Microscopy. I: A Review

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INTRODUCTION

Since the origination of the cell theory, its establishment by Schleiden and Schwann, and its application to pathology by Virchow, the microscope has played an important and historic role in biology and medicine. The microscope has now been developed to utilize the several properties of the light wave—the amplitude, the frequency, the phase and the plane of vibration—and to employ, in addition to electromagnetic radiations, other forms, or carriers, of radiant energy such as an electron beam. It is the purpose of this review: (a) to describe some of the recent advances in methods of microscopy of the cell and (b) to indicate the results obtained and their manifest or potential significance in cancer research.

The plan of the presentation is:

Part I:

a) To present the microscopic methods of analysis that utilize the phase (phase, interference, and polarizing microscopy), the plane (polarizing microscopy), the dispersion (dispersion microscopy), and the amplitude and the frequency (microspectroscopy) of light waves in the optical spectrum.

b) To describe the use of other carriers of radiant energy in microscopy (electron microscopy and historadiography).

c) To discuss briefly the automatic counting and measurement of microscopic particles (microscanning).

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Finally, Parts I and II will attempt:

d) To indicate the application of these technics in the study of the size, shape, mass, elementary and organic composition and ultrastructure of the nucleus, nucleolus, chromosomes, mitotic apparatus, and cytoplasmic particles.

In general, the analytical methods reveal local structural and optical inhomogeneities which are accompanied by alterations in the optical path length (product of thickness and refractive index) or by differences in the absorption and the scatter of photons, electrons, and X-rays. The requirements for good imagery include the presence of contrast and the resolution of fine detail in cellular formed elements which have several orders of dimensional magnitude that are very approximately given by: 10μ for nuclei; 1μ for nucleoli, chromosomes, and mitochondria; and 0.1μ for microsomes. It will be shown subsequently that for certain types of quantitative work a departure is made from conventional methodology by the preparation and the analysis of optically homogeneous cellular material. An excellent general review on recent progress in practical microscopy is that of Barer (8).

PHASE MICROSCOPY

Principles.—Upon passing through a transparent object in an ideal microscope, a light wave, which may be treated as a sinusoidal wave motion, does not undergo a change in amplitude as compared to that of a wave passing through the adjacent medium or surroundings. Consequently, photosensitive surfaces, such as the retina, photographic emulsion, and cathode of a photo-cell which are amplitude- or intensity-detectors, record no difference between the object and its surroundings. However, if there is a difference in the re-
fractive index$^1$ of the object and the medium, then the optical path (linear path multiplied by refractive index) will be dissimilar for light waves that pass through the object (deviated wave) and the medium (undeviated wave), and there will be a displacement in the phase of one wave in relation to the other. With annular illumination, the deviated and the undeviated waves pass through different areas of the back aperture of the microscope objective, and there, upon transmission through the appropriate phase-altering and amplitude-decreasing plates, the two waves which initially differed in phase but not in amplitude are brought into phase with differing amplitude. When focused at the image plane of the objective, the waves may then interfere constructively (addition of amplitudes) or destructively (subtraction of amplitudes) to produce either bright or dark contrast in the object in relation to the surrounding medium. A conversion from phase-to-amplitude-differences underlies the phase contrast principle which was originally formulated by Zernike and applied by him to phase microscopy (252–254).

The most important attribute of the phase microscope is to enhance the visibility of particles which, relative to their surrounding medium, have a small difference in optical path. The difference, $\Delta$, in optical path is a linear distance given by the expression

$$\Delta = (n_o - n_m) l,$$

where $n_o$ and $n_m$ are, respectively, the refractive indices of the object and the medium and $l$ is the thickness of the object. It is customary to express $\Delta$ as a number or a fraction ($\Phi$) of wavelengths ($\lambda$) so that

$$\Delta = \Phi \lambda,$$

and

$$\Phi = \frac{(n_o - n_m)}{\lambda}$$

where $\Phi$ is called the phase difference or the phase change.

For a given thickness, the value of $\Delta$, which is of the order of $\lambda/20$ or less (17) for many cellular particles, depends upon the difference between $n_o$ and $n_m$; and the contrast of a transparent particle relative to the medium is determined by this difference, together with the nature of the phase-altering and amplitude-reducing plates of the microscope. In a dark-contrast microscope, the particle is dark for $n_o > n_m$ and light for $n_o < n_m$; and in a bright-contrast microscope the particle is light for $n_o > n_m$ and dark for $n_o < n_m$.

The literature on the theory and the more practical aspects of phase microscopy includes, aside from the work of Zernike that of Köehler and Loos (190), Burch and Stock (39), Bennett et al. (16, 17), Payne (179, 180), Osterberg (174, 175), Barer (5, 6, 9, 10), Oettlé (169), Richards (193) and Francon (88).

Applications.—The necessity for brevity precludes a listing of publications relating to the phase microscopy of viral inclusions, rickettsia, bacteria, fungi, algae, higher plants, protozoa, and higher invertebrate animals and requires a selection of work pertaining, for the most part, to the living cells of higher vertebrate animals (Figs. 1, 8).

The unique value of the phase microscope is that it, as no other method, provides a means for the structural analysis of the nucleus and the cytoplasm of living cells without the hazard of injury or the uncertainty of artifact. A typical, living normal interphase cell in its medium of growth, such as a mouse fibroblast in tissue culture, is observed in dark contrast to have: a delicate, or nearly invisible cell membrane; formed elements in the cytoplasm which are of two structural categories—smaller, pale gray, rod-shaped and filamentous structures (the mitochondria) and larger, dark gray to black, spherical elements ("fat" droplets, etc.); occasionally, a juxtanuclear, spherical or crescentic, granular mass designated as the central body (181, 189) and thought by some (139) to form the Golgi apparatus after fixation; a delicate nuclear membrane; spherical nucleoli; irregular masses of chromatin not always distinguishable from nucleoli (see 257, however); and a ground substance in the nucleus and the cytoplasm, the details of which are at, or below, the limit of optical resolution. (See section “Electron Microscopy” to be published in Part II.)

The mitotic division of living embryonic cells of the chick in tissue culture is recorded on film with time-lapse phase photomicrography by Hughes and Swann (112). The sequence of events in mitosis includes: the dissolution of the nucleolus and the nuclear membrane within a period of one minute; appearance of the chromosomes as radially disposed threads during the next 5 minutes; irregular motion of the chromosomes as radially disposed threads during the next 5 minutes; and polarity formation of the metaphase plate; partial rounding of the cell body and withdrawal of its processes; movement of the chromosomes in anaphase, after one or more "false starts," producing a separation between the sets of chromosomes of the order of 10 $\mu$ or more within 5 minutes; and telophase, with flattening and migration of the daughter cells and the appearance of nucleoli and nuclear membranes within 10 minutes after cytoplasmic cleavage. During mitosis the filamentous mitochondria shorten to become thick, sometimes branched, rods which, together

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$^1$ The refractive index of a medium is equal to the ratio of the velocity of light in air to that in the medium, $n_m = V_m/V_a$ at a particular wave length and temperature and is a property dependent upon and predicted from elementary composition.
with other cytoplasmic elements, bound the nuclear region. These elements encroach upon the spindle area and constrict the interzonal region prior to the formation of the cleavage furrow. The spindle itself is not seen. (See section on "Polarizing Microscopy.") There is a characteristic formation of cytoplasmic bubbles, often prominent in late anaphase and telophase, which has been observed by earlier workers.

In a study of mitosis in living embryonic cells of the mouse in tissue culture Fell and Hughes (79) observed the formation of chromosomes, traced the course of anaphase movement, described the swelling and unraveling of the chromosomes in the reconstruction of the daughter nuclei, and demonstrated the origin of polyplody by the mitosis of binucleate cells. Other notable studies of mitosis in the living cell include those of Michel (157), Hughes (109, 110), and Firor and Gey (80), utilizing the phase microscope and the classical observations of Lewis (158) with the ordinary light microscope.

Viable cells in spreads of mouse tissues mounted in physiological solutions were studied in the phase microscope by Hux- ford and Smiles (159, 160). The fibroblasts of subcutaneous connective tissue and tumor stroma are compared to sarcoma cells of the rapidly growing Rb tumor of the RIII strain of mice. There is an increased content of cytoplasmic mitochondria, a greater amount of nuclear chromatin, typically larger nucleoli, and commonly an enlargement of the central body in the sarcoma cells. The mitochondria are more numerous in the cytoplasm of fibroblasts derived from tumor stroma than from subcutaneous connective tissue. Mitosis is studied particularly in the sarcoma cells, in which it appears that both the nucleolar and the chromatin material enter into the formation of the chromosomes at the prophase. The mitochondria of the majority of dividing cells are spherical or rod-shaped, and they may form clusters as though originating by the segmentation of filaments into strings of granules. In the later stages of cell cleavage, the mitochrondia return to a more filamentous form, and, while the chromosomes are still visible in the daughter nuclei and before the appearance of the nucleoli, there is a concurrent increase in the number of the mitochondria and in the volume of the cytoplasm.

Observations such as the foregoing are of considerable interest in view of the fact that the mitochondria, a structural unit whose isolation and early analysis is due to the work of Claude (45, 47) and of Benasly and Hoerr (80), is a complex unit whose isolation and early analysis is due to the work of Michel (157), Hughes (109, 110), and Firor and Gey (80), utilizing the phase microscope and the classical observations of Lewis (158) with the ordinary light microscope.

Polarizing Microscopy

Principles.—The refractive index of a transparent object may be identical in all directions, as in an isotropic object, or the refractive index may vary with the direction of propagation and the plane of vibration of light, as in a birefringent or anisotropic object. There are two or more values, \( n_d, n_v, \) or \( n_\alpha, \) for the refractive indices of a birefringent object, and the term, \( n_d - n_v, \) which is called the birefringence is an optical constant that depends upon the space orientation and other characteristics of the molecular structure of the object. It is a characteristic property of a birefringent object that when it is rotated between a crossed polarizer and analyzer, the object appears alternately light and dark (position of extinction) at 90° angular intervals. It can be shown that plane-polarized light, when incident upon a birefringent object, is divided into two rays, designated as the ordinary and the extraordinary ray, which vibrate in mutually perpendicular planes with differing refractive indices, \( n_d \) and \( n_v, \) respectively. After passing through a birefringent object of thickness, \( t, \) and emerging, the two rays recombine to form an elliptically polarized ray with two components which have a difference, \( \Delta, \) in optical path called the retardation and given by the expression

\[
\Delta = (n_d - n_v) t. \tag{4}
\]

Utilizing equations (4) and (2), the difference in optical path may be expressed as the phase difference, \( \Phi, \) by the relation

\[
\Phi = \frac{(n_d - n_v) t}{\lambda}. \tag{5}
\]

Polarizing microscopy permits the visualization of birefringent objects, the establishment of the optical axes, the characterization of the birefringence, and the measurement of the retardation and the refractive indices. The optical axis serves as an axis of reference and is the direction in which light passes through a birefringent object without alteration and, in an oriented biological object, usually
cations of polarizing microscopy includes the
coincides with a distinctive direction or axis of
symmetry, such as an axis parallel to or perpen-
dicular to the length.

Birefringence is designated as being either positi-
ve or negative with respect to the optical axis,
depending upon whether the refractive index par-
allel to that axis is the greater or the lesser one. In
the case of biological objects of interest, the sign
of birefringence is conventionally referred to the
long axis, even though this may not be the optical
axis. Birefringence is further characterized as in-
trinsic birefringence in which the anisotropy is due
to a preferential asymmetrical alignment of the
oscillating atoms and molecules within the object
and is totally independent of the refractive index
of the surrounding medium; form birefringence in
which the anisotropy occurs when asymmetrical
particles, small with respect to the wavelength
of light and not in themselves necessarily aniso-
trropic, are preferentially oriented in a medium of different
refractive index; and strain birefringence in which
a normally isotropic object is subjected to distort-
ing forces that produce preferential orientation of
the oscillating elements. Protein fibers, with one
important exception, have both an intrinsic and a
form birefringence which are positive with respect
to the long axis and attributed to the longitudinal
orientation of the protein chains (108—200). An
exceptional property is shown by nucleo-protein
fibers: while the form birefringence is positive as in
other protein fibers, the intrinsic birefringence is
negative due to the nucleic acid moieties which are
presumably oriented perpendicularly to the protein
chains.

The conventional polarizing microscope de-
signed for petrography is, in general, not suitable
for the detection and the measurement of the lesser
magnitudes of retardation, the range of which is of
the order of \( \lambda/5,000 \) to \( \lambda/200 \) \((0.1—5 \text{ m\( \mu \)) for
the constituents of many living cells (114). Recent
improvements in instrumentation, notably those by
Swann (225, 226), Swann and Mitchison (227), and
Inoué (113, 114) make the detection and the measure-
ment of such magnitudes of retardation possible and provide new potentialities for the
biological application of the polarizing micro-
scope. The literature on the theory and the appli-
cations of polarizing microscopy includes the
publications by Schmidt (200), Schmitt (198, 199),
Bear (15), Chamot and Mason (44), Frey-Wyss-
lung (88) and the excellent review by Bennett (19).

Applications.—Hughes and Swann (112), by means of “bi-
frame recording,” which is a term applied to alternate serial
photography by phase and polarizing microscopy, studied the
relations between chromosomal movement and the changes
within the spindle in the living, dividing chick embryo cell. The
chromosomes but not the spindle are visible in the phase mi-
roscope, and the birefringent spindle and, less readily, the
chromosomes are discerned in the polarizing microscope. The
spindle is first observed in early metaphase as a small circular
or oval region of positive birefringence (with respect to the long
axis of the cell) which enlarges rapidly and soon assumes the
typical spindle configuration. A small birefringent structure,
the aster, radiates out from the centrosome at either pole. The
retardation of the fully developed metaphase spindle has an
average value of about \( \lambda/500 \) and a maximum value at the
poles. Soon after anaphase begins, the spindle starts to elon-
gate, and the retardation in the equatorial region decreases.
During cytoplasmic cleavage as the equatorial region of the
spindle is constricted, positive birefringence develops in the
cytoplasmic bridge and attains a retardation of \( \lambda/200 \). Ulti-
mately the cytoplasmic strand appears to snare, and the bire-
fringence fades away. The actual constricting collar is also bire-
fringent with a positive sign. Quite often there occurs near one
end of the main body of the cell a roughly circular area of nega-
tive birefringence with respect to the long axis of the cell.

Among the possible interpretations of the foregoing ob-
servations, the most logical (118) is that the spindle is fairly
completely oriented near the poles and less well oriented at the
centrosome; that the centrosomes at the poles exert an orient-
ing force through which a co-ordinated contractile mechanism
for chromosome movement is built up from isotropic proto-
plasm. Schmidt (200), who originally observed the decrease in
the retardation of the spindle of the sea urchin egg as the
chromosomes moved toward the poles, emphasized the analogy to a
feature of a muscular contractile mechanism in which the re-
tardation decreases during isotonic contraction. The modern
interpretation of the spindle fibers is that of an oriented mass
of elongated molecules formed by molecular aggregates, or
micelles, and having the property of contractility which is re-
lated to the folding of molecular (polypeptide) chains (108, 184)
during anaphase.

Swann (225, 226) observed a close correlation between the
position of the chromosomes and the regions of decreasing bi-
refringence in the sea urchin egg and advanced the hypothesis
that the change in birefringence was brought about indirectly
by the chromosomes themselves. Accordingly, the chromo-
somes would be responsible for their own movement by con-
trolling the structural changes in the spindle. The mediation of
this action might be due to the release of an “active substance”
which diffuses away from the chromosomes and produces its
effect as it goes.

Inoué (113, 114), with an improved polarisation microscope
and an ingenious method (114) for the measurement of small
retardations, studied the structure of the mitotic spindle in the
living cells of the animal (Chaetopterus egg) (Fig. 2) and of the
plant (Easter lily pollen mother cell). The spindle was ob-
served to be composed of several fibrous components, some of
which correspond to categories proposed by Schrader (205):
“continuous fibers” connecting the two poles; “chromosomal
fibers” connected with the kinetochores of the chromosomes;
and “interzonal connections” between the separating chromo-
somes in anaphase and telophase.

The effect of colchicine on the spindle was investigated by
Inoué (118). When the Chaetopterus egg is immersed in col-
chicine sea water, the spindle length begins to shorten; the bire-
fringence of the continuous fibers and the astral rays decreases
and then completely disappears, and finally the thick chromo-
somal fibers lose their birefringence. The chromosomes remain
on the equatorial plate until the birefringence of the spindle
completely disappears, and then the chromosomes begin to
scatter. This indicates that there is a close relation between the
mechanical integrity of the spindle and the maintenance of the
metaphase plate of chromosomes.
INTERFERENCE MICROSCOPY

Principles.—If a beam of monochromatic light at nearly perpendicular incidence to a microscope field undergoes multiple reflections between two plane, parallel semi-reflecting slides enclosing a medium of refractive index $n_m$, the intensity of the light which is transmitted will go through maxima and minima depending upon the separation of the reflecting surfaces. When the field is bright, the optical path difference between an incident ray and a reflected ray is such that constructive interference (addition of amplitudes) occurs; and when the field is dark destructive interference (subtraction of amplitudes) is present. If a small object of different refractive index, $n_o$, is placed in the medium between the slides, the optical paths for the rays which pass through the object and through the medium will differ; and, if the spacing of the slides provides a bright background, the object will appear in dark contrast, or, if the background is dark, the object will be bright as in phase contrast. Furthermore, if there are differences in optical paths through the object, then loci of identical paths will be shown by alternate bright and dark lines, called interference fringes, which form a contour-like map of the object. This is a method of multiple-beam interference microscopy as originated by Frederikse (87), developed by Merton (154, 155) for biological application, and used by Richards (195) and Barer (10).

The work of Tolansky (237, 238) defines the mathematical conditions which are to be fulfilled in order to permit the interpretation of the images in multiple-beam interference microscopy. The physical requirements include the use of: an axial bundle of monochromatic illumination with a very small angular aperture; slides of high reflectivity; very close spacing and small angle of inclination and an objective of sufficient angular aperture. Under these circumstances a necessary mathematical condition is satisfied, and the following interpretation of the interference fringes is permitted. The difference in total optical path represented by two bright fringes, or two dark ones, is $\lambda$; but in each interreflection there is a double passage through the object or the medium so that the optical path difference for one passage is $\lambda/2$. Therefore, an optical path difference, $\Delta = \lambda/4$, occurs between an adjacent bright and dark fringe.

Applications.—As shown above, the difference, $\Delta$, in optical path is related to the refractive indices, $n_o$ and $n_m$, of the object and the medium, respectively, and the thickness, $t$, of the object by the expression

$$\Delta = (n_o - n_m) \frac{t}{\lambda}.$$  \hspace{1cm} (6)

The observation of fringe patterns by multiple-beam interferometry makes possible an estimation of the optical path difference. In the case of an optically homogeneous (isotropic) object, the term $(n_o - n_m)$ is a constant for a given condition, and the optical path difference is related directly to variations in the thickness of the object. The resolution of the interferometric method is extremely high (237). For example, if the separation of two bright fringes is 5 mm. in a cell-image, then a tenth of the distance between fringes is readily estimated (0.5 mm.) and is equivalent to a path difference of $\lambda/20$ which for green light is 0.027 $\mu$. Now, for the interferometry in air ($n_o = 1$) of an essentially aqueous ($n_o = 1.33$) cell-object, the expression $(n_o - n_m)$ in equation (6) becomes 0.33, and the thickness, $t$, which corresponds to an optical path difference of 0.027 $\mu$, is 0.081 $\mu$. A resolution of 0.061 $\mu$ and a magnification of 2000 $\times$ (0.0008 $\mu$ in the third, or the vertical, dimension of a homogeneous cell object, therefore, falls in the range of that achieved by the electron microscope for the lateral dimension (see section on "Electron Microscopy," to be published).

An important use of interference microscopy in biology is described by Barer (11) and Davies and Wilkins (63). Since the organic mass of a cell is principally protein, then the term $(n_o - n_m)$ in equation (6) may be considered to be the refractive index increment between a protein solution with index $n_o$ and its solvent with index $n_m$. For proteins such as serum albumin and globulin it has been shown by Adair and Robinson (1) that the relation between the refractive index increment and the concentration is given by

$$\alpha = \frac{n_o - n_m}{C},$$  \hspace{1cm} (7)

where $\alpha$ is a constant called the specific refractive increment and $C$ is the number of gm dry protein/100 ml of solution.

Combining equations (6) and (7), the optical path difference is

$$\Delta = \alpha \cdot C \cdot t,$$  \hspace{1cm} (8)

where $t$ is the thickness of the cell in cm.

The equivalent mass, $M$, of dry protein in gm/cell is given by

$$M = \frac{V \cdot C}{100},$$  \hspace{1cm} (9)

where $V$ is the volume of the cell in ml. and $C$ is expressed in gm/100 ml.

As an approximation,

$$V = A \cdot t,$$  \hspace{1cm} (10)

where $t$ is the thickness in cm. and $A$ is the area of the cell in square centimeters.

Combining equations (9) and (10), we have

$$M = A \cdot t \cdot \frac{C}{100};$$

R. C. Mellors, unpublished observations.
and, substituting the value of \(C\) from Equation (8), this becomes

\[
M = \frac{A + \Delta}{100a},
\]

(11)

where \(M\) is the equivalent mass of dry protein per cell in gm., \(A\) is the area in square centimeters, \(\Delta\) is the optical path difference in cm., and \(a\) is the specific refractive increment.

The mean value of \(a\) is about 0.0010 for a variety of proteins and for deoxyribonucleic acid and does not vary a great deal for many organic constituents of the cell. Thus, the equivalent dry mass computed by equation (11) is not markedly influenced by reasonable, qualitative variations in organic composition. By the foregoing method of analysis, Barer (11) obtains a value of \(31 \times 10^{-12}\) gm of hemoglobin/red blood cell and \(120 \times 10^{-12}\) gm of protein/nucleus in the epithelial cell of the oral mucosa.

It can be shown that a more general expression for the equivalent dry mass of protein in a cell is given by

\[
M = \frac{\Delta_1A_1 + \Delta_2A_2 + \ldots + \Delta_nA_n}{100a},
\]

(12)

which, for \(\Delta_1 = \Delta_n = \lambda/2\), becomes

\[
M = \frac{\lambda}{2} \sum n_0A_n, \quad (13)
\]

wherein \(\Delta_1 = \Delta_n = \lambda/2\), is the path difference between successive bright fringes, \(A_0\) is the area enclosed by each bright fringe, and \(\sum A_n\) is the summation of the areas enclosed by the bright fringes. If the optical path difference between neighboring structures is but \(\lambda/4\), as is frequently observed between the aqueous medium and the cytoplasm or between the cytoplasm and the nucleus, then equation (13) takes the form, \(M = \lambda/4 \times \sum A_n/100a\), where \(A_n\) refers to the area of the structure whose anhydrous mass is computed.

Of considerable interest is the fact that the hydrous protoplasmic mass of the nucleus and the cytoplasm of a cell can be measured by multiple-beam interferometry in air, a method readily applicable to living cells in tissue culture (Figs. 4-9). If the assumption is made that the density (gm/ml) and the refractive index (44) of the immersion medium and the object have the same re

The proximity to the matching index is also indicated by color. If the refractive index of the object is slightly above the matching index, the object appears predominantly red; for successively higher increments in index, the object is orange, yellow, and pure white, respectively; if the object is very slightly below the matching index, it is colored pure blue; and for successively lower indices, the object is light blue and pure white, respectively. By the use of polarized light in dispersion microscopy, birefringent objects, if close in index to that of the mounting medium, exhibit two contrasting colors that are dependent upon the relation of the two refractive indices to that of the medium. Isotropic objects have but one color under the same condition of observation.

The method of dispersion microscopy, therefore, provides a means for the determination of the refractive index and the birefringence of a biological object without reference to the optical
path or to the object thickness. The difference in index between certain structures such as collagen and elastin fibers, or cell nuclei and other tissue components, is such that these structures appear in contrasting color, depending upon whether they are equal to, above or below, the refractive index of the medium. It may be possible to identify the toxic dusts such as free silica, asbestos, and beryllium oxide by dispersion staining. The further biological application of this method is awaited with considerable interest.

MICROSPectROSCOPY

Principles.—The extensive treatment elsewhere (33) of various aspects of the subject precludes more than a brief presentation of the fundamental basis of spectroscopy. Molecular spectra arise when the motions of the constituent electrons and atoms of a molecule are energized by incident radiant energy: the electrons move about the positively charged atomic nuclei, and the atomic nuclei themselves move together in a linear translation and rotate and vibrate periodically about their center of gravity. The frequency of the absorbed radiant energy must be equal, or very nearly equal, to the frequency of the molecular vibrations that it excites: ultraviolet and visible light energize the motions of electrons and infrared light excites the vibrational-rotational motions of atomic nuclei. It is found empirically and substantiated theoretically that certain groups of atoms and chemical bonds in a molecule absorb particular frequencies in the infrared, visible, and ultraviolet regions, and it is on this basis that the identification of a molecule can be made from its characteristic absorption spectrum.

By the measurement of the attenuation, or the absorption, of a parallel beam of monochromatic radiation as it rectilinearly traverses a solution of an isotropic absorbing material, or solute, which is molecularly dispersed in a nonabsorbing medium, the concentration, C, of the solute is given by the Lambert-Beer equation,

\[ C = \frac{E_\lambda}{k I} \]  

(14)

where \( C \) is in gm/liter; \( E_\lambda \) is the extinction or the optical density given by \( E_\lambda = \log_{10} I_0/I \), with \( I_0 \) the intensity of the incident light and \( I \) that of the transmitted light; \( k \) is the specific extinction coefficient which is characteristic of the absorbing material and is a function of the wave length, \( \lambda \); and \( I \) is the path length in cm. This equation is derived under the assumption that the absorption per molecule of the absorbing material is uninfluenced by the concentration, a condition which is by no means universally applicable.

An instrument for the analysis of spectra is composed of a dispersive element ( prism or grating) for the separation of the various frequencies or wavelengths of light and a photosensitive element—retina (as in a spectroscope), photographic plate (spectrograph), and photoelectric or radiometric device (spectrometer)—for the measurement (spectrophotometer) of spectral intensities. The use of a microscope in spectral analysis constitutes microspectroscopy. The pioneer development and biological application of ultraviolet absorption microspectroscopy was accomplished by Caspersson (40—42). In recent years, there has been an extension of spectral microanalytical methods to include absorption microspectroscopy in the ultraviolet (Figs. 10—13), visible, and infrared regions (the entire optical spectrum), together with emission and fluorescence microspectroscopy. This is due in part to the development of a reflecting microscope by Burch (88), Brumberg (94), Grey (96, 97, 98, 100), Kavanagh, and others (69, 116, 154, 167), (Chart 1) which utilizes pairs of reflecting mirrors rather than refracting elements and, therefore, remains in focus at the same mechanical setting throughout the entire optical spectrum; and to activities in research at Oxford (7, 12, 18), King’s College (86, 167, 168, 192, 242), Massachusetts Institute of Technology (136, 207, 248), Columbia (185—190), and Memorial Center (145—148, 152) and in commercial development by Willcocks, Ltd., Bausch and Lomb, Polaroid (24, 25, 125), and the American Optical Companies.

The usefulness of the microscope in systems for absorption microspectroscopy lies in the small order of magnitude of the analytical sample. In accordance with diffraction theory, the resolving power, \( d \), of a microscope is given by

\[ d = \frac{1.2 \lambda}{NA_0 + NA_e} = \frac{0.6 \lambda}{NA_e} \]  

(15)

where \( NA_e \) is the numerical aperture of the objective, is the same as that of the condenser, \( NA_e \), and \( \lambda \) is the wavelength. The wavelength in microns is 0.2—0.4 \( \mu \) in the ultraviolet, 0.4—75 \( \mu \) in the visible, 0.75—2.5 \( \mu \) in the very near infrared, and 2.5—15 \( \mu \) in the near infrared regions; and \( NA_e \) is of the order of unity. While the theoretical minimum transverse diameter of the analytical sample as set by the resolving power of the objective is of the order of 0.15 \( \mu \) in the ultraviolet at \( \lambda 0.25 \mu \), and 6 \( \mu \) in the infrared region at \( \lambda 10 \mu \), other limiting factors (24, 25) such as the spectral radiance of

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the source, the detection power of the microspectrometer, and the magnitude of the extinction coefficients determine the minimum size of the sample. At present, the spectral analysis of various compounds of biochemical interest requires a weight of sample of the order of $10^{-8} - 10^{-12}$ gm. in the ultraviolet and $10^{-6} - 10^{-7}$ gm. in the near infrared region (12, 24, 147). The theoretical detectable concentration of biochemical compounds which will give an optical density of 0.1 in a path length of 1 $\mu$m can be computed from equation (14) using their specific extinction coefficients for absorption maxima in the ultraviolet and the visible regions. The values when computed as the concentration in gm/ml, or the quantity in $10^{-15}$ gm/$\mu^3$, are of the following order: for purines and pyrimidines, 0.01; nucleic acids, 0.05; cyclic amino acids, 0.05–0.09; porphyrins, 0.002; steroids, 0.02–0.10, and phosphoric acid groups for the estimation of nucleic acids. Kurnick (124) comprehensively evaluated the selective staining of nucleic acids by methyl green and pyronin. Dempsey and collaborators (64) have investigated the affinity of a variety of tissue components for basic and acidic dyestuffs by the method of micro-absorption colorimetry.

Recent attempts are made to circumvent the unfavorable factors (91, 172) inherent in the cell object as well as in the optical system of microspectroscopy. Methods designed to avoid the errors in the analysis of inhomogeneously dispersed systems (117) and objects of indeterminate thickness for ultraviolet absorption microspectroscopy include the preparation of isolated, unfixed cells or nuclei (129), suitably mounted (8) and proved (151) by phase, interference, and absorption microscopy to be optically homogeneous; and the fluorescence microphotometry (149, 172) of individual, not necessarily homogeneous, cells that are stained with a basic fluorochrome. The use of coated (166) and reflecting optics (147) and the limitation of fields of illumination in micro-absorption colorimetry (166) and microspectroscopy (147) to diameters of a few microns are measures taken to minimize the defects that lead to an anomalous distribution of energy by light scatter at the interfaces of optical elements and by diffraction effects in the object.

**Ultraviolet and visible absorption.**—As shown by the extensive work of Caspersson and his collaborators (41), as well as by the earlier observations of Lucas (137, 138) and Wyckoff (249–251), the nucleus of a typical cell, when photographed in the ultraviolet region, is transparent at longer wavelengths (> 300 mp) and absorbing at shorter (260–280 mp) wavelengths. While Soret (216) originally suggested that the ultraviolet absorption in the region of 280 mp shown by protein solutions was due to the constituent cyclic amino acids, and Dhéré (65) attributed the ultraviolet absorption of yeast cells at 260 mp to the purine and the pyrimidine moieties of ribonucleic acid,
it remained for Caspersson to show that a greater absorption maximum in cells at 260 μm and a lesser maximum at 280 μm are due, respectively, to the presence of nucleic acids and proteins. By such means as (a) the ultraviolet absorption method, (b) the use of the visible Feulgen reaction for desoxyribose, which is a constituent of desoxyribonucleic acid (DNA) but not of ribonucleic acid (RNA), (c) the employment of specific nucleases (128, 145) for hydrolysis, and (d) the use of isolation procedures, the work of Caspersson (40—42), Claude (45—48), Bruchet (89—91), Mirsky and co-workers (158—160, 196, 197), and others (59—61, 90, 204) demonstrate that in the interphase cell there are appreciable quantities of DNA in the nuclear chromatin and of RNA in the nucleolus and in the cytoplasm and that in the dividing cell, particularly at metaphase, there is a great concentration of DNA in the chromosomes.

The observations that nucleic acids are prominent constituents of biological elements such as chromosomes, nucleoli, mitochondria (45—47) and, presumably, genes, and of viruses (917), growth organizers (90) and mutation or transforming factors (9) focus great interest upon the role of these compounds and their chemical moieties in the mechanisms of growth, reproduction, heredity, and the genesis of cancer. The majority of the applications of ultraviolet and visible microspectroscopy in biology and cytology are in these fields of interest. Since the subject of ultraviolet microscopy is thoroughly covered in the comprehensive article by Fitzgerald and Engstrom (61), this review will present only those observations which appear to be of immediate pertinence to cancer research. Caspersson and co-workers (41, 42), as a result of microspectroscopic studies of nucleic acids, have presented a theory of cellular growth. Caspersson and Santesson (48) observed an increased ultraviolet absorption at 250 μm in the nuclear chromatin, nucleolus, and cytoplasm of epithelial cancer cells of human tissues in fixed sections and interpret this as a manifestation of an altered and accelerated growth mechanism in which the DNA of the nucleus has a special and determinative role. Stowell (821, 823), using the Feulgen reaction and visible micro-absorption colorimetry of fixed sections, found a greater amount of DNA in the cells of human epithelial carcinomas as compared to adjacent homologous normal tissues, and observed analogous differences in experimental carcinogenesis. Opie and Lavin (171) determined by ultraviolet absorption and enzymatic digestion that RNA accumulates in the cytoplasm of liver cells that are hyperplastic and precursors of tumors produced by feeding butter yellow to the rat; Stowell (822) reported substantially similar observations. Catchpole and Gersh (48), in an ultraviolet microabsorption analysis of isolated cellular components, found an increased amount of nucleic acid in small cytoplasmic particles in cells of hepatoma, as compared to normal liver. The ultraviolet method is applied to the study of tissue sections of human sarcomas by Moberger (41) and by Santesson, Moberger, and Caspersson (41); sections of brain tumors by Moberger, Hakanson, and Ringertz (161); marrow cells of acute leukemia by Thorell (234); marrow cells of multiple myeloma by Olhagen (170); and exfoliated cancer cells in the vaginal smear (145, 146, 151). The results of these studies indicate an alteration in the quantity and the distribution of nucleic acids in the neoplastic cell.

There is confusion in the literature concerning the ultraviolet absorption properties of the living cell in the region of 260 μm. Larionow and Brumberg (88, 180) maintained that nuclear structural elements (except nucleoli) do not absorb at 254 μm, unless the cell is damaged or killed. Ris and Mirsky (196) stated that the living interphase nucleus of uninjured cells absorbs diffusely at 254 μm and that chromosomal (chromatin) structures are not visible in the ultraviolet unless they are also distinct in the visible region. The qualitative and the quantitative observations of others (62, 75, 148, 241), however, show beyond a doubt that there is localized absorption at 260 μm in the nuclear chromatin, nucleolus, and cytoplasm of living, uninjured interphase cells. The maximum tolerated dose of ultraviolet radiation imposes a considerable restriction on the type and number of observations permissible in this wave length region (62, 155, 148). Injury by radiation or by other means modifies the light-absorbing properties of both nucleus and cytoplasm, and, as shown by Bradfield (38), the response varies with the type of cell.

Ludford, Smiles, and Welch (140—149) have some remarkable ultraviolet photographs at 257 and 275 μm of fresh tissue spreads prepared from a variety of sarcomas and carcinomas of the mouse. These show that the sarcoma cells, in comparison with stromal fibroblasts, are larger; their nuclei are larger, have more chromatin, and bigger nucleoli; and their cytoplasm has a greater mitochondrial content which absorbs intensely. The cells from the most rapidly growing tumors exhibit the most intense ultraviolet absorption.

Recently, the results obtained by the microspectroscopic analysis of individual nuclei for nucleic acids are being compared to the data obtained by the biochemical analysis of known numbers of isolated nuclei. The one method gives the frequency-distribution of values for individual nuclei, while the other yields the average for many nuclei, which, depending upon their tissue of origin, may be cytologically very diverse. The concept of Boivin, Vendrely, and Vendrely (88, 236, 246) and the confirmatory observation of Mirsky and Ris (160) that the quantity of DNA in each set or fundamental number of chromosomes is constant in accordance with the genetic requirements of relative constancy in the composition of chromosomes has been extensively investigated by Pollister and his associates (180), notably, Swift (229, 230) and Leuchtenberger (197, 198), and by Pasteels and Lison (178) with the method of Feulgen-staining and micro-absorption colorimetry. The relative quantity of DNA so determined in the nucleus of an interphase somatic (diploid) cell is very similar for different species of mammals and is twice that of a germinal (haploid) cell; and some tissues, notably the liver and the pancreas, contain nuclei with 1, 2, and 4 times the diploid amount of DNA, although in some groups of nuclei the DNA does not follow this geometrical relationship (175). High order polyploidy and correlating DNA values are observed in the tissue cells of lower animals (229); studies on the cells of plants show either inconstancy (95) or constancy (928) of the DNA per nucleus; and under certain conditions the nuclear DNA is observed to be altered spontaneously (29—51), experimentally (59—61, 121, 122), and functionally (130). Among the most interesting observations is the finding (180) that in rapidly dividing tissue the synthesis of DNA for the new set of chromosomes takes place not in prophase, as customarily supposed, but in interphase, when there is no morphological evidence of mitosis. Moses (163) studied the nuclear acids and the proteins of Paramecium by visible ultramicrocolorimetry and has substantiated the genetic evidence that the macronucleus contains a multiple of the genetic elements possessed by the micronucleus in the diploid state. Naora (164, 165), in contrast with the work of many and using an improved optical system, found an arithmetical rather than a geometrical progression in the quantity of Feulgen-stained DNA in hepatic nuclei.

The quantitative estimation of DNA in isolated individual...
nuclei from the liver, the thymus, and the sperm of the beef and the rat is made by the Leuchtenbergers and the Vendrelys (129) with the ultraviolet absorption method of Caspersson. The DNA per nucleus, expressed in units of 10^{-10} gm., is found by this method to be of the order of 6 for diploid and 3 for haploid cells—which is in excellent agreement with the results obtained by chemical analysis. Leuchtenberger, Klein, and Klein (188) found by means of ultraviolet microspectroscopic analysis that the DNA content of nuclei of the Ehrlich ascites tumor cell of the mouse is twice that found in normal diploid nuclei, whereas the quantity of DNA per nucleus in the DBA ascites lymphoma is similar to that of diploid nuclei. These results are in accord with the chromosome counts of Hauschka and Levan (107) on similar material. The average RNA per nucleus (128), as estimated by the difference in ultraviolet absorption before and after ribonuclease digestion, is markedly increased, however, in both types of ascites tumor cells, in comparison with that of normal cells. Petermann and Schneider (107) observed by biochemical analyses an increase of RNA and DNA in the nuclei of transplanted mouse leukemia over that of normal splenic nuclei; and Cunningham, Griffin, and Luck (58) found no difference in the chemically determined DNA per nucleus in normal (see 106) and neoplastic tissues of the rat.

The advantages of using isolated nuclei mounted in glycerin for the microspectroscopic determination of DNA are (129) a more favorable distribution of absorbing materials and the elimination of the necessity for the measurement of thickness. Recently, by means of simplified procedures for the conversion of tissues into suspensions of nuclei, and with proper mounting (5), for which glycerin alone is less than optimal, nuclei are prepared that are optically homogeneous, as shown by phase and interference microscopy as well as by ultraviolet absorption microspectroscopy. A factor of uncertainty in quantitation for which no immediate answer is in sight is the magnitude of the specific extinction coefficient for DNA as it exists in the nucleus.

Ultraviolet micro-absorption spectroscopy is used to estimate the quantity of total nucleic acids in interphase nuclei of squamous-cell carcinomas and of normal epithelial cells that exfoliate from the mucosa of the human cervix uteri (151). There is a trimodal distribution of values in a group of abnormal cells and cancer cells (classes III, IV, and V of Papanicolaou [176, 177] combined), with an average and a modal quantity of total nucleic acid per nucleus which is 2, 4, and 8 times, respectively, that for the unimodal distribution given by a group of normal and atypical epithelial cells (classes I and II combined). A geometrical progression in values is consistent with the general concept of growth (72) by the reduplication of nuclear formed elements (92, 121, 286) containing DNA and RNA, and the data have cogent bearing upon the development of methods for the cytological diagnosis of cancer (149).

Infrared absorption.—The infrared spectrum of an organic compound is a physical property that may be used in the identification of the compound and in the investigation of its molecular structure (38, 244). The potentialities of the infrared spectra, as compared to spectra obtained in other spectral regions, are shown by the fact that biochemical compounds, such as the carbohydrates and the lipids (which are essentially non-absorbing in the ultraviolet and the visible regions) and the proteins (which have one similar broad absorption band in the ultraviolet region), are characterized by distinct, many-banded spectra in the infrared. Moreover, a study of the absorption spectra with polarized infrared radiation (12, 74, 85, 108, 181, 224) reveals the direction, if such exists, for the preferred arrangement (orientation) of certain chemical bonds. By this means, for example, it is observed that in certain types of fibrous proteins the carbonyl bonds (C = O) are oriented perpendicular to the axis of the fiber, and, from this fact, and the accepted nature of the peptide bond, it can be deduced that the polypeptide chains are predominately extended and oriented parallel to the fiber-axis.

In the application of infrared spectroscopy to the study of the biochemical constituents of tissues and the tissues themselves, the cross-sectional area of the sample required in current systems of microspectroscopy (12, 24, 25, 86, 105, 147, 246) is of the order of a square 10—20 μ on an edge, with the smaller sample analyzable only in the very near infrared, while for macrospectroscopy the linear dimension is 10 or more times this value. Barer, Cole, and Thompson (12) were the first to describe a system of infrared microspectroscopy and to record the spectra of minute quantities of antibiotics, therapeutic factors, biochemical compounds, fibers, crystals, and tissue cells. Blout and his co-workers (27) determined the infrared spectra of dehydrated tissue sections and smears by macrospectroscopy and observed the presence of an intense absorption band, which lies at the same position as a strong band in the nucleic acid spectra (26), in a section of a rapidly proliferating cellular mammary carcinoma, as contrasted with the lesser absorption of a benign adenoma of this gland; and they (25—26) recorded the spectra of nucleohistones fibers, steroid crystals, and tissue sections with both a macro- and a microspectrometer.

Wood (247) observed the infrared microspectrum of living muscle cells (in the spectral region beyond that in which water has intense absorption) and found the spectrum to be closely duplicated by that of the protein, myosin. Morales at al. (16%) studied the infrared absorption of proteins and other constituents derived from the contractile system of skeletal muscle. Fraser and Fraser (85, 248) presented data on the molecular structure of DNA obtained by the microspectroscopic study of oriented samples with polarised infrared radiation. Schwartz (205) and co-workers found by macrospectroscopy that the spectra of anhydrous sections of “visceral” tissues (liver, muscle, heart, kidney, adrenal, thymus, spleen, and lymph node) and of neural tissue have two different types of absorption bands: namely, bands which are common to all tissues and so-called “finger print bands” which are characteristic for the individual tissue. Stevenson and Bolduan (316) discussed the use of infrared spectroscopy in the identification of dried films of bacteria. Woerley (246), in an investigation of the infrared macrospectra of dehydrated tissues and tissue components, attributed the absorption pattern of DNA and RNA in the wavelength region, 8—11 μ, to the pentose and phosphoric acid moieties. He observed a strong resemblance in
this region between the spectra of nucleic acids and that of films of isolated nuclei, and correlated, in a general way, the intensities of the absorption bands with the concentrations of nucleic acids in the tissues. Thus, the infrared and the ultraviolet absorption spectra may provide complementary and substantiating information about cellular nucleoproteins. A universal microspectrometer for the infrared, visible, and ultraviolet regions is described (15%) and a ratio automatic recording microspectrophotometer for the entire optical spectrum, which is under design and construction by the Perkin-Elmer Corporation, will be a most versatile instrument with manifold applications in biology and medicine.

**Fluorescence.**—When a molecule is brought to an excited state by the absorption of electromagnetic radiations and by the transfer of energy to a particular part of the molecule, a transition from this to a lesser energy level may be accompanied by an emission of light which, if instantaneous, is called a fluorescence spectrum (191) and for complex molecules consists of a continuous emission band with several peaks of higher intensity. While the relation between the chemical structure and the fluorescence of an organic molecule is but poorly understood, it is known that a closed ring structure favors luminescence, as is observed in the majority of aromatic hydrocarbons composed entirely of condensed benzene rings, and in many heterocyclic compounds.

The use of fluorescence spectroscopy in the investigation of complex mixtures of hydrocarbons in carcinogenic tars is a historical methodological step in the isolation and identification of benzopyrene as a cancer-producing agent (5%). Graff (98) was the first to employ the fluorescence microscope in the study of the uptake of chemically pure carcinogens by living mammalian cells in tissue culture. The localization and distribution of cutaneously applied carcinogenic hydrocarbons is a subject of investigation which is facilitated by the fluorescence of carcinogens and their derivatives. Doniach, Mottram, and Weigert (97, 68) observed a brilliant violet fluorescence spectrum in the skin of mice painted with 3,4-benziprene and in certain organs of animals receiving intravenous injections of suspensions of the carcinogens. Simpson and Cramer (112, 115) examined frozen sections of the skin of mice with a fluorescence microscope and found that fluorescing material appears in the epidermis within a day after a single painting with 20-methylcholanthrene and persists for several weeks. Setalä (209, 210) used polyethylene glycols rather than organic solvents as vehicles for the carcinogens, 20-methylcholanthrene, 1,2,5,6-dibenzanthracene, 8,10-dimethyl-1,3-benzanthracene, and 1,2-benzanthracene; and he studied their cutaneous localization by fluorescence microscopy. Berenblum, Holiday, and Jope (91) discussed the possibility of seeking carcinogens in individual cells by fluorescence microspectroscopy, an approach which is now a reality in that this analytical method is used (180) in the study of fluorescing material that localizes in epithelial cells of the bladder after the ingestion of the carcinogen, β-naphthylamine. Sjostrand (114, 215) described the use of fluorescence microspectroscopy in the detection of riboflavin and thiamin in tissue cells.

Cells and tissues may be stained with fluorescent dyes (fluochromes) which, because of their basic or acidic chemical properties and other factors, localize in and differentiate nucleolar and cytoplasmic structures, as do conventional dyes. The subject of fluorescence microscopy and the employment of fluochromes are discussed in the comprehensive monograph of Haitinger (108) and in articles by Barnard and Welch (14), Ellinger (78), and Richards (194).

An ingenious method for the microscopic localization of antigenic material in tissue cells, employing fluorescence microscopy and antibody conjugated with the fluochrome, fluorescein, is described by Coons (53) and his collaborators.

Friedman (89) described the use of three fluochromes in the study of exfoliated cells in the vaginal smear: berberine sulfate, a basic dye that imparts a brilliant yellow-white fluorescence to nuclei; acid fuchsin, an acidic dye that combines with the cytoplasm to provide a red-purple fluorescence; and acridine yellow, which acts as a nuclear and cytoplasmic fluorescent stain. Cancer cells are observed in general to fluoresce more brightly than do normal cells and to have cytoplasmic bright orange or red-orange fluorescence which differs in color from that of normal cells.

The development of a quantitative scanning method for the preliminary screening of smears for the presence of cancer cells has been undertaken by workers at the Memorial Center (149, 153). The underlying chemical and physical principles are as follows: cells are stained with a basic fluochrome, such as berberine, under conditions (101, 156) that favor selective combination of the dye with chemical constituents, such as DNA, which are located in greater concentration in the nucleus; squamous cancer cells, relative to normal cells which exfoliate from the cervix uteri, combine with more fluochrome and emit on the average per unit nuclear area twice the quantity of light energy of normal cells. The light energy derived from the scan of a cell is converted by a photocell into a voltage pulse which, in turn, is analyzed in terms of its ability to energize an electronic counting circuit that is set to respond to a certain voltage input and to register automatically thereby the presence of certain types of cells—e.g., a cancer cell, as differentiated from a normal cell. If it is shown by microspectroscopy that cancer cells with a characteristic fluorescence hue, when stained by the polyfluorochrome method of Friedman (89), have a distinct difference in the fluorescence spectrum when compared to the normal cell, then this additional discriminative property may be conveniently utilized by the scanning device.

**SUMMARY**

A description has been given of some of the recent advances in methods of microscopy of the cell which utilize the phase (phase, interference,
and polarizing microscopy), the plane (polarizing microscopy), the dispersion (dispersion microscopy), and the amplitude and the frequency (microspectroscopy) of light waves in the optical spectrum. The applications of these methods to the study of the mass, the elementary and organic chemical composition, and the structure of the nucleus, nucleolus, chromosomes, mitotic apparatus, and cytoplasmic particles are presented with a view toward the manifest or potential significance of the results in cancer research.

The unique value of the phase microscope is that it, as no other method, provides a means for the structural analysis of living cells without the hazard of injury or the uncertainty of artifact. Outstanding among the applications are the study of the events during mitosis, the formation and the movement of chromosomes, and the structural alterations in the mitochondria.

Recent improvements in the polarizing microscope make it possible to investigate the spindle apparatus in the living, dividing cell and provide information which indicates the spindle to be an orienting force through which a co-ordinated contractile mechanism for chromosome movement is built up from protoplasm. It has been shown by a study of the effects of colchicine that there is a close relation between the mechanical integrity of the spindle and the maintenance of the metaphase plate of chromosomes. The contractility of spindle fibers is interpreted in terms of the folding of molecular (polypeptide) chains during anaphase.

By means of interference microscopy, loci of identical optical paths through a cell-object appear as alternate bright and dark lines, called interference fringes, which form a contour-like map of the object. This effect may be interpreted in terms of variations in thickness of the cell-object, with a resolution of the order of 0.03 μ, or it may be used to compute the anhydrous organic (protein) mass and the hydrous protoplasmic mass of a living, or unfixed, cell-object.

Dispersion microscopy provides a method for the determination of the refractive index of a biological object. The difference in index between certain cellular structures is such that, by the use of this method, structures appear in contrasting color due to optical effects.

The observations that nucleic acids are prominent constituents of biological elements such as chromosomes, nucleoli, mitochondria, and, presumably, the genes focus great interest upon the role of these compounds and their chemical moieties in the mechanisms of growth and the genesis of cancer. Applications of ultraviolet and visible microspectroscopy in these fields of interest are discussed. The quantitative estimation of nucleic acids in individual nuclei of cancer cells of the mouse and the human cervix uteri yields values that are distinctly greater, by a geometrical progression, than those of the normal cell.

The use of infrared microspectroscopy in the analysis of the biochemical constituents of tissues and the tissues themselves is discussed in terms of the identification of organic compounds and the investigation of molecular structure.

Fluorescence microscopy and microspectroscopy provide a means for the investigation of the localization and the distribution of fluorescent carcinogens in tissues and cells. Fluorescent dyes (fluorochromes) may be used to differentiate nuclear and cytoplasmic structures, as do conventional dyes. Exfoliating cancer cells of the vaginal smear have distinguishing properties relative to normal cells when stained with fluorochromes. One or more of these discriminative properties may be utilized by an automatic scanning device for the detection of cancer cells.

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Fig. 1.—Living fibroblast cell in tissue culture of embryonic mouse skin. Phase photomicrograph, dark contrast, 1,500 X, N. A. 1.25. The formed elements in the nucleus are nucleoli; and those in the cytoplasm are dark spherical 'fat' droplets and elongated filamentous mitochondria.

Fig. 2.—Living oocyte of Chaetopterus pergamentaceus. Photomicrograph taken with a special polarisation microscope, approximately 2,000 X, N. A. 0.84. The metaphase spindle is vividly displayed because of its birefringence. The position of some of the chromosomes can be identified in the equatorial plane. (Photograph obtained through the courtesy of Dr. Shinya Inoué, School of Medicine, University of Washington.)

Fig. 3.—Living sarcoma cell in tissue culture of the mouse tumor, Ma 357. Phase photomicrograph with same magnification and optical conditions as in Figure 1. The nucleus is large and contains numerous aggregations of chromatin; and spherical and elongated mitochondria are present in abundance. (Photographs obtained through the courtesy of Dr. John Biesele, Sloan-Kettering Institute for Cancer Research.)
Figs. 4-7.—Living sarcoma cells in tissue culture of the mouse tumor, Ma 387. Multiple-beam interference photomicrographs with monochromatic light, $\lambda$, 0.546 $\mu$, 1,000 $\times$, N.A. 0.85, and with the cells temporarily suspended in air. The interference fringes appear as bright topographical or contour lines, some of which are concentric with the nucleus, and they may be interpreted in terms of minute variations in the thickness of the cell or be used to compute the hydrous mass of protoplasm in various parts of the cell. For example, the nuclei of these sarcoma cells have an average hydrous protoplasmic mass of $40 \times 10^{-12}$ gm., a value substantially greater than that found in embryonic fibroblast nuclei.

Figs. 8, 9.—Squamous epithelial cells of the human buccal mucosa. Multiple-beam interference photomicrographs in monochromatic light, $\lambda$, 0.546 $\mu$, 1,000 $\times$, N.A. 0.85, and with the cells suspended in air.
Figs. 10–13.—Sarcoma cells and nuclei of the mouse tumor, Ma 387. Ultraviolet photomicrographs at λ, 0.265 μ, 1,500 ×, Bausch and Lomb (Grey design) reflecting objective, N. A. 0.72.

Fig. 10.—Living cell in tissue culture.

Fig. 11.—Cell in tissue culture fixed with acetic acid-alcohol and mounted in glycerin.

Fig. 12.—Cells and nuclei isolated in 0.88 M sucrose with 0.0018 M CaCl₂ and mounted in glycerin.

Fig. 13.—Similar procedure to Figure 12 except cells and nuclei are mounted in 0.88 M sucrose, pH 7.0 buffer. The nucleic acid content per nucleus is computed from the product of the ultraviolet absorption (extinction) and the area of nuclei in optically homogeneous preparations such as Figure 13 and is 17.1 × 10⁻¹² gm. for these sarcoma nuclei, a value very much greater than that found in embryonic fibroblast nuclei.
Microscopy. I: A Review

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