METHODS IN MICROBIOLOGY

VOLUME 5A

METHODS in MICROBIOLOGY

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Detailed acknowledgments are given in the legends to figures.

PREFACE

Volume 5 of "Methods in Microbiology" is concerned with the microbial cell—methods of observing it, of studying its properties and behaviour, of analysing it chemically and immunologically, and of purifying and characterizing its various "organelles" and macro-molecular components. Wherever possible, the emphasis has been placed on quantitative methods.

We have tried to cover relatively new techniques such as reflectance spectrophotometry, isoelectric focusing and polyacrylamide gel electrophoresis which appear to us to have considerable future potential in microbiology in addition to more generally used techniques such as those for cell disintegration and hybridization of nucleic acids which are not fully described in a concise form elsewhere.

As with earlier Volumes in the Series we have left the treatment of the different topics largely to the individual authors, restricting our editorial activity to ensuring consistency and avoiding overlaps and gaps between the contributions.

As contributions accumulated it became obvious that there was too much material for a single Volume and the content was divided. Volume 5A contains Chapters concerned with the direct observation or study of whole cells or organelles while Volume 5B is concerned with the disintegration of cells, their chemical analysis and the techniques used to separate and characterize their components.

Our thanks are due to the pleasant way in which our authors have co-operated with us and particularly to those who agreed to update their contributions when delay in the publication process made it necessary.

J. R. NORRIS
D. W. RIBBONS

April, 1971

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CHAPTER I

Microscopy and Micrometry

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I. INTRODUCTION

The microscope is an essential tool for the microbiologist; without it he is working in the dark. Modern research microscopes are precision instruments designed with great skill and dedication, yet they are used by many in an indifferent, almost haphazard, way. It is impossible in the space of a single Chapter either to study the microscope historically or to study the scientific theory which lies behind its design, but only to provide the basic knowledge required for the intelligent and accomplished use of the instrument as a tool of microbiological investigation. Basic theory will be discussed only in so far as it is required for the realization of the full design potential of the modern microscope.

The main features of a modern microscope are illustrated in Fig. 1.

II. LIGHT AND ITS BEHAVIOUR

The visible spectrum used in light microscopy forms a very small portion of a much larger spectrum of electromagnetic radiations. This restricted spectrum is of particular use in microscopy because the biological sensitivity of the eye to radiations in this range enables us to use the brain as the "recording" or "display" stage of the process, albeit a temporary record. This does not mean that other sections of the electromagnetic spectrum cannot be used for microscopy—they can—but when invisible radiations are used in the image-forming process a display which "generates" wavelengths in the visible range must be used, e.g. photographic emulsions in U.V. microscopy, fluorescent screens in electron microscopy. The relationship between the visible spectrum and the electromagnetic spectrum (E.M.) is shown in Fig. 2. While the E.M. spectrum includes wavelengths of radiations from about 3000 metres to less than 10^{-12} centimetres visible light fills the very small range from about 7×10^{-5} cm down to 4×10^{-5} cm, or, in the units usually used to define wavelengths, from 7000 Angstrom units (Å) to 4000 which is equivalent to 700 nanometers (nm) to 400 nm (1 Å = 10^{-8} cm; 1 nm = 10^{-7} cm).

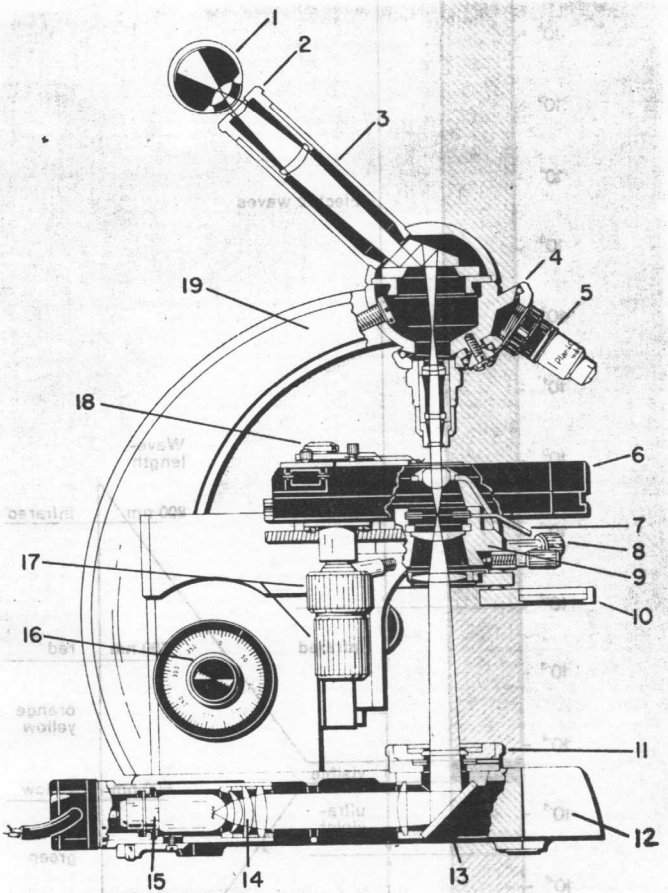

FIG. 1. A modern microscope with inclined oculars and built-in lamp—the Zeiss (Oberkochen) Standard RA Routine and Research Microscope. (Figure by courtesy of Carl Zeiss, Oberkochen, W. Germany.)

1. Eye
2. Eyepiece
3. Eyepiece tube
4. Revolving nosepiece
5. Objective
6. Mechanical stage
7. Condenser diaphragm—control lever
8. Control knob for swing-out top element of condenser
9. Condenser
10. Filter carrier
11. Diaphragm insert
12. Base
13. Mirror
14. Lamp collector lens system
15. Built-in low voltage lamp
16. Concentric coarse- and fine-focus control knobs
17. Concentric x-, y-axis control knobs for slide holder movements
18. Slide holder
19. Limb

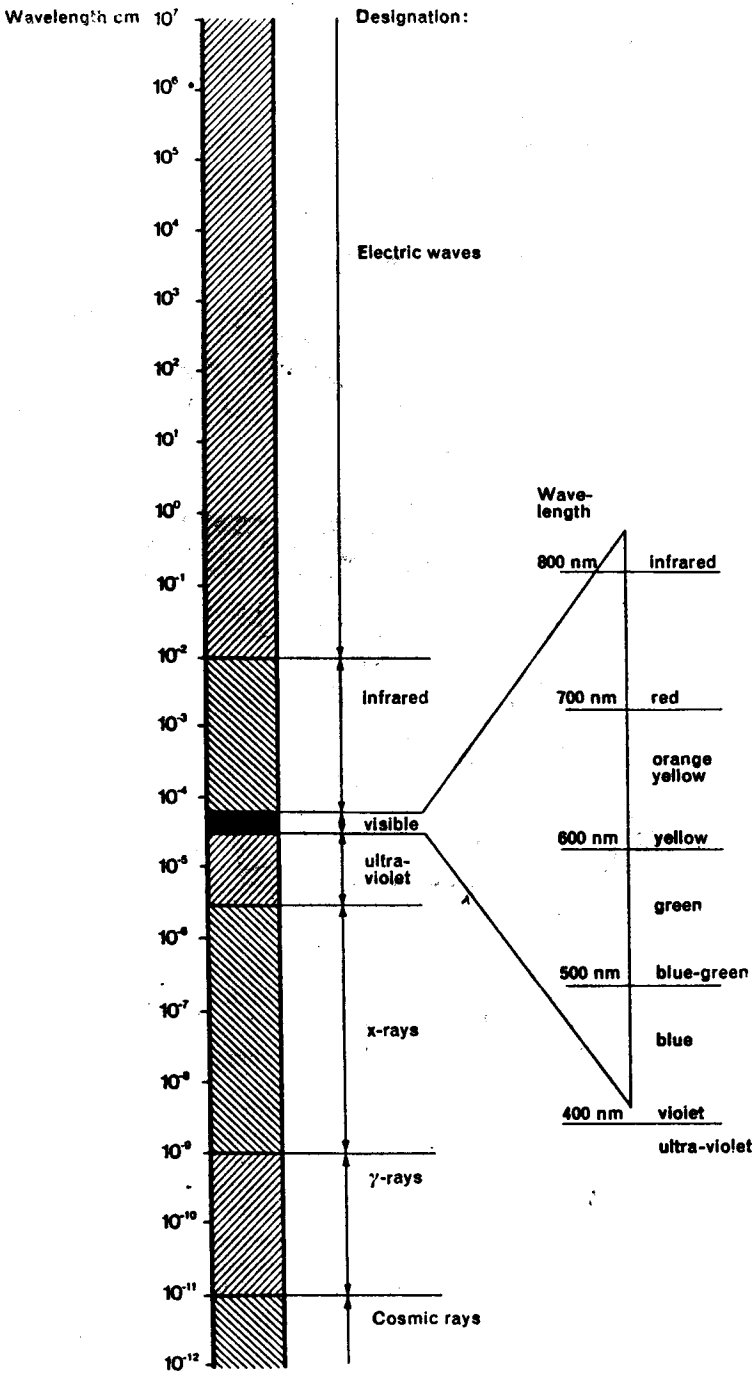

FIG. 2. The electro-magnetic spectrum of radiation (Figure by courtesy of Wild Heerbrugg, Switzerland).

Each wavelength of the electromagnetic spectrum can be associated with a wave number—the reciprocal of the wavelength in cm; and since they are all propagated with the same velocity each will have a different frequency, ν. Each wavelength is also associated with a specific energy E, where $E = h\nu$ is the energy in ergs of a quantum of frequency, ν, where h, is Planck's constant; or the energy value may be expressed as V, the energy in electron volts. In general terms we can say that the shorter the wavelength the higher the energy content and the more penetrating (and dangerous) the radiation; while the longer the wavelength the lower the energy and the less penetrating the radiation. Thus we find a progressive increase of energy from ultraviolet through X- and γ-rays to cosmic rays the most penetrating of all.

A. Theories of light

The earliest theories of light were attempts to explain the phenomenon of vision rather than the nature of light itself. As early as 500 B.C. Greek thinkers had put forward the "tactile" and "emission" theories; the former postulated that the eye sent out invisible sensors or sensitive probes which were able to "feel" objects too distant to be touched physically, while the latter postulated that the object itself emitted something which entered the eye and affected some sensitive part of the eye which was responsible for sight. For reasons obvious to us now, the "emission" theory eventually completely displaced the "tactile" theory, and by giving the emission the term "visible radiation" we can provide a reasonable explanation of the visual process as follows.

Visible radiation emitted (e.g. on heating), reflected or scattered by a body, on entering the eye, is focused by the eye lens onto the retinal surface which contains special sensitive cells connected by nerves to the brain. When light falls on these a chemical and physical reaction takes place involving the transformation of the pigment visual purple and resulting in the emission of electrical impulses to the centre of the brain where the visual image is "recomposed" and results in the sensation of sight. The physiology of vision will not be discussed here.

Quite apart from any physiological consideration it is possible to show experimentally that visible radiation is associated with the transfer of energy and any theory of light must accommodate the energy phenomena associated with it. By analogy with other known methods for the propagation of energy we could propose that light conveys energy in the form of "waves" (as the sea transports energy through its wave motion), or the energy may be conveyed as discrete quantities associated with the movement of particles (as moving billiard balls, for example, possess kinetic energy). It should be noted that the transfer of energy in waves need not involve

the physical translocation of the medium through which the wave passes, either of water, or, in the case of light, of air. These considerations led to two basic theories of the nature of light: the "wave" theory and the "corpuscular" theory.

From the 17th century it has been known that the propagation of light could be represented by rays and simple experiment showed that those rays travelled in straight lines translating energy along a path from source to receptor. From the study of the interference phenomena associated with "Newton's Rings", Newton recognized that there was some sort of periodicity associated with light which was evidence for a wave theory; on the other hand, he could not reconcile this with the rectilinear propagation of light and, incorporating the concepts of his laws of motion, preferred to explain light as a procession of corpuscles which either possessed an internal vibration of their own or were in some sense controlled by waves or vibrations of the medium through which the light travelled. This objection to a simple wave theory was removed when it was discovered that light is not propagated in a strictly linear fashion, and that the rays of a beam of light which impinged on the edge of an object were bent away from the direction of propagation—the phenomenon known as *diffraction*.

As a result of many experiments on interference and diffraction a set of determinations of the wavelength of light were made and it was shown conclusively that different wavelengths were always associated with different spectral colours. The wavelengths of the different spectral colours are shown in Fig. 2.

While the simple wave theory could be used to describe the behaviour of light under many circumstances there were many inadequacies of the theory which were resolved when Maxwell formulated the equations of electromagnetism and showed how these could be used to describe the behaviour of light. An essential feature of the theory was the propagation of transverse electromagnetic waves (a vector quantity) as distinct from the simple theory of longitudinal waves in which the direction of vibration is always the same as the direction of propagation, so that longitudinal wave motion could be represented as variations of a scalar quantity.

The work of Maxwell soon led to the enormous expansion of the spectrum and the realization that "light waves" were only a tiny section of a very much larger spectrum of electromagnetic radiation associated with wavelengths from over 3000 m to less than 10^{-13} m.

Even Maxwell's electromagnetic explanation of the nature of light was inadequate to explain certain phenomena such as the energy transferred to the electron of an atom which had been excited by radiation with subsequent ejection of the electron (ionization). The failure resulted from the implication that the energy of electromagnetic radiation was continuously

distributed. This difficulty was surmounted by Einstein's explanation that the energy of the radiation was concentrated into separate discrete packets, each packet being called a photon, and that for any particular wavelength all the photons had the same energy value. Coupled with Plank's realization that the energy is emitted in multiples of a single unit, with no fractions of a unit being possible, the unit of energy was called the quantum and the relationships between the energy level of a radiation and the wavelength were given by—

$$E = hc/\lambda$$

Where h is Plank's constant ($6{\cdot}6 \times 10^{-34}$ joule sec), c is the velocity of light and λ the wavelength.

If ν is the frequency then,

$$c = \nu\lambda$$

and

$$E = h\nu$$

The modern theory of light is a composite theory which incorporates both the electromagnetic (wave) theory (which has no place for photons) and the photon (particle or corpuscle) theory which has no place for the waves. The former describes adequately the phenomena of interference, diffraction and polarization while the latter is required for an explanation of the observed interactions of radiation and matter.

Modern quantum mechanics constitutes a single theory incorporating the appropriate parts of the electromagnetic wave theory, the quantum theory and the theory of relativity in a composite explanation of the properties of light and matter.

For an elementary understanding of the behaviour of light in relation to microscopy we can disregard the quantum theory. The quantum aspects are of importance, however, in understanding the phenomenon of fluorescence which lies at the heart of the fluorescence microscopy techniques which are now used to such great effect. Fluorescence microscopy is dealt with elsewhere in this Volume (Walker *et al.*, this Volume, page 219).

B. Light waves

For our present purposes we may depict light as consisting of waves such as that represented by the sine curve shown in Fig. 3.

This form of simple harmonic motion is describable by the trigonometric function—

$$y = A \cos 2\pi \left(\frac{x}{\lambda} - \frac{t}{T}\right)$$