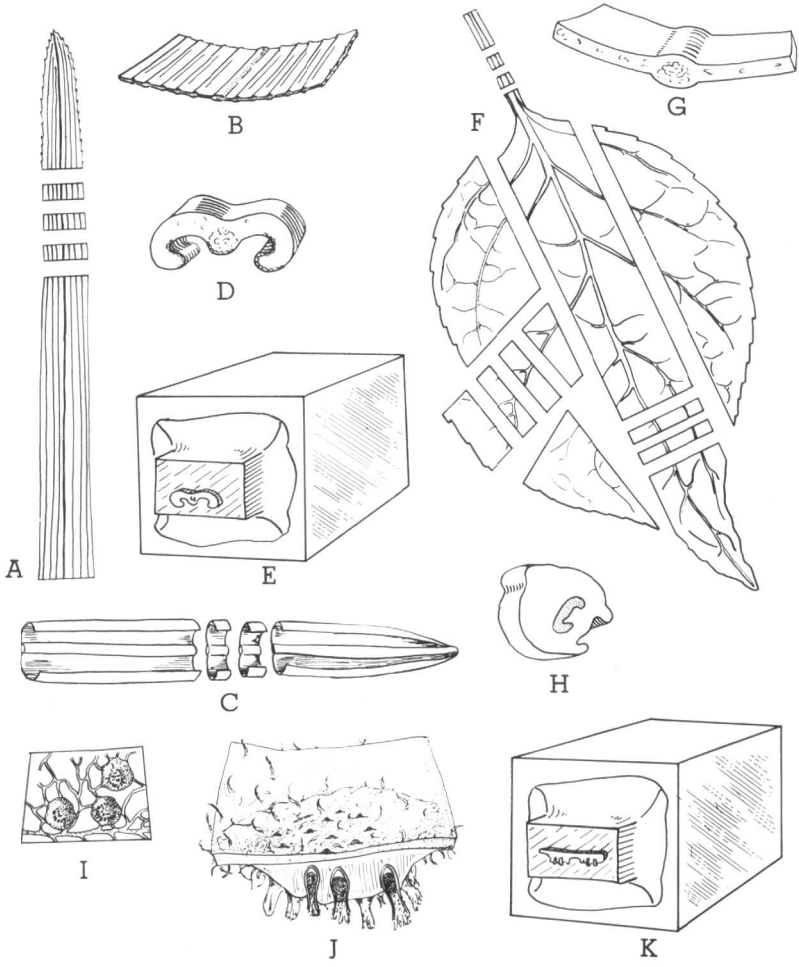

FIG. 2.1—Methods of subdividing leaves for embedding: *A–D,* long narrow leaves and transverse pieces removed from such leaves; *E,* embedded piece of leaf fastened to mounting block; *F–H,* large broad leaf and excised pieces of blade and petiole; *I,* portion of leaf with fungus pustules; *J,* enlarged view of excised aecia; *K,* embedded piece of leaf bearing aecia, fastened to mounting block.

cells contain ingredients that are toxic to protoplasm. In order to stop life processes quickly and without distortion of structure, the killing fluid must reach the innermost cells of a piece of tissue before disintegration takes place. Most reagents penetrate very slowly through the cuticle or cork on the surfaces of plant organs, but

penetration is much more rapid through cut surfaces. Therefore, it is desirable to cut the organ being studied into the smallest pieces that will show the necessary relationship of parts.

The subdividing of soft fresh material is best done with a razor blade, with the material placed on a sheet of wet blotting paper or held carefully against a finger. Excessive pressure against the support is likely to rupture delicate tissues as in the mesophyll of leaves (Fig. 11.1) or the chlorenchyma of a stem (Fig. 11.2). Such damage does not become visible until the sections in the ribbon are examined or possibly not until the finished slide is examined. The usual results are peeling of the epidermis and distortion of the crushed tissues.

Leaves are almost invariably cut into small pieces for processing. Narrow leaves that are not much over 5 mm. wide, may be cut into complete transverse pieces measuring 2 to 4 mm. along the rib (Fig. 2.1 *A–D*). Examples of this type are bluegrass, garden pinks, hedge mustard, and some narrow-leaved milkweeds. Broad leaves should be cut into small pieces, selected to include midrib, lateral veins, fungus pustules, fern sori, or other desired structures (Fig. 2.1 *F, G, I, J*). The enlarged views of the pieces of leaf (Fig. 2.1 *B, D, G, I, J*) and the pieces of embedded tissue mounted on blocks ready for sectioning (*E* and *K*) will aid in visualizing the orientation of pieces. Particular care should be used in subdividing pathological material (Fig. 2.1 *I, J*). If it is necessary to know which is the long axis of the leaf, cut all pieces so that the shorter dimension is along the long axis of the leaf, or vice versa, and record the method in your notes.

Herbaceous stems, roots, petioles, and other more or less cylindrical organs are usually cut into short sections or disks. When cutting out sections or subdividing pieces, do not roll or press the pieces. Keep the material moist, and work rapidly. After the final subdivision, drop the pieces into the killing fluid promptly. By means of descriptions and sketches, like those in Figs. 2.1 and 2.2, keep an accurate record of the part of the plant from which the pieces of tissue were obtained.

Figure 2.2 gives additional suggestions for subdividing organs. A stem that does not exceed 2 mm. in diameter should be cut into sections 2 mm. long if highly cutinized, but may be as long as 10 mm. if the surface is permeable. An organ 5 mm. in diameter should be cut into 5-mm. lengths. An organ 1 cm. in diameter should be cut into disks 2 to 5 mm. thick. Stems of larger diameter are usually cut into 5-mm. disks that are halved or quartered longitudinally or divided into wedge-shaped pieces.

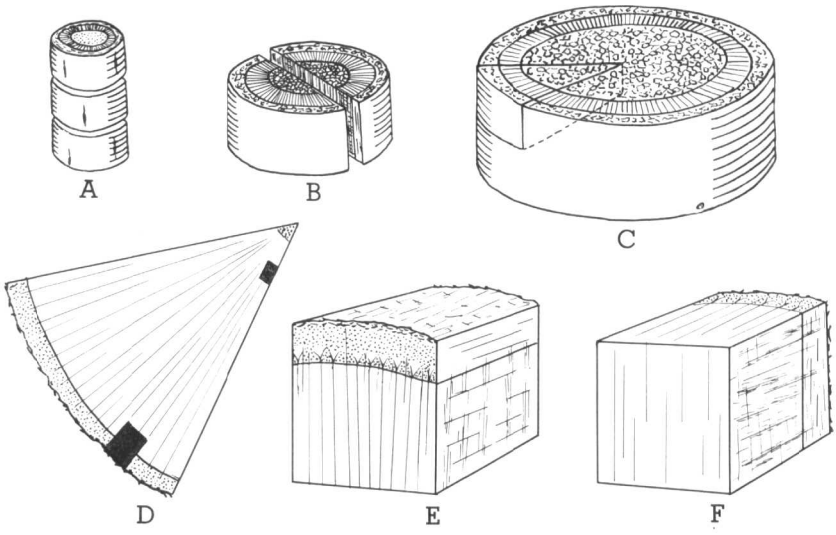

FIG. 2.2—Methods of subdividing massive cylindrical organs: *A–C*, sample includes portions of all tissues in the axis; *D* shows the position of pieces removed from a large log; *E* and *F*, enlarged views of trimmed pieces removed from large log.

Woody twigs having a diameter up to 5 mm. should be cut into 15-mm. lengths. Larger twigs should be cut into shorter pieces because the impermeable cork makes penetration by reagents difficult, except through the cut ends. Do not cut the twigs into pieces with pruning shears or a knife. Rough handling will bruise the cambium, phloem, the fragile primary cortex and cork cambium, resulting in the separation of the outer layers during sectioning or during staining. Use a razor blade and cut through the twig by chipping a groove deeper and deeper around the twig until it is cut through. An excellent tool for cutting twigs into sections is a fine-toothed high-speed saw, such as a rotary dental saw or a jig saw, especially the vibrating diaphragm type.

To make slides of transverse, radial, and tangential sections in the region of the cambium of old trees, use tissues removed from newly felled logs or limbs having a diameter of at least 10 cm. Cut disks 2 to 3 cm. thick from portions of the log that were not bruised in felling. Wrap the disks in wet burlap and take into the laboratory