THE ARCHITECTURE OF LIVING CELLS—RECENT ADVANCES IN METHODS OF BIOLOGICAL RESEARCH—OPTICAL SECTIONING WITH THE ULTRA-VIOLET MICROSCOPE

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In papers[1,2,3] presented elsewhere during the past few years, the development and application of the ultra-violet microscope to the science of metallography has been described.

Metallography, at first thought, appears wholly unrelated to histology or other branches of biology but the two branches of science do have many points in common. Both deal in the last analysis with the structure of matter and, in each, the microscope is an indispensable tool. Improvements in microscopic vision which enlarge the world of vision in one branch of science inevitably have a reflection in the other.

It is not the purpose in this paper to enter into a discussion of structures of living cells as revealed by the ultra-violet microscope. More particularly, the object is to present a tool for biological research; a tool which enables us to photograph the structure at different planes or levels within a single cell or group of cells; one which enables us to see the living cell with a degree of precision and clarity not heretofore possible by any other known means and with a potential resolving ability at least twice that of the best apochromatic system using visible light.

We are now able to take a series of photographs at slightly different focal planes or levels through a single cell or group of cells. These photographs result in a consecutive record of the internal architecture of the cell or cells. This accomplishment we have called "optical sectioning."

Nearly thirty years ago,[4] Dr. A. Köhler of the Zeiss Scientific Staff developed the ultra-violet microscope for use in the field of biology. It has a potential resolving ability twice that of the best optical system using visible light. The equipment was made available to scientists by the firm of Carl Zeiss and some sporadic work was done by different investigators but no outstanding accomplishment has appeared to its credit. Difficulty in manipulation and inability to focus the equipment seem to have reacted against the widespread use of the ultra-violet microscope.

Nearly ten years ago, following the development of high power metallography and the utilization of the full potential resolving ability of the best apochromatic systems using visible light, Bell Telephone Laboratories arranged with the Zeiss Scientific Staff to design an ultra-violet microscope suitable for metallography and for transparent specimens.

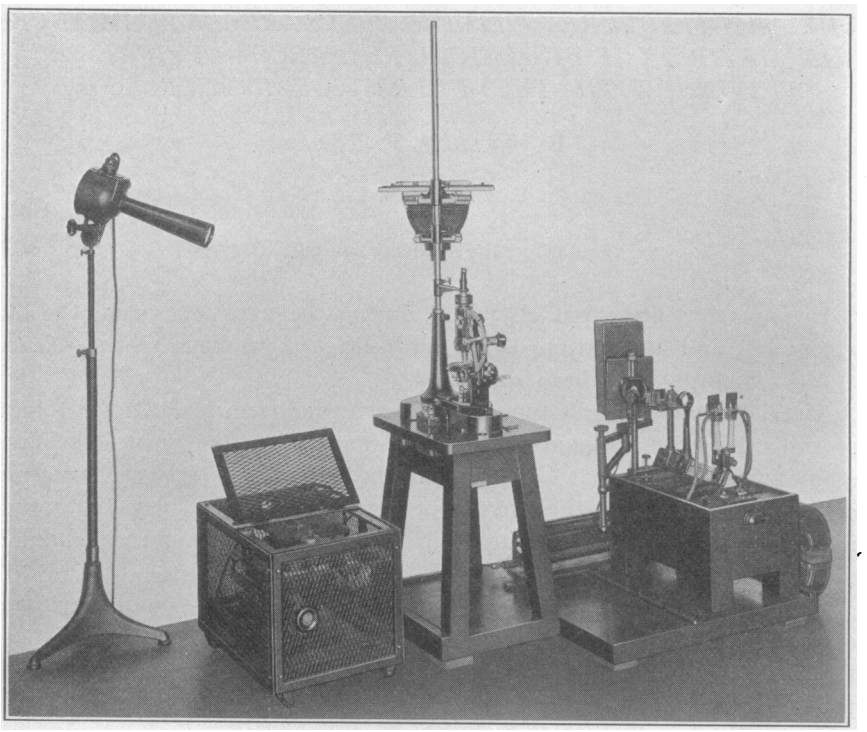


FIGURE 1

ULTRA-VIOLET MICROSCOPE ARRANGED FOR OPTICAL SECTIONING OF LIVING CELLS

The combined sound and fume hood is shown removed from the spark generating apparatus and the searcher eyepiece is in position above the ocular of the microscope.

The ultra-violet microscope is a monochromatic system corrected for wave length—275$\mu\mu$. The optical parts are of quartz. Monochromatic light is necessary due to lack of suitable optical materials with which to correct the objectives for chromatic differences. The source of illumination is a spark gap and a quartz slit. Cadmium electrodes are used. The potential at the electrodes is 10,000 volts secured from a primary source of 220 volts at 60 cycles by means of a step-up transformer. The light passes through a collimator and two 60° prisms to emerge from the prism or field diaphragm in the form of a line spectrum. The desired line (λ 2748.67) enters a prism at the base of the microscope and is directed upward into the substage condenser.

Since we are dealing with invisible light, the optical image cannot be seen in the ordinary way. It must either be photographed or visualized on some sort of a fluorescent screen. The fluorescent screen takes the form of an artificial eye, which is a uranium glass wedge incorporated in a small optical system and mounted interchangeably with a camera just above the ocular. The surface of the wedge on which the image is to be received has two cross lines. These cross lines are brought into focus by a small magnifier. Then the fluorescing image of the object is focused on the plane of the cross lines by the adjustments of the microscope in the usual way. When the image is once in focus in the artificial eye or searcher eyepiece as it is called, the calculations of the optical system are such that it will be in focus in a plane 30 centimeters above. The camera is then substituted for the searcher eyepiece and a photograph taken.

After working with the equipment as a metallurgical microscope, it soon became evident that a more exact method of focusing must be devised than the "searcher eyepiece" method of Köhler. In most cases it was impossible, except by pure chance, to secure an exact focus. The solution of this problem was discussed in a lecture before the American

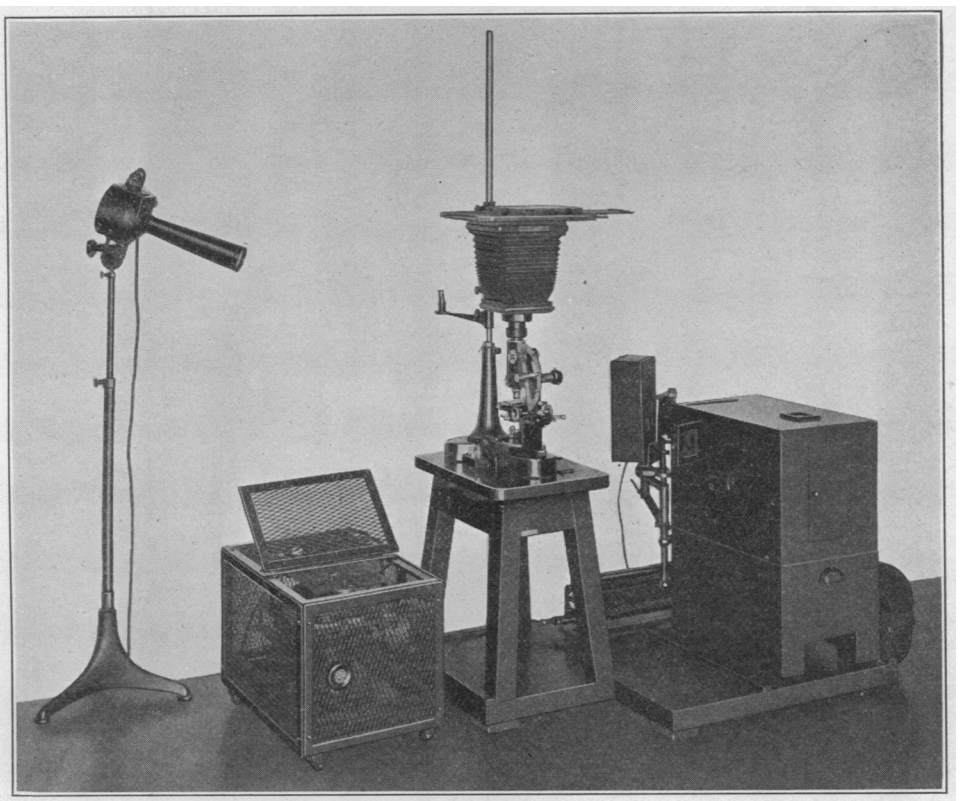


FIGURE 2

ULTRA-VIOLET MICROSCOPE ARRANGED TO PHOTOGRAPH OPTICAL SECTIONS

The sound and fume hood is in place. The light emerges through the open window shown. The window is fitted with glass and when swung closed excludes the ultra-violet light. In this way, the specimen can be protected from the ultra-violet light except during intervals of exposure. The fumes generated by the spark are carried away by an exhaust fan. The metal exhaust outlet, cushioned to prevent vibration, is shown at the extreme right of the spark generating apparatus. The camera is swung around to replace the searcher eyepiece.

Society of Mechanical Engineers and appears[3] also in published form to which those interested are referred. The details are briefly recounted here.

As a metallurgical microscope, it was found that the focus must be exact to at least one-quarter micron. The graduations on the drum of the slow motion adjustment of the microscope are by increments of one

or two microns, depending on the construction of the particular slow motion mechanism used on the microscope. It was necessary to increase the sensitivity of the fine adjustment. The peripheral motion of the drum was amplified by a pointer fitted to the thumb-screw of the fine adjust-

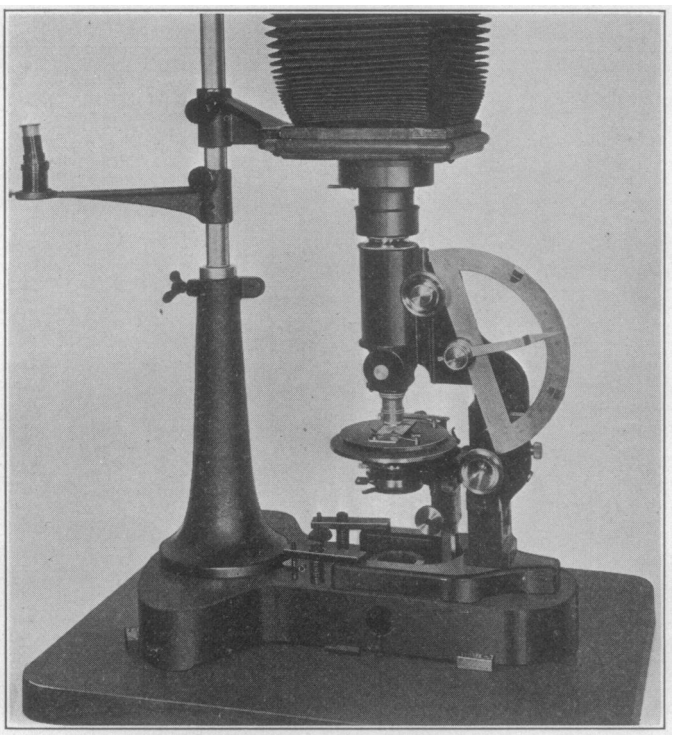
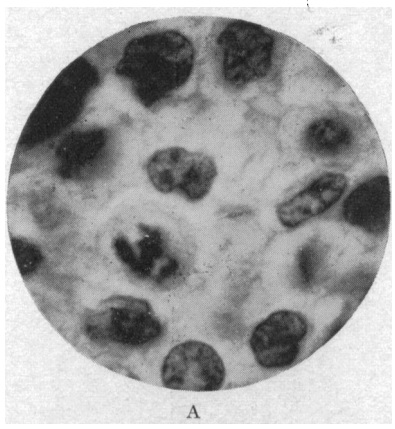


FIGURE 3

ULTRA-VIOLET MICROSCOPE

Showing the microscope fitted with the graduated half-circle, graduated slider and the aluminum pointer attached to the slow motion thumb-screw. In this particular assembly, one degree on the protractor corresponds to a change in focal planes of one-quarter micron. A sensitivity of one-half degree in adjustment may easily be attained. By means of a different slow motion mechanism, a spacing in focal planes of about one-sixteenth micron may be secured. A spacing of one-quarter micron seems adequate for most work.

ment. The pointer functioned with a graduated half-circle attached to the microscope stand. An ordinary draftsman's protractor was used for the graduated half-circle. The graduated rim of the protractor was fitted with a slider which also was graduated, each division equaling four degrees.



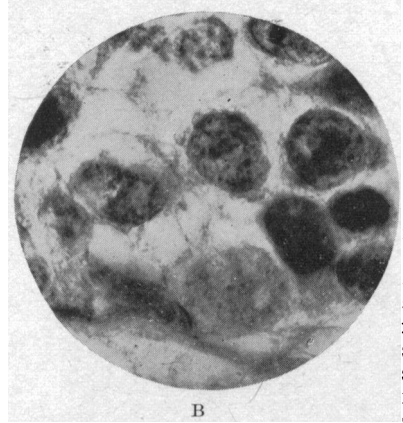


FIGURE 6

A fixed and embedded specimen, carcinoma of the appendix, photographed by visual light methods and by the ultra-violet microscope.

The ultra-violet microscope has an enormous increase in resolving ability over the visual light system and much higher magnifications may be employed. Figure 6-A was taken with the best apochromatic system (NA 1.40) using visual light. The magnification is 1000×, reduced $^1/_3$ in reproduction. On this particular specimen, an actual loss in definition occurred at 1500×. Figure 6-B shows an unstained section from the same paraffin block photographed at 1500× with the ultra-violet microscope, reduced $^1/_3$ in reproduction. The sections were identical except one was stained and mounted on glass for the visual light microscope. The other was unstained and mounted on quartz for the ultra-violet microscope. Figure 6-B is one of a group of optical sections.

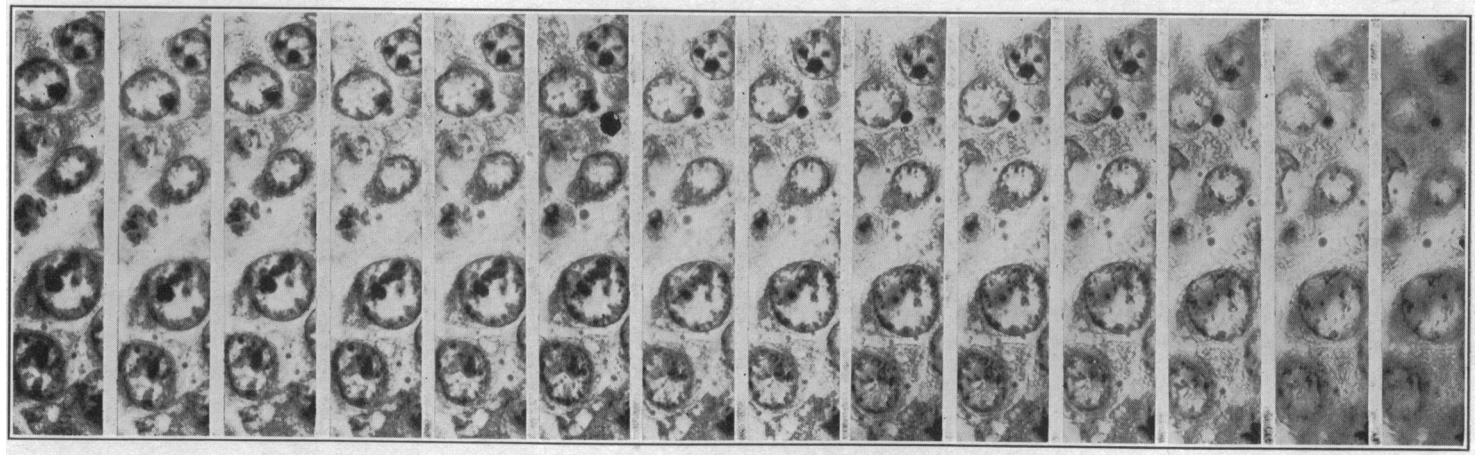


FIGURE 7

Optical sections of a fixed but unstained specimen. The specimen is a mouse tumor photographed on planes spaced one-quarter micron apart. The original magnification is 1800×, reduced $^1/_2$ in reproduction.

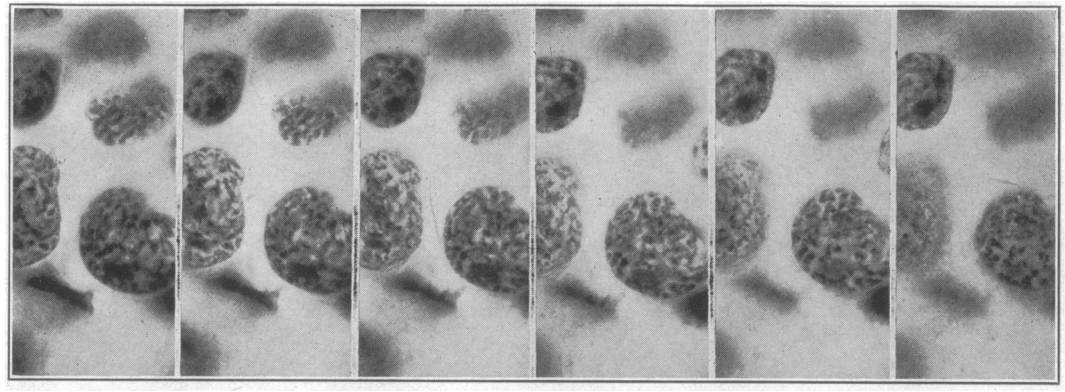


FIGURE 8

Optical sections of living mouse tumor cells. Neither fixative nor stain was used. The focal planes are spaced one-quarter micron apart. The magnification is 1800×, reduced $^1/_2$ in reproduction.

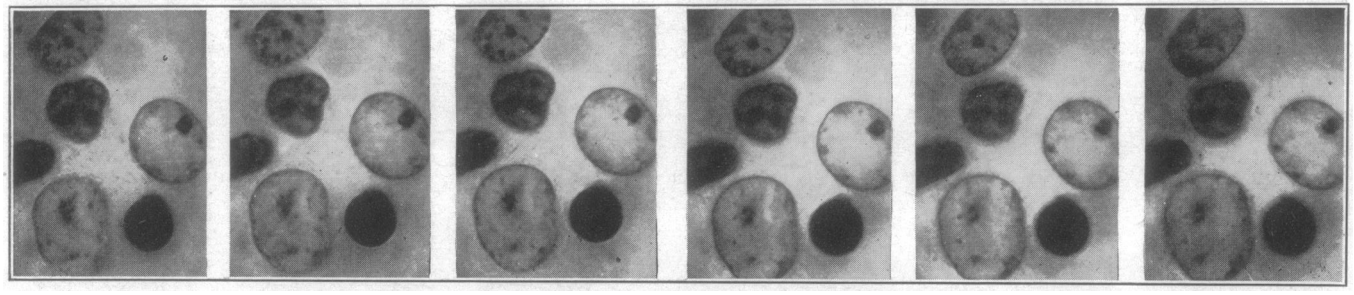


FIGURE 9

Six optical sections of mouse tumor cells. The focal plane spacing is one-quarter micron and the magnification is 1800 × reduced, $^1/_3$ in reproduction. Locke's solution was used as a mounting medium. No stain was used. Observe the progressive changes in the very fine structural details.

The method of operation is as follows: The image in the searcher eyepiece is brought into approximate focus by the usual methods. The slow motion is then turned so that the instrument passes downward through the focus and then it is very gradually brought back. In this way all lost motion in the slow adjustment is compensated for by gravity. A very nice mechanical balance must be obtained in the moving parts, otherwise, the method will fail. Any "creep" or change in focus, even though very slight, will upset the whole method. The personal element perhaps is still most important because the method relies on the keenness of vision of the operator to bring the image in the searcher eyepiece just

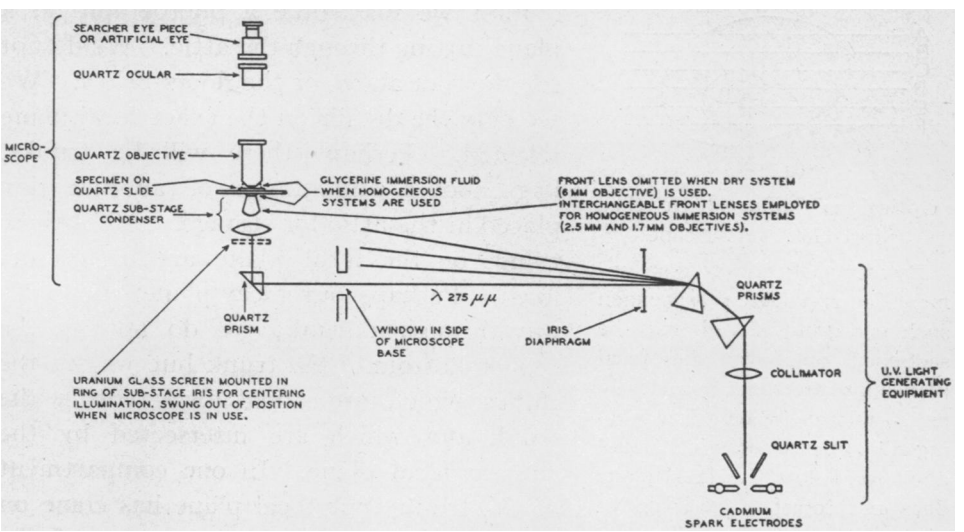


FIGURE 4

Schematic diagram of optical systems of the ultra-violet microscope as arranged for transparent specimens.

to the stage of coming into focus. The pointer is then set near the middle of the scale and the slider moved so that the first graduation coincides with the pointer. A photograph is taken with the apparatus in this position. The pointer is then lowered one degree which corresponds to a change of one-quarter micron in elevation of focus, the mechanical arrangements of the parts being arranged accordingly and a second photograph taken and so on. Four photographs taken in this way of a metallurgical specimen are sufficient to provide one photograph in exact focus.

Any optical system which will yield a very sharply defined focal plane to an accuracy of about one-quarter micron should prove to be of great value. For example, it appeared reasonable to suppose that we might substitute a transparent preparation for the opaque metallurgical specimen, and, because of the transparency of the specimen, we should be

able to photograph details of structure on different planes. Obviously if the focus is confined to a focal plane of inappreciable depth, detail above or below the exact focal plane should not interfere with the image.

From the solution of one problem dealing with the focus of a metallurgical microscope has evolved a method of optically sectioning living cells.

Dr. E. E. Free has written of this development by likening a single cell to a house and he has imagined what he would see if the house were divided by equally spaced cutting planes. I shall adopt Dr. Free's analogy to make clear what actually does happen when living cells are so sectioned.

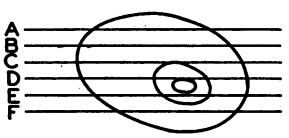


FIGURE 5

Optical sectioning with the ultra-violet microscope

By means of this new development a transparent specimen such as a group of cells may be sectioned optically, the focal planes may be spaced as closely as $^1/_{16}$ micron—generally a spacing of $^1/_4$ micron suffices. Detail above or below the focal plane does not interfere. Successive photographs taken on planes A, B, C, etc., give a progressive record of the structure.

Magnifications as high as 5000 diameters result in crisp brilliant images with a degree of resolution (ability to reveal fine detail) surpassing by far that achieved with any other known optical system.

First we may take a photograph on a plane cutting through the attic. We do not see the roof above or the floors below. We see only the details on the exact focal plane selected. Perhaps there will be trunks, bags, boxes and things of another day placed in the attic for storage. All of these things on the focal plane are clearly defined. Perhaps our focal plane cuts midway through a trunk; we do not see the top or bottom of the trunk but we see the things which are stored away within the trunk and which are intersected by the cutting focal plane. In one compartment of the trunk, our focal plane has come on some woolens. We see the texture of the cloth and observe that it shows wear and then we come on the handiwork of the moth larvæ. In another compartment are some queer-looking sections of straw and cloth and wire and we decide that it must be a hat on which the sun has risen and no doubt set for the last time. And so we could investigate the contents of all the trunks, bags and boxes without opening them, disturbing their contents or doing the slightest damage.

The next optical section intersects the sleeping and bathrooms of the second floor. At a glance, we note the number and arrangement of the rooms. We recognize the furniture and its disposition about the room. One room we conclude is a nursery because children are at play with things which we recognize as toys and the play and movement goes along entirely oblivious of the scrutiny from above. In another room, our optical section coincides with the top of a dressing table. We see all of

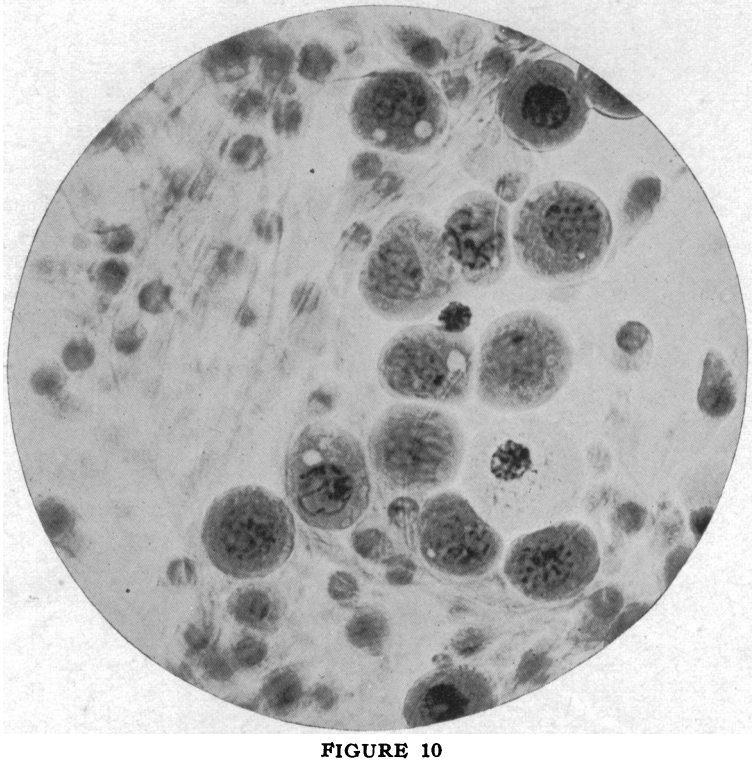


FIGURE 10

Living sperm cells of the grasshopper, photographed with the ultra-violet microscope. One of a series of optical sections. Ringer's Solution was used as a mounting medium. No stain was used. The magnification is 500×, reduced $^1/_3$ in reproduction.

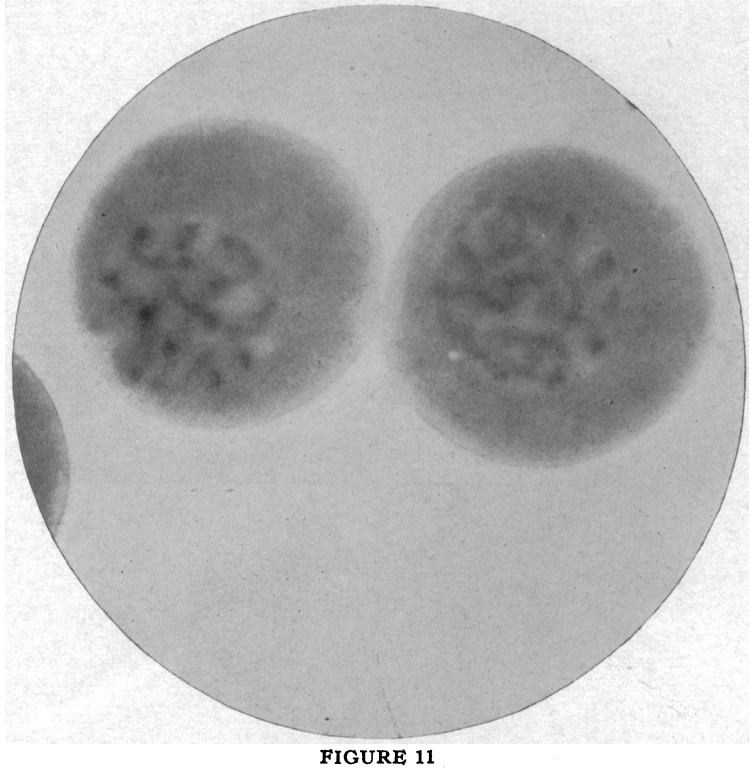


FIGURE 11

Individual cells of the same specimen as Figure 10. Magnification is 1800×, reduced $^1/_3$ in reproduction.

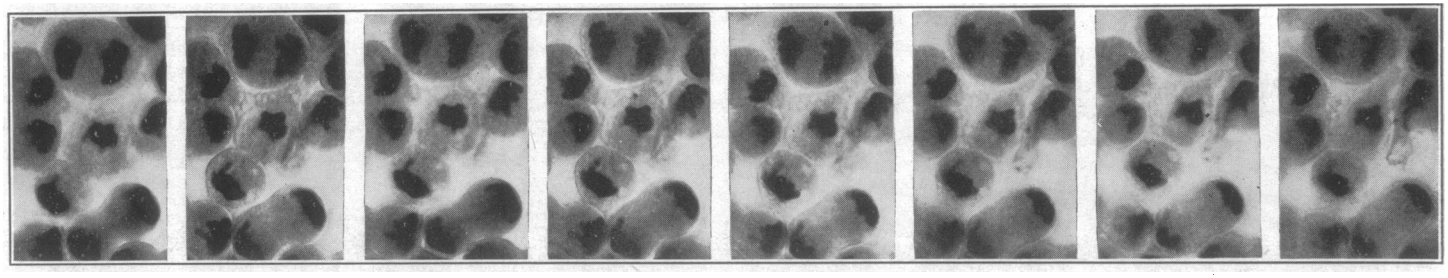


FIGURE 12-A

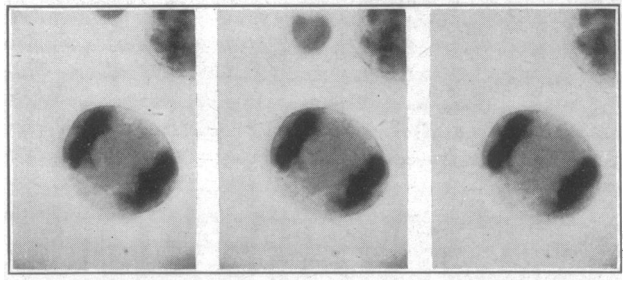


FIGURE 12-B

Figures 12-A and 12-B. Living sperm cells of the grasshopper. The focal planes are spaced one-quarter micron. The magnification is 1800×. Figure 12-A has been reduced $^2/_3$ in reproduction, 12-B reduced $^1/_2$. Note absorption of light by the chromosomes and absence of spindle fibers. The preparation was mounted in Ringer's Solution. No stain was used.

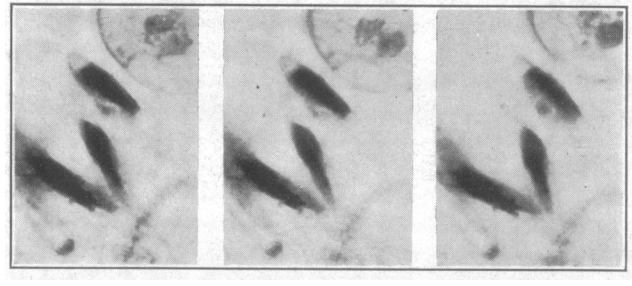


FIGURE 13

Living unstained spermatozoa of the mouse. The optical sections are spaced one-quarter micron. The magnification is 3600×, reduced $^1/_2$ in reproduction.

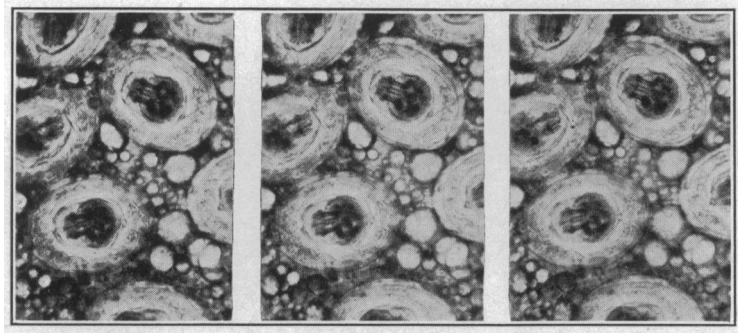


FIGURE 14-A

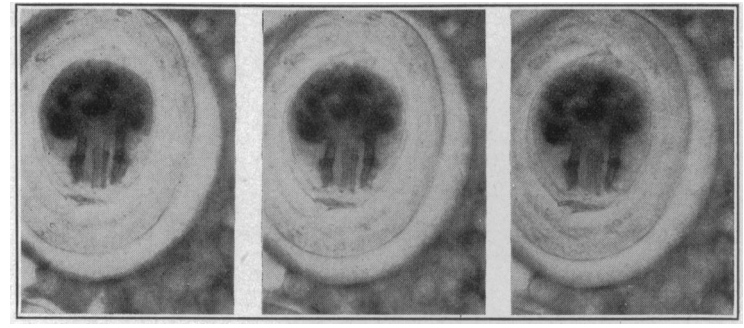


FIGURE 14-B

Embryos within the segments of a small tapeworm found within the body cavity of a mouse. Identification by Dr. Stark.
Figure 14-A shows three optical sections at 500× and Figure 14-B three optical sections at 1200×, both reduced $^1/_3$ in reproduction. The focal plane spacing is one-quarter micron. The preparation was mounted in Locke's Solution and was unstained.

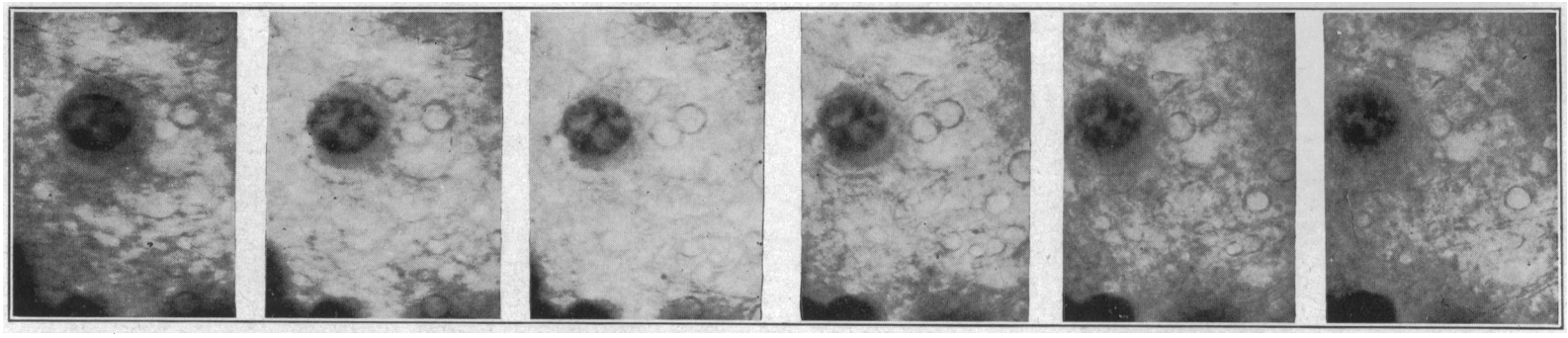


FIGURE 15-A

Golgi cells of the cerebellum of the mouse. Magnification is 1800×, reduced $^1/_3$ in reproduction. Cells mounted in Ringer's Solution with faint neutral red stain. Optical spacing one-quarter micron.

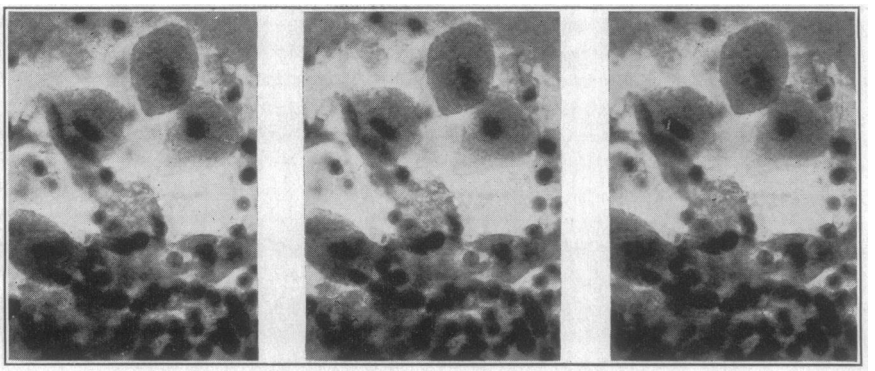


FIGURE 15-B

Purkinje cells of the cerebellum of the guinea pig. Magnification is 500×, reduced $^1/_4$ in reproduction. Cells mounted in Ringer's Solution with faint neutral red stain. Optical spacing one-quarter micron.

FIGURE 16-A

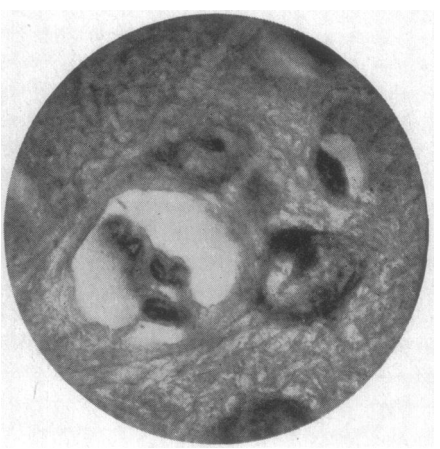


FIGURE 16-B

Cerebellum of the cat. The specimen was fixed but not stained. Illustrating the application of the ultra-violet microscope to the study of fixed but unstained brain cell structures. Original magnification is 1800×, reduced $^1/_2$ in reproduction.

the toilet articles and they have apparently been put down in haste as things are in disorder. In a jewel case cut by the sectioning plane, are many pieces of jewelry which we pause to admire. A large diamond ring which has been itself sectioned strikes our fancy because we look within and see a black carbon spot which we surmise the owner does not know is present. We discover something wrong with the contour of the stone. It is altogether too broad for its depth and we call it a "fish eye" and pass on.

And so we may go from room to room and from floor to floor observing the life and habits of the occupants. If we take photographs as we go, they may be laid out in order from top to bottom and a working model of the house constructed.

Fortunately, our private lives are not open to such scrutiny as the hypothetical example just cited. Though it does describe on a large scale what we are now able to do with many living things. A transparent specimen such as a single living cell or a group of living cells may be sectioned optically. The focal planes may be spaced as closely as $^1/_4$ micron. Detail above or below the focal plane does not interfere. Photographs taken on successive planes give a progressive record of the structure within the cell.

Dr. Köhler pointed out many years ago that one advantage of the ultra-violet microscope lay in the fact that organic specimens are differentiated in structure by virtue of the selective absorption which they manifest toward ultra-violet light. When using visual light microscopes, the need for staining biological specimens is too well known to need comment here. Unstained specimens respond under the ultra-violet microscope much as though they were stained.

It is generally recognized that the structure of organic material is apt to be profoundly altered by the treatment in preparing it for microscopic examination. The trend in cytological research appears to be toward the study of living material thus avoiding artifacts induced by fixation, staining and mounting. In the final analysis, biologists are interested in the structure, functions and behavior of the living undisturbed cell.

It may be argued that any cell removed from its normal living habitat and placed in some artificial medium is no longer a normal cell though it may be a living cell. In some cases, well justified doubts may be entertained as to whether the cell is actually alive. By what criteria shall we decide matters in this borderland? Shall we answer by opinion or careful laboratory observations or by both? Ultra-violet microscopy of living cells is in its infancy. We have observed by experimental methods on fresh material and aged material that changes do occur. In some cases the material undergoes quite rapid changes. In other cases, a surprisingly long interval may elapse before any change or degeneration can be de-

tected. Cells of the nervous system are known to be most delicate of all and we have found them more difficult to work with than other types of cells. With respect to cells of the nervous system, our results have not been fully studied, nevertheless the photographs perhaps will be found of interest since they are believed to represent at least a close approximation to normal.

If we are to employ the ultra-violet microscope in such researches, there are two reservations which must be borne in mind. The ultra-violet light itself must not have apparent injurious effects on the organisms and the organisms must not completely absorb the ultra-violet light. Things which completely absorb the ultra-violet light cannot be studied by these methods.

Ultra-violet light of the intensity and wave length used in conjunction with the ultra-violet microscope appears to have little if any harmful effect so far as some types of living cells are concerned. It is true that some single cell organisms are destroyed almost instantly; others of another species in the same mixed culture appear mildly excited and others immune. From some preliminary observations, it appears that living cell cultures may be exposed under the ultra-violet microscope for as long as forty-five minutes and, when returned to the incubator, they appear to grow and to show no ill effects from the exposure.

One wonders what effects wave-lengths other than $275\mu\mu$ would have on certain standard cell cultures. Will monochromatic ultra-violet light of one wave-length cause a living cell to disintegrate immediately and similar light of another wave-length have little or no effect? If so, then a very powerful means of dealing with cells will be at hand.

To illustrate the application of the methods used, I have chosen a variety of cell structures. The tissues were taken from the animals and transferred to physiological salt solutions. Cell smears were quickly made on quartz slides; a drop of the salt solution added and the preparation covered with a quartz cover slip. The cover slip was sealed with vaseline to prevent evaporation from the preparation. The salt solutions were chosen with particular regard to their suitability for the purpose, the object being to maintain isotonic conditions with reference to the cell. In some cases we have used the body fluids of the cells themselves instead of the artificial salt solutions.

It will be observed that the results with the ultra-violet microscope often show marked departures from corresponding structures photographed with visual light systems. This is particularly noticeable not alone because of the wealth of detail revealed by ultra-violet methods but also because of the apparent lack of some details. An example of this is found in some mitotic figures where the spindle fibers appear wanting or only faintly suggested by ultra-violet methods.

In the preparation of the material and the interpretation of the results, I have had the coöperation of Dr. Mary B. Stark of the Flower Hospital Staff and the assistance of Miss A. K. Marshall of the Bell Telephone Laboratories.

The photographs which accompany this paper are sufficiently described in the titles and need not be specifically dealt with here.

[1] Lucas, "An Introduction to Ultra-Violet Metallography," *Trans. Am. Inst. Mining and Metallurg. Eng.*, February, 1926.

[2] Lucas, "A Resumé of the Development and Application of High Power Metallography and the Ultra-Violet Microscope," **1**, *Proc. Int. Cong. Test. Materials*, Amsterdam, Holland, 1927.

[3] Lucas, "Photomicrography and Its Application to Mechanical Engineering," *Mech. Eng.*, **50**, pp. 205–212, March, 1928.

[4] Köhler, "Microphotographic Examinations with Ultra-Violet Light," *Zeit. Wissensch. Mikroskopie und für Mik. Tech.*, **21**, 1904, pp. 129–165 and 273–304.

A FUNDAMENTAL THEOREM ON ONE-PARAMETER CONTINUOUS GROUPS OF PROJECTIVE FUNCTIONAL TRANSFORMATIONS[1]

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Communicated August 11, 1930

Let L^x, L^x_s, L^x_1, L^1_s be real bounded integrable functions of the real variables x and s as indicated ($a \leq x, s \leq b$) and let us denote Riemann integration on (a, b) by the repetition of a superscript and subscript in the same term unless one of them is enclosed in a parenthesis.

The regular infinitesimal projective transformation in function space

$$\varphi^x = \bar{\varphi}^x + \delta t[L^x\bar{\varphi}^x + L^x_s\bar{\varphi}^s + L^x_1 - \bar{\varphi}^x L^1_s\bar{\varphi}^s] \tag{1}$$

will generate by continuous application a family of projective functional transformations[2]

$$\bar{\varphi}^x(t) = \frac{K^x(t)\varphi^x + K^x_s(t)\varphi^s + K^x_1(t)}{K^1_s(t)\varphi^s + K^1_1(t)} \tag{2}$$

where $\bar{\varphi}^x(t)$ satisfies the integro-differential system

$$\left.\begin{aligned} \frac{\partial\bar{\varphi}^x(t)}{\partial t} &= L^x\bar{\varphi}^x(t) + L^x_s\bar{\varphi}^s(t) + L^x_1 - \bar{\varphi}^x(t)L^1_s\bar{\varphi}^s(t) \\ \bar{\varphi}^x(0) &= \varphi^x \end{aligned}\right\} \tag{3}$$

Dines[3] has shown that in order for $\bar{\varphi}^x(t)$ in (2) to satisfy (3) the following equations hold