Principles and Practice in PLANT CYTOLOGY

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FOREWORD

The preparation of this guide book was motivated by a desire to provide students with a book which would assist them in the preparation and interpretation of their own laboratory materials in plant cytology. Toward this end a plan of laboratory studies is presented together with a brief review of the current status of information in this field and of laboratory procedures of proven effectiveness.

Emphasis has been placed on chromosome cytology at the expense of extra-nuclear structures and reproductive processes because the latter are usually rather well covered in physiology, anatomy or morphology courses.

Most of the photomicrographs were made from slides prepared by students in our cytology course at Utah State Agricultural College whose contribution is gratefully acknowledged. They are included primarily to illustrate principles discussed in the text but in addition are representative of the quality of work which students in elementary cytology can be encouraged to produce.

The subject matter is designed for a one-semester or one-quarter course and assumes that the students already possess a firm foundation in general botany. Since intelligent use of the microscope and effective laboratory techniques are prerequisites for successful cytological investigations the first two chapters are concerned with these problems.

The writer is greatly indebted to Dr. M. V. Bradley of the University of California for critically reading the manuscript and making many valuable suggestions.

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Chapter I

USE OF THE MICROSCOPE

The serious student is obliged to learn how to use the microscope as effectively as possible in view of the fact that most cytological work requires the use of oil immersion objectives and very frequently demands the attainment of the greatest possible resolving power of which the instrument is capable. The purpose of this chapter is to acquaint the student with some fundamental principles and procedures toward this end.

The quality of a microscope is dependent upon its resolving power, i.e. its ability to disclose the component details of an object to the observer. The resolving power of a lens system such as an objective can be calculated from its Numerical Aperture. This is expressed mathematically: N.A. = n X sine $\frac{AA}{2}$ where "n" represents the medium of lowest refractive index between the condenser and the objective which may be air (1.0), water (1.33), cedar oil (1.5) etc. and "AA" (angular aperture) represents the angle of the cone of light entering the lens. It will be apparent therefore that an N.A. greater than 1 can only be achieved by immersing the objective and condenser in a medium of greater refractive index than 1, such as water or immersion oil. By the same reasoning the use of an oil immersion objective potentially capable of a 1.3 N.A. in which the operator omits immersion of the condenser or the objective will reduce the actual N.A. of the objective to approximately 1. It is of course necessary to use a substage condenser having an N.A. at least equal to that of the objective.

Unwarranted importance is frequently attached to magnification. It is quite without value as a means of increasing resolution. Magnification is of course necessary to provide a sufficiently large image to be resolved by the human eye but beyond a certain point (1000 X N.A. or less) additional magnification provides no increase in resolution. It is possible by means of a microprojector to magnify an image many thousands of times with an inexpensive microscope. The maghification however is not accompanied by increased resolution. The approximate magnification can be estimated by multiplying the magnifying power of the objective by that of the ocular. Accurate determination of magnification requires use of stage and ocular micrometers.

The objective is the most critical component of the optical system in the microscope since the quality of the image is very largely dependent upon the quality of the objective. There are three principal types of objectives: achromatic, "fluorite" and apochromatic. The major differences between them are largely concerned with the degree to which they are corrected for spherical and chromatic aberration. A lens which possesses spherical aberration will not be able, for example, to focus the light rays passing through the periphery of the lens at the same point as those passing through the center of the lens. A lens possessing chromatic aberration will be unable to focus all colors of the spectrum at a given point.

Achromatic lenses have spherical aberration corrected for one color and chromatic aberration corrected for two colors. Apochromatic lenses have spherical aberration corrected for two colors and chromatic aberration corrected for three colors. Apochromats permit attainment of higher N.A. and increased magnification compared to achromats. However, unless they are properly used the image they produce will be inferior to that of achromats. "Fluorite" objectives are thus named because they contain this mineral which has special properties of refraction not found in a single piece of glass. They possess a higher degree of color correction than achromats but are not equal to the apochromats.

The main function of the ocular is to magnify the image formed by the objective. The ocular may also function as a component of the correction system. There are two principal types: Huygenian and Ramsden. The former are found on most inexpensive microscopes and have a practical magnification limit of approximately 10 X. Ramsden oculars are compensating and should always be used with apochromatic objectives. These oculars are usually marked "compens" or "K". Hyperplane, orthoscopic, periplan and holoscopic are trade names for types which are not completely compensating but are more fully corrected than the Huygenian and are best used with achromats.

The minimum requirements in a microscope and accessories for general cytological work can be considered to be the following: (1) An achromatic oil immersion objective with an N.A. of at least 1.3, (2) a low power, dry, objective, (3) an intermediate power, dry, objective, (4) a substage condenser with an N.A. equal to that of the oil immersion objective, (5) a 10 X Huygenian ocular, (6) a plane mirror, (7) substage iris diaphragm, (8) coarse and fine tube adjustments, (9) mechanical stage, and (10) a microscope lamp which will permit the use of "Critical" or Kohler illumination.

For most research work the following equipment will be desirable and in many cases indispensable: (1) three apochromatic objectives: 16 mm. 10X dry; 4 mm. 45X dry, 2 mm. 90X oil immersion, the last with an N.A. of at least 1.3, (2) two or three compensating oculars (or ocular pairs if a binocular instrument is used) approximately 10X, 15X and 25X, (3) an aplanatic or preferably an acromatic-aplanatic, centerable, substage condenser with an N.A. of at least 1.3, (4) a mechanical stage with vernier scale, (5) a high quality microscope illuminator, (6) camera-lucida, (7) stage and ocular micrometers, (8) neutral and green filters to assist in controlling light intensity and for improvement of contrast in stained preparations.

While it is quite true that a binocular microscope provides no increase in resolving power compared to a monocular instrument most investigators agree that the ease of observation and absence of eyestrain are significant advantages accompanying use of the binocular microscope. A dissecting binocular will be found very useful. An inexpensive microscope will assist in observing stain differentiation in the paraffin method.

Illumination

Probably no other aspect of technique and equipment is so frequently abused by biologists as is the proper illumination of the microscope. Faulty illumination can reduce the effectiveness in performance of the fines microscope to that of a much inferior instrument.

Proper illumination requires the use of an illuminator possessing the following features: 1) an intense light source of sufficient size to illuminate the front lens of the condenser, 2) a condensing lens preferably with a focusing mechanism, 3) an iris diaphragm, 4) a means of centering the light source in the condensing lens, 5) means for holding filters, and 6) an inclination joint.

Methods of illumination can be broadly grouped into two general types: "Critical" and Köhler. There has been some confusion in the literature concerning the definition of these types of illumination, (see Schillaber, 1944, Munoz and Charipper, 1943). In the following discussion the sense in which these terms are used is as follows: In Köhler illumination the light source is focused at the level of the iris diaphragm of the substage condenser. In "Critical" illumination the light source is focused at the level of the object. There are of course other important distinctions but this will serve to clarify our terminology.

The type of illumination that the investigator should use is determined by the type of observations he wishes to make. It would be foolish, for instance, to painstakingly prepare your microscope and lamp for Köhler illumination if you wish simply to observe the gross structure of an anther section. On the other hand, when your work demands the greatest possible resolving power in the microscope no effort should be spared to obtain the best possible illumination.

For those observations not demanding maximum resolving power probably the most satisfactory type of illumination will be "Critical" illumination used with a ground-glass plate on the lamp. Directions for this follow:

"Critical" Illumination Using Ground Glass

1. Adjust the lamp for proper angle of inclination and height so that the beam of light is centered on the mirror. The plane mirror is always to be used if the microscope has a substage condenser.

2. Place a slide in position and focus on the object with the low power objective. Adjust the mirror to obtain maximum illumination.

3. Focus the condenser so that the grain of the ground glass is visible in the plane of the object. Now throw this slightly out of focus so that the grain does not interfere with the image.

4. Remove the ocular and while looking down the tube adjust the substage iris diaphragm to a diameter which will completely illuminate the back lens of the objective. To open it wider will introduce unnecessary glare into the optical system.

5. If the high, dry, objective is needed, revolve the nosepiece until this objective clicks into position (provided the instrument is parfocal; see p. 10). Adjust the substage iris diaphragm as described in #4.

6. To use the oil immersion objective the safest procedure is the following: Raise the tube and rotate the low power objective into position. Lower the condenser. Place a drop of immersion oil on the

top lens of the condenser (distilled water can be substituted at a slight loss in resolution). Re-focus the condenser in the manner described in #3. Accurately center object in the field. Raise the tube approximately ½ inch. Place a drop of immersion oil on the slide over the point of light visible. Turn the oil immersion objective into position. Now hold your head to one side of the microscope in order to observe the level of the objective and slowly lower the tube with the coarse adjustment until the objective lens just contacts the drop of oil. A slight flash of light can be observed at this point. While looking into the ocular, slowly and most carefully focus downward with the fine adjustment (NEVER the coarse adjustment) until the image comes into focus. Adjust substage iris diaphragm as described in #4. Check focus of condenser for adjustments as in #3. It is most important that the back lens of the objective be completely illuminated.

Intensity of illumination should be controlled by filters and/or a rheostat on the lamp. Lowering the condenser will also decrease intensity of illumination but inevitably at the expense of resolution.

"Critical" Illumination Without Ground Glass

The procedure here is basically the same except that the true light source---the tungsten coil or ribbon filament of the lamp is focused by the condenser at the level of the object rather than the ground glass which served as a secondary light source.

Since this is a much more precise method of illumination and one which is capable of providing superior illumination when properly used additional care should be taken to precisely center the substage condenser with the objective, to center the light source in the lamp and to more precisely align the lamp with the microscope.

Probably the simplest method of centering the condenser in the absence of a specialized equipment is by focusing on the substage condenser iris. Focus on a specimen slide with the low power objective and a low power ocular. Raise the substage condenser to its top position. Close the substage iris diaphragm to its smallest diameter. Slowly raise the tube with the coarse adjustment until the image of the substage iris diaphragm comes into focus. By means of the centering screws adjust this image until it is in the center of the field.

To center the light source in the lamp: open the iris diaphragm of the lamp. Move the condenser lens of the lamp away from the light source. Insert a piece of paper over the opening of the iris diaphragm. By means of the centering screws center the illuminated area in the iris diaphragm.

Köhler Illumination

This system of illumination is to be preferred when maximum resolution is required and especially for photomicrography. (Refer to Chamot and Mason, 1939, Schillaber, 1944 for discussion of theoretical considerations). It is distinguished from other systems in the focusing of the light source and condenser: the light source is focused at the level of the substage iris diaphragm and the iris diaphragm of the lamp is focused, by the condenser, at the level of the object. Direc-

tions for this method follow:

1. Properly center the light source in the lamp and the substage condenser with the objective as previously described.

2. Place the lamp at a distance from the microscope which will insure complete illumination of the condenser lens (approximately 10" or more, depending upon the light source).

3. Focus the light source (ribbon filament or coil) in the plane of the substage iris diaphragm. The simplest method to accomplish this is to insert a piece of stiff, white paper into the filter holder against the substage iris and by means of the focusing knobs on the lamp or by moving the lamp, focus the filament directly on this paper. The lamp will of course be adjusted for proper inclination and height so as to place the image centrally on the mirror.

4. Properly adjust the mirror so that the light source is reflected into the substage condenser.

5. Place a slide in position and focus on the specimen with the low power objective.

6. While looking into the microscope, adjust the position of the substage condenser so that the iris diaphragm of the lamp is in focus. This probably will not be precisely in the center of the field and a slight movement of the mirror will be necessary to bring this about.

7. Now bring the oil immersion objective into position and focus it as previously described.

8. Remove the ocular and look down the tube. The back lens of the objective should be completely illuminated. Quite probably the light source will not be precisely centered in the back lens. To correct this, raise, adjust inclination, or lower the lamp to center it.

9. Repeat the adjustments of the mirror and inclination of the lamp as described in #6, #8 until the back lens of the objective is completely illuminated and the image of the light source is correctly centered and fills the aperture, which should appear as a brilliant, evenly illuminated disc.

10. Adjust the diameter of the substage iris diaphragm as described for "Critical" illumination. Adjust the diameter of the lamp diaphragm so that the field is just completely illuminated, not wider.

When the microscope is thus properly illuminated for oil immersion work it can be observed that the low power objective will not be completely illuminated. Since ordinarily the most important observations will be carried on with the oil immersion objective this will not be serious, since the low power objective is used chiefly to locate and center the object in the field. Complete illumination of the low powered objective can be achieved, however, by removing the top element of the concenser providing it is of the dismountable type.

General Rules to be Constantly Observed

In Using the Microscope

1. Always handle the instrument with great care. Carry it in an upright manner. Place it on the desk or in the cabinet very gently.

2. It is imperative that the lenses and mirror be kept absolutely clean. They should be cleaned with a special camels hair brush used for no other purpose and lens paper only. First remove the dust with the brush then gently wipe the lens with lens tissue moistened with a drop of xylol. Excessive xylol may soften the cement between the lenses.

3. Focus in an upward direction whenever using the coarse adjustment. Only the fine adjustment should be used when focusing downward. Never allow the objective to touch the slide.

4. Before changing to a higher powered objective by rotating the nosepiece first determine whether the instrument is parfocal. A microscope is parfocal if the objectives can be interchanged by rotating the nosepeice and are each approximately in focus without raising or lowering the tube. The safest way to determine this is as follows: Focus on a specimen slide with the low power objective. Insert a sheet of lens paper between the objective and the slide. Carefully begin to rotate the high power objective into position. Meanwhile keep the lens paper in horizontal motion and if the high power objective makes contact with the slide it can immediately be detected before any damage occurs. Such objectives must be focused separately.

5. Never use alcohol on any part of the microscope. The cement between the lenses and frequently the finish on the microscope is alcohol-soluble.

6. Remove immersion oil promptly after use. Gently wipe off the bulk of the oil with lens paper followed by use of another sheet slightly moistened with xylol.

7. Completely cover the instrument with a protective hood or preferably a plastic bell jar when not in actual use.

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Chapter II

CYTOLOGICAL MICROTECHNIQUE

The purpose of the following discussion will be to describe some of the most basic and useful techniques available to the student for the study of the structure and behavior of chromosomes. Proficiency in these techniques may be considered essential even for routine work and a basis upon which the advanced student may build and modify according to the specialized needs which inevitably arise.

The Aceto-Carmine Technique

The aceto-carmine technique was developed by John Belling (1926) and has proved to be of inestimable value. Despite its simplicity this technique is most useful and effective with a wide range of plant and animal materials. It has been the basis of the technique used during the course of discovery of some of the most important principles in biological science.

The stain is prepared by adding 0.5 grams of certified carmine dye to 100 ml of boiling, 45%, glacial acetic acid. Many technicians continue boiling the solution for several minutes or longer interval in a reflux condenser. After rapid cooling, the stain is filtered and is then ready for use. For many types of materials it is desirable to add several drops of 45% acetic acid saturated with ferric acetate. This intensifies the stain in the chromosomes. It also usually induces some precipitation of the carmine necessitating occasional filtration.

Since aceto-carmine can act as a fixative as well as a stain it is entirely feasible to smear fresh material immediately. However, fixation before staining usually results in a superior preparation and this method will be described in detail.

A. Technique for Anthers

A fixing solution which has proved satisfactory for a wide range of plant materials consists of 3 parts absolute ethyl alcohol and 1 part glacial acetic acid (Farmer's fixative). Superior fixation is obtained if the anthers are dissected out and dropped into the fixing solution. Fixation of whole buds or young inflorescences may prove satisfactory if the surrounding tissues do not appreciably retard penetration of the fixing solution. The material should remain in the solution overnight or for 15 minutes at 60 C. If the material is to be stored for any length of time it should be transferred to 70% alcohol. Several changes of 70% alcohol at intervals of several hours increases the length of time anthers can be stored without undue deterioration in staining quality in many plants. Obviously, freshly fixed material will yield the best results. Another fixing solution that has wide application consists of absolute alcohol, chloroform and acetic acid in the proportions 4:3:1 or 6:3:1. The latter are known as Carnoy's fixatives.

Select an anther, place it momentarily on a blotter to absorb adhering liquid and mount in a small drop of aceto-carmine on a slide. With a scalpel cut the anther in two transversely. Turn one anther-half so that the two cut ends are together, the halves lying side by side. With a rolling motion of the scalpel gently force the contents of the anthers into the stain. Discard the empty anther sacs. If the anthers are small it will be necessary to carry out the above operations under a dissecting microscope.

Examine the slide under the low power objective. If the desired stage is not present clean the slide and select another anther, smaller or larger, depending on the stage desired. If the correct stage is obtained, push the sporocytes or pollen grains into the center of the drop. Heat very gently over an alcohol lamp. Too much heat will induce precipitation of the stain. Now affix the cover glass. Add additional stain if the area under the cover glass is not filled. Heat gently once more and place slide on a blotter in preparation for flattening.

The amount and type of pressure to be applied on the cover glass will vary widely depending on the type of material and stage of development and can only be determined by trial and error methods. In any case care must be taken to prevent lateral movement of the cover glass. The most common method is to hold down one corner of the cover glass by means of a folded blotter with one hand and apply vertical pressure with the other hand by means of a cork, eraser-end of a pencil, handle of a dissecting needle etc. In smearing pollen mother cells with large numbers of chromosomes it is frequently helpful to tap the cover glass with the blunted tip of a dissecting needle directly over a group of sporocytes or pollen grains.

A satisfactory temporary seal can be made by dipping a hot, bent wire into a sealing compound composed of equal parts of paraffine and gum mastic and applying it to the edge of the cover glass. Such temporary mounts may last for weeks if the seal remains unbroken. The stain is frequently better the following day. Subsequent time usually results in darkening of the stain in the cytoplasm as well as in the chromosomes. Observations should be made from such temporary slides within at least a day or two following their preparation. Permanent mounts can be made from these temporary ones in a manner to be described later in this chapter.

There are of course innumerable minor variations in the technique just described. The student is referred to Smith (1947), LaCour (1947), Darlington and LaCour (1942), Johansen (1940) and others listed in the references for additional discussion and details.

A well prepared slide should have the tissues spread in a very thin layer preferably not over one cell layer thick, the chromosomes intensely stained, well separated, arranged in the same plane and the cytoplasm colorless or lightly stained. Naturally such a preparation is ordinarily obtained only with the most painstaking technique. Such skill demands intelligent practice. Students should not begrudge the time and skill necessary to produce a first class slide. Since interpretation is the goal much more time might be spent in attempting to interpret the chromosomes of an inferior, carelessly made slide.

B. Technique for Root Tips

The preparation of root tip smears by the aceto-carmine method is basically similar to that described for anthers. They do however frequently require additional treatment between fixation and smearing.

Plants which can be stimulated to produce roots in water cultures, such as onions, furnish the most easily available material. Onion bulbs for example are simply placed atop a beaker with the water level reaching the lower portion of the bulb. Roots are produced usually in 3-4 days and meristematic activity is most pronounced during their first inch of growth.

Root tips are easily obtained from potted plants provided that growth conditions are satisfactory. To obtain the root tips invert the pot and while supporting the plant and soil, gently tap the edge of the pot upon the bench. The compact soil will usually slip away from the pot, exposing the root tips. Roots from pot-bound plants or those otherwise not in a healthy condition are usually unsatisfactory for study. Germinating seeds supply an excellent source of root tips.

With forceps carefully break off the last $\frac{1}{4}$ inch and drop into the fixing solution. Fixing solutions and procedures described above for anthers are applicable here. Cut off the last 1-2mm. of the root tip and mount in aceto-carmine. If the root tip is large it may be helpful to cut it lengthwise or transversely into smaller portions and smear only a portion at one time. By means of a scalpel spread the tissue in a thin layer. Heat, flatten and seal as described for anthers.

The smearing of root tips of many plants is facilitated by Warmke's (1935) modification of the aceto-carmine technique. Hydrolysis by hydrochloric acid breaks down the middle lamella between the cells thus permitting the tissue to be more easily flattened in a thin layer. With this technique the root tips are removed from the fixing solution and immersed in a macerating solution consisting of equal parts of 95% alcohol and concentrated hydrochloric acid for 2-10 minutes. Wash in several changes of 50% alcohol. Smear as before. Root tips of some plants, particularly grasses, may require maceration in concentrated HCl for 1-2 minutes.

Another helpful modification (Meyer, 1945) facilitates study of gross chromosome structure and number by prefixation in paradichlorobenzene. This seems to prevent excessive clumping of the chromosomes and stimulates contraction of the chromosomes in some plants. Pretreat the root tips in a saturated aqueous solution of paradichlorobenzene for 1-4 hours. Fix in 65% acetic acid, 3:1 alcohol-acetic or Carnoy for 12-24 hours. Transfer to 10% hydrochloric acid by volume in distilled water for 10-30 minutes. Wash 2-3 times in distilled water. Smear as before.

Pretreatment with 8-hydroxyquinoline at 0.001 to 0.003 M. concentrations has recently been reported by Tjio and Levan (1950) and Schreiber (1951) to be very effective. The treatment results in clarification of chromosome morphology and facilitates spreading by inactivation of the spindle and contraction of the chromosomes.

The use of colchicine (Darlington and LaCour, 1942), enzymes (McKay and Clarke, 1946), section smears (Warmke 1941) are other modifications which are very helpful with some materials.

Meristematic tissue other than root apices, such as young leaves, stem apices, anther walls etc., are sources of mitotic divisions readily adaptable to the aceto-carmine technique and in fact are frequently more readily available than root tips.

Temporary mounts can be made permanent by two principal methods depending on the type of mounting medium used. The simplest method is a modification of Burrell's (1939) schedule and consists of the following steps: 1) carefully pare off the sealing compound, 2) soak slide in a solution consisting of equal parts acetic acid and absolute alcohol until cover glass floats off, 3) carry both slide and cover glass through two changes of absolute alcohol, one or two minutes in each, 4) mount in a drop of Euparal or diaphane.

If balsam or other xylol-soluble mounting medium is to be used the method of Buck (1935) has wide application: 1) soak slide in a solution of equal parts absolute alcohol, xylol and acetic acid. Rinse for five minutes after the cover glass floats off, 2) carry through two changes of equal parts absolute alcohol and xylol for 5-10 minutes each, 3) 10 minutes in xylol, 4) mount in balsam. This method is basically similar to that described by McClintock (1929).

Another method widely used for graminaceous plants utilizing tertiary butyl alcohol is recommended by Sears (1941) and described in more detail by Smith (1947).

A very useful method for making aceto-carmine smears permanent without removal of the cover glass has been designed by Bradley (1948) utilizing the vapor exchange technique. Quite frequently in making mounts permanent by previously described methods much valuable material is lost or overlapped in the process. This difficulty is eliminated with Bradley's method although it requires considerably more time. The procedure is summarized as follows: 1) if the preparation is a sealed temporary one, carefully pare away the sealing compound and remove remaining traces of it with xylol, special care being taken to wipe off xylol and dissolved seal, 2) if necessary, destain either freshly made or previously sealed preparations with 5 parts 50% acetic acid plus 1 part aceto-carmine. A drop of this mixture is placed at the edge of the cover glass and allowed to penetrate the mount. After warming the slide gently a blotter is placed over the slide and gentle pressure exerted to press out excess liquid. If the preparation is still too dark repeat this procedure, 3) place the slide end-on in a closed alcohol vapor chamber for 4-6 hours. A vapor chamber is prepared by lining a jar with paper towels saturated with 95% ethyl alcohol, 4) remove slide from the vapor chamber and allow a few drops of absolute alcohol to run underneath the cover glass, then press out the alcohol, 5) allow several drops of diluted diaphane or Euparal (thinned with alcohol) to run underneath the cover glass, 6) place slide in a second alcohol vapor chamber prepared by adding 10-15 drops of absolute alcohol on a blotter in a Petri dish. This facilitates entrance of the mounting medium. It should remain here about 24 hours, 7) permit slide to remain at room temperature for 3-4 weeks to allow mounting medium to dry slowly.

It should be explained that strictly speaking, the term "smear" refers to the procedure where the tissue to be examined is spread onto the slide before fixation and staining in contrast to the "squash" method in which the material is fixed and stained before spreading. Since the difference is not a profound one especially in terms of end results the term smear is frequently used to cover both techniques.

The Feulgen Technique

The Feulgen stain is the most specific nuclear stain known. When properly used it stains only the chromosomes and not the cytoplasm or nucleolus. It was originally introduced by Feulgen and Rossenbeck (1924) as a microchemical test for the demonstration of desoxyribose nucleic acid in tissues and is based on the Schiff reaction for aldehydes.

The basic technique has been subjected to innumerable modifications for smears, squashes and sections on a wide variety of plant and animal materials. The discussion here will be restricted to the Feulgen squash method for root tips originally based on Heitz' (1936) method and adapted from LaCour (1947) with minor modifications.

The stain is prepared in the following manner: Pour 200 ml. distilled, boiling water over 1 gram of basic fuchsin. Shake 5 minutes. Cool to 50 C. and filter into a brown stock bottle. Add 30 ml. normal HCl. Add 3 grams potassium metabisulphite ($K_2S_2O_5$). Allow solution to decolorize for 24-48 hours in a stoppered bottle in a dark place. Add 0.5 gram decolorizing vegetable charcoal. Shake for approximately a minute and filter rapidly. Store the stain in the dark. Keeping qualities are improved if stored in a refrigerator. If it turns red it is worthless. Often old but still colorless stain is worthless.

Fix root tips in 3:1 absolute alcohol-acetic for 2-24 hours at room temperature or at 60 C, for 10-15 minutes. Transfer to vial of normal HCl at 60 C. for hydrolysis for 5-15 minutes. (Many dicotyledonous plants with small chromosomes require up to 30 minutes hydrolysis.) Handle carefully after this stage since the tissues will be soft and fragile. Stain in the leuco-basic fuchsin for 30 minutes to 2 hours. Divide the meristematic region into small sections or fragments. Place section on a slide in a drop of 45% acetic acid, spread in a thin layer and apply cover glass. Warm slide over alcohol lamp. Flatten gently, allowing no lateral movement of cover glass. The preparation can be temporarily sealed or made permanent by methods described for the aceto-carmine method.

When the Feulgen stain is used for tissues which resist maceration such as root tips of graminaceous plants Schreiber (1951) suggests fixation in 45% acetic acid.

Attainment of consistent success with the Feulgen technique is not easy. The type of fixative used, duration of hydrolysis, variations in the stain plus the varying responses of different organisms and tissues together present a formidable number of variables that are very difficult to control. Probably the most important single source of difficulty is attainment of optimum hydrolysis. Achievement of this depends very largely on trial and error methods.

Although the paraffine method is an indispensable part of cytological microtechnique a detailed discussion of it here is outside the intended sccpe of this chapter. Students are referred to Johansen (1940), Sass (1950) and LaCour (1947) for fundamentals of this method.

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Chapter III

THE LIVING CELL

Although most of our attention in these laboratory studies will be devoted to the behavior and structure of the chromosomes it is imperative that such studies be fortified with an understanding of basic cell structure and functions. In all probability advancements in our knowledge on such fundamental problems in cytology as the nature of the gene and its duplication and modification, chromosome structure and movement and the general biochemistry of transmission genetics are likely to come from cytochemical investigations rather than expansion of the rather well developed descriptive approach. Neyertheless, an understanding of the descriptive aspects of cytology is a prerequisite for advanced study involving the more experimental aspects.

A representative living plant cell is bounded by a cellulose wall which encloses the living substance of the cell, the protoplasm. Within the protoplasm are found one or more nuclei, plastids, mitochondria and various inclusions. The protoplasm outside the nucleus constitutes the cytoplasm. In mature cells there is usually a large central vacuole containing a watery solution in which salts, sugars and various other substances are dissolved or dispersed. In undifferentiated cells there are usually several much smaller vacuoles. All surfaces of the cytoplasm are probably bounded by differentially permeable membranes.

It is beyond the intended scope of this chapter to consider in detail the structure of protoplasm and the cell wall. It is most important however to emphasize the dynamic and extremely complex nature of protoplasm. Although it appears simply as a transparent, somewhat viscous, substance it possesses most remarkable powers of synthesis, reproduction, assimilation, and growth. Protoplasm has an extremely complex colloidal structure and consists principally of molecular aggregates termed micelles, proteinaceous in nature, dispersed in an aqueous medium. In addition to the protein components there may be carbohydrates, fats and fat-like compounds, and inorganic salts of various kinds.

Among the most conspicuous structures in the cytoplasm are the plastids. Three types of plastids are conventionally recognized: chloroplasts which are green, chromoplasts which are commonly red, orange or yellow, and the colorless leucoplasts. Such a classification is of course an artificial one since a given plastid may become a leucoplast, chromoplast or chloroplast depending on many factors in the differention of the cell and ontogeny of the plant.

In the higher plants chloroplasts usually appear as somewhat flattened elipsoidal bodies approximately 5-10 microns in length. Chloroplasts in the green algae on the other hand may be extremely elaborate structures. The structure of chloroplasts remains a controversial problem. A concept favored by many investigators is the presence in the chloroplasts of disk-like bodies, the grana, impregnated with chlorophyll and embedded in a proteinaceous mass, the stroma (Weier and Stocking, 1952). They are probably bounded by a membrane.