Principles and Technique of Fluorescence Microscopy

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With four plates (figs. 3 to 6)

CONTENTS

SUMMARY

Interest in fluorescence microscopy has greatly increased in recent years. Technical considerations have to some extent prevented even wider application of the various fluorescence techniques now available for microscopical study of biological specimens. This paper outlines the basic requirements for optimal image quality, for the benefit of biologists and others who may not be conversant with the optical principles involved. The central problem of illumination is reviewed in some detail, and an assessment given of the two methods in current use, namely the bright-field and dark-field systems. Ratios of fluorescent to activating light received by the objective aperture, given by the two systems, have been compared, and measurements have been made of their relative light-concentrating power.

Available light sources and their suitability for the excitation of fluorescence are discussed, with the problems of selecting appropriate light filters for use with the alternative systems of illumination.

It is concluded that the dark-field system has decided advantages in practice and in theory for the following reasons:

(1) The dark-field condenser serves as an efficient primary filter, contributing to a black background and hence good contrast.

(2) The equivalent focal length is less than that of the bright-field condenser and it concentrates energy in a smaller area; this compensates in part for the loss of energy inevitably caused by the central stop.

(3) It permits the use of wide-band primary filters of maximum transmission because contrast in the fluorescent image is affected only by a weak superimposed dark-field image produced in the object-plane by scattered residual activating light passed by the primary filter. With blue-light activation the visible dark-field image is effectively eliminated by means of a weak blue-absorbing secondary filter.

(4) The loss of contrast due to veiling glare is minimized.

A rational layout for fluorescence microscopy and methods for accurate alignment of the microscope in the vertical and horizontal positions are described. Factors influencing the choice of suitable objectives and eyepieces and some details of methods for mounting specimens are given.

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Introduction

THE possibility of studying the distribution and morphology of autofluorescent structures in biological specimens was recognized by Köhler (1904 *a*, *b*) during early experiments in ultra-violet microscopy. Light from the cadmium spark at a wavelength of 275 mμ was used. Many objects exhibited fluorescence when illuminated in this way, or by an intense emission in the region of 280 mμ from the magnesium spark. Köhler further envisaged the possibility of treating microscopical objects to make them self-luminous, so anticipating the use of fluorescent dyes which were later introduced by Prowazek (1914).

Ellinger (1940) records how Köhler and Siedentopf in some pioneer work in 1908 tried out a dark-field condenser for concentrating ultra-violet light on to the specimens. The sub-stage illuminating components, specimen slide, and coverglass were made of quartz; observation was with normal glass objectives and oculars. A fluorescence microscope incorporating these features but with a carbon arc as the light source was devised soon afterwards by Heimstadt (1911); but in spite of this early recognition of the value of dark-field illumination most investigators were satisfied with bright-field condensers for fluorescence work.

However, in 1937 Barnard and Welch used the Beck–Barnard ultra-violet microscope and a special dark-field condenser designed by Smiles (1933) for investigation of some fluorescent components in bacterial cells, and were responsible for renewed interest in the dark-field system. They showed subsequently that adequate low-power fluorescence microscopy could also be carried out with a bright-field condenser fitted with a central stop to prevent direct light entering the objective.

Today there is increasing interest in fluorescence microscopy, encouraged by introduction of the labelled-antibody technique (Coons and Kaplan, 1950) and by the application of fluorescent dyes as tracers and in histochemical techniques. Utilizing the special affinity of aminoacridine compounds for nucleic acids, Armstrong (1956) developed a sensitive fluorescence technique in which acridine orange is used for the identification of DNA and RNA in mammalian cells; this has been applied profitably to the study of cytochemical aspects of virus cytopathology (Anderson, Armstrong, and Niven, 1959). For routine detection of mycobacteria in smears or tissue sections, fluorescence microscopy after auramine staining has replaced the standard Ziehl–Neelson method in some laboratories; and fluorescent dyes are now similarly employed in diagnostic exfoliative cytology (Friedmann, 1950; Bertalanffy, Masin, and Masin, 1956, 1958).

Many variations on the basic optical equipment for fluorescence microscopy are described in the literature, but in every case the apparatus falls into one of two categories depending upon the condensing system employed for illumination of the specimen. For the studies with acridine compounds, referred to above, the apparatus used was one developed for general fluorescence work at

the National Institute for Medical Research; this incorporates a dark-field condenser of the cardioid type. Coons and his associates (1955) have evidently favoured a very similar arrangement for their more recently published work with the fluorescent antibody technique. On the other hand, Ellinger (1940) considered the dark-field system to be obsolete on the grounds that it naturally limited the intensity of the exciting radiations; essentially the same view was expressed in a recent and authoritative review by Richards (1955), and has been accepted by many microscopists. A primary object of the present paper, therefore, is to focus attention upon definite advantages of the dark-field system, deduced both from a consideration of the optical principles involved and from practical experience with many of the current uses of fluorescence microscopy in biological research.

Methods of Illumination

Fluorescence occurs when a substance absorbs light of specific wavelengths and simultaneously re-emits part of this energy at longer wavelengths, usually in the visible region of the spectrum. In order to observe fluorescence through the microscope it is necessary to illuminate the object with light of high intensity and of specific wavelengths, generally in the region between 300 mμ and 500 mμ.

A fundamental difference between the ordinary light microscope and one adapted for observing fluorescence lies in the mode of formation of the visible image. Normally, the image is formed by the modification of light passing through the specimen, and to obtain well-resolved images with good contrast the aperture of the condenser should not exceed that of the objective. A fluorescent image, on the other hand, is due to visible light emanating from the specimen itself, and the illuminating beams which excite fluorescence do not contribute directly to the formation of the image. In view of the low intensity of most forms of fluorescence and inevitable light losses of up to 90% in the microscope, it becomes essential to employ the most efficient possible light source and optical system for illumination of the specimen. In the fluorescence microscope therefore the illuminating aperture must be as large as working conditions will permit to ensure that the maximum amount of light will reach the specimen.

The quality of the fluorescent image, and the precise requirements for optimal observing conditions, depend ultimately on physical properties of the specimen itself. The most satisfactory results can be anticipated only when such properties have been taken into account in the layout of the optical system. When fluorescent materials are irradiated with light, maximal excitation occurs in the regions where there is a high level of energy absorption. The most suitable exciting wavelengths for fluorescence microscopy will be determined therefore mainly by the absorption characteristics of the specimen under investigation, e.g. porphyrins naturally occurring in tissues having specific absorption maxima at 400 mμ and 550 mμ; but much of the recent work on biological specimens has involved the use of fluorescent dyes or

labelling reagents and in these circumstances it is the absorption properties of the reagents (or, more precisely, of the tissue-reagent complex) which determine the appropriate conditions for microscopical observation. Absorption curves of some of the fluorescent dyes in general use are shown in fig. 1. Apart

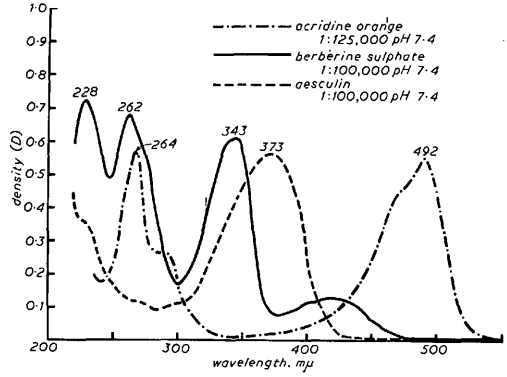

FIG. 1. Spectral absorption curves of fluorochromes.

from the natural limitations imposed by the specimen itself, resolution and image quality obtained in fluorescence microscopy depends on two factors:

image brightness, which is determined by the intensity of the exciting radiations;

image contrast, expressed as

$$\frac{\textit{intensity of object}-\textit{intensity of background}}{\textit{intensity of object}}$$

The latter is controlled by the light-condensing system employed to illuminate the specimen and by light filters which may be incorporated in the optical system.

Two subsidiary but significant considerations are the light losses at the various air-to-glass surfaces in the microscope, and the degree of what is known as 'veiling glare' in the system which reduces the contrast and true colour values of the fluorescent image.

Filtration. Several commercially-manufactured light sources with suitable intensity and wavelength emission are adequate for most purposes (see Light Sources, p. 435). It is necessary, however, to select the wavelengths required by interposing suitable primary coloured liquid or glass filters between the source and the condensing system which only transmit light in the region required to excite fluorescence. A suitable secondary filter which absorbs activating rays but transmits the fluorescent light emitted by the specimen has

to be placed between the objective and the observer (often for convenience in the eyepiece). The filter prevents interference with the definition of the image and also protects the eyes from ultra-violet radiations that may enter the microscope. Incorrect filter systems may be responsible for a considerable loss of brilliance and contrast in the final image.

Evaluation of these factors, as presented below, is based upon several years' practical experience in biological applications of fluorescence microscopy. It has been possible to compare the advantages and shortcomings of bright-field and dark-field condenser systems and the different forms of light filtration which must be used in conjunction with these, in relation to the study of specific problems by some of the techniques in current use. In particular, attention has been given to the need for obtaining a final image of high intensity with good resolution, contrast, and colour preservation, allowing routine photomicrographic records to be made on colour film.

Practical systems for fluorescence microscopy

The bright-field condenser system. In this arrangement the substage optical system is virtually that used for ordinary bright-field microscopy. If wavelengths below 360 mμ were to be used to excite fluorescence, it would be essential to employ a quartz condenser and lamp collector lens to ensure maximum transmission of ultra-violet light, but these shorter wavelengths are rarely employed at the present time. Abbe and aplanatic design condensers are adequate for low and medium magnifications, but to obtain the best results, especially when using immersion objectives, an achromatic condenser, NA 1·30 to 1·4, is recommended, immersed in oil to the undersurface of the slide to ensure the maximum angle of activating light. In general the highest illuminating aperture possible should be used, providing this covers the extent of the field with the objective and eyepiece in use.

A proportion of the light, or all of it, depending on the ratio of the objective aperture to the focal length and aperture of the condenser, will pass through into the observing system of the microscope. Consequently with this system the whole waveband of activating light must be 'selectively' absorbed by means of an appropriate secondary filter in the eyepiece of the microscope. Typical filter combinations recommended for general observation purposes in the study of tissue preparations are shown in table 1. The filters quoted are adjusted to the intensity and spectral transmission of the 250-watt high pressure mercury lamp. Sources with different characteristics will, of course, require their own carefully-matched pairs of primary and secondary filters, appropriate to the wavelengths required.

There are several theoretical disadvantages inherent in the bright-field condenser system; these must always be considered if the equipment is to give results which are acceptable from the optical standpoint and also biologically meaningful. The primary filter should, so far as possible, transmit only the wavelengths needed for excitation of fluorescence, and the loss of intensity caused by the denser filtrations required to achieve this end inevitably result

TABLE I

Bright-field condenser

Primary filters			*Secondary filters*	
Stain technique	*Type*	*Maximum transmission*	*Type*	*Maximum absorption*
Primulin dye for distinguishing between living and dead cells	*Wratten No. 18A (glass)	365 mμ (52% transmission—ultra-violet excitation)	Wratten gelatin No. 3 plus No. 2B	at wavelengths shorter than 460 mμ
Fluorescein-labelled antibody (Coons technique)	*Wratten No. 50 (glass-mounted gelatin)	436 mμ (10% transmission—blue light excitation)	Wratten gelatin No. 8 plus No. 9	at wavelengths shorter than 500 mμ
Acridine orange nucleic acid staining method (Armstrong)	*Wratten No. 35 (glass-mounted gelatin)	436 mμ (37% transmission—blue light excitation)	Wratten gelatin No. 15G	at wavelengths shorter than 500 mμ

* A cell, 2 cm thick, containing a 10% copper sulphate solution, is always used in conjunction with these filters.

Note. The transmission of OX7 and Wratten No. 50 filters diminishes with continued exposure to ultra-violet radiation.

The selection of filters. It is advisable to check with a spectroscope the transmission of all filters used in conjunction with a light source that is suitable for the observations to be undertaken. Slight variations in density of the secondary filters will modify the fluorescent colours. Autofluorescent violet, blue, and white light emitted by activated unstained tissues, &c., may be appreciably altered in colour by certain filters that absorb ultra-violet and blue-violet light.

A correctly balanced colour range with blue light excitation should permit the unstained, non-fluorescent structures to be observed only as very weak blue dark-field images when employing induced fluorescence techniques.

An alternative filter combination giving an 80% transmission from 350 mμ to 410 mμ with the bright-field condenser is prepared by dissolving 0·75 g of iodine in 100 ml of carbon tetrachloride and using at 1 cm cell thickness for the primary filter. Secondary filters are Wratten No. 9 with Wratten No. 2B. This system cannot be used for blue and white light fluorescence emission.

Details of liquid and glass filters for the isolation of specific wavelengths of the mercury discharge lamp are given by Bowen (1946).

in diminished activation of the specimen. In addition, the need for a strongly absorbing secondary filter with fairly wide spectral absorption characteristics, results in a further sacrifice of intensity in the fluorescent image and also some degree of colour degradation. The latter may be serious, as interpretation of the fluorescent emission from a biological specimen may require an accurate identification and measurement and depend upon recognition by the observer of specific polychromatic effects, i.e. differential fluorescence.

The most serious light losses, however, occur at the specimen itself. Fluorescent light is emitted equally in all directions and it may be assumed that this is so with biological material as it is for a self-luminous body. Since objectives are used at full aperture in fluorescence microscopy the amount of light reaching the image plane, after refractions at the interfaces in the object plane, is primarily determined by the angle of the illuminating cone.

It will be seen from fig. 2, A that it is possible to utilize to advantage the maximum aperture of the bright-field condenser at shorter wavelengths (350 to 420 mμ). When the aperture of the objective is reduced in relation to the illuminating aperture, i.e. medium and low powers, the system becomes more efficient and is equivalent to using a combination of condensers, one giving a bright-field of NA equal to that of the objective and the other a dark-field system with a high aperture. The efficiency of this system depends on the low visible intensity of the light transmitted into the objective. A dry 4-mm objective of NA 0·95 will thus receive a 143·6° ($u = 71{\cdot}8°$) cone of ultra-violet radiations, as well as fluorescent light from the object, when illuminated with a condenser of NA 1·30 oiled to the specimen slide. If now an 8-mm objective of NA 0·45 is used with this condenser under the same conditions, the angular cone of rays received will be 53·4° ($u = 26{\cdot}7°$). Although the energy concentrated in the object plane will be the same as for the 4-mm objective, the proportion of activating light entering the 8-mm objective will be considerably less than half. Secondary filtration can therefore be proportionately reduced and an image intensity, relative to the illuminating aperture used with the 4-mm objective, maintained against a darker background. The ratio of light concentrated on the specimen to light received by the objective reaches unity when oil-immersion objectives of the same numerical aperture as the condenser are used. Filtration will then be at a maximum to absorb the high proportion of exciting radiations superimposed on the fluorescent image. Approximately 10 times as much light is collected by an objective of NA 1·30 as by one of NA 0·45 (fig. 2, B), and the degree of filtration necessary at any wavelength for these high-aperture objectives reduces the final image intensity to below that obtained with the dark-field condenser system. When the bright-field condenser is used for excitation of fluorescence with visible light it becomes even less efficient. Advantages of the objective/condenser aperture ratios, gained with ultra-violet illumination, are lost because of the high intensity of the background and the inability of the secondary filter to absorb these wavelengths completely without modifying the colour of the fluorescent image. In this instance the system is unsatisfactory when lower magnifications

are used since the intensity of the illuminating beams in the primary image plane will vary as the reciprocal of the square of the magnification and stronger secondary filtration will be required to absorb residual blue light.

Another complication, arising from the large illuminating apertures necessary to maintain a high fluorescence intensity, is an appreciable amount of 'veiling glare'. In the fluorescence microscope, unlike other microscope systems

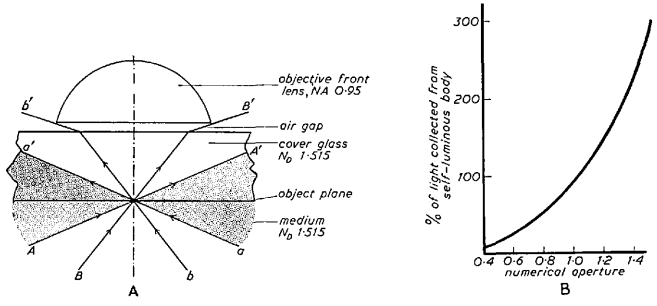

FIG. 2. A, section through the object plane, cover-glass, and objective front lens for a dry system. Illuminating cone for bright field condenser, *A*, *a*. Fluorescent light and direct radiations entering objective, B^1, b^1. B, relationship of the light concentrated on an object and the energy emitted by a self-luminous body to the light-gathering power of the objective. Calculation based on objective of NA 1·0 receiving 100% energy.

with the exception of the ultra-violet absorption microscope, glare can originate from the autofluorescence of glass lenses in the illuminating apparatus as well as from the specimen mount and lenses of the observing system. Apart from the presence of glare due to light reflections during visible light excitation, there is sometimes appreciable autofluorescence from glass slides, immersion oil, and lens components; this is most likely to occur when ultra-violet light is being used. Stray visible light from these various causes will mask the image of the specimen with a resulting loss in contrast and resolution. For these reasons, and also because of the low ultra-violet-transmitting properties of glass, it is advisable to employ a quartz condenser and collector lens when using wavelengths below 360 mμ to excite fluorescence. Tests have been carried out in this laboratory with glass and quartz lenses in the illuminating system. Results demonstrating two forms of veiling glare, often present in the fluorescence microscope, are shown in fig. 5. Total glare in a system originating from numerous causes may present a serious problem when the bright-field condenser is used and must be taken into account when assessing the performance of the microscope. Autofluorescence of the objective and eye-piece lenses can be eliminated by mounting a special ultra-violet absorbing filter on the front lens of the objective. Many of the yellow or minus blue filters that are available transmit a high proportion of the ultra-violet at 360 mμ and are quite unsuitable for this purpose or for the protection of the eyes. The

spectral qualities of these filters must be carefully checked before they are put into general use.

In spite of the various disadvantages associated with the bright-field system, experience has shown that in one respect it may have definite advantages over the alternative dark-field method. This applies to low-power microscopy (up to ×75), provided that excitation is by ultra-violet and not visible light wavelengths. With a well-corrected long focus bright-field condenser, having as high a numerical aperture as possible, extremely good results are attainable at these magnifications, whereas under the same circumstances the efficiency of the dark-field condenser system falls off markedly with the area of specimen requiring even illumination. There are instances when it may be necessary to examine or record large sections of material, e.g. in the study of naturally-occurring prophyrins in sections of bone and teeth, when only low magnifications (×20 to×30 diameters) are required. In these circumstances the bright-field condenser gives superior results at 360 mμ and is useful for rapid screening or counting of specimens in which fluorescence is activated by ultra-violet light.

Dark-field condenser systems. Since visibility is dependent on contrast it is desirable in fluorescence microscopy, when image brightness is often low, to aim for maximum image contrast. Dark-field illumination has proved superior in this respect to other methods precisely because it ensures a black background to the image, irrespective of whether ultra-violet or visible light is used for excitation. This is also true when incident annular oblique illumination is used for the study of the fluorescent surface structures of opaque specimens. The complications associated with the bright-field system are largely avoided, since all direct radiations from the light source pass from the top surface of the condenser outside the aperture of the objective lens and so cannot interfere with the formation of the fluorescent image. This also permits wide spectral bands for excitations, thus ensuring a high intensity level together with enhanced contrast.

The underlying principles are illustrated by a simple diagram (fig. 2, A). Here the larger angle *A*, *a* represents the entrance aperture to a bright-field condenser. By introducing a circular opaque stop centrally in this aperture, a hollow cone of light *A*, *a*, *B*, *b* will come to a focus in the object plane. Provided that the obstructed aperture of the condenser is larger than the objective aperture in use, direct light will not enter this lens and the object will appear bright on a black background. It is advisable to use the specially designed high-power dark-field condensers of the cardioid or bispherical forms for this purpose; these must always be immersed in oil to the undersurface of the slide. They can be used with all visible wavelengths, and down to 360 mμ in the ultra-violet, without loss of efficiency. They illuminate a field of sufficient size for use with both oil-immersion and dry objectives having focal lengths up to 8 mm, but the standard form of dark-field condenser is unsuitable for lower magnification than this. However, objectives of 16 mm (×10) focal length and ×10 eyepiece may be effectively used in the following way.

A special condenser for the purpose can be obtained by removing the front and intermediary lens components from an achromatic bright-field condenser and fitting a stop in the anterior focal plane, just large enough (i.e. one-tenth larger than the objective aperture) to prevent any direct light from entering the objective. Such a system gives results comparable in intensity and colour to those obtained with a 4-mm objective and cardioid condenser.

The dark-field condenser may be regarded, in this context, both as an illuminator and as an efficient primary filter, enabling the use of intense exciting radiations. Its efficiency depends largely on the relative refractive indices No/N_m of the structures in the specimen, where No is the index of the object and N_m that of the surrounding medium. When $No = N_m$ for a particular wavelength, no light of that wavelength will be scattered by the non-fluorescing parts of the object, and the image plane will be uniformly black. In fig. 3, A the object is represented by a black ink-mark. If the wavelength used can activate fluorescence in the specimen, only the light emitted by the specimen will enter the objective and reach the image plane. Clearly, an ideal specimen would be one in which the light dispersions of the object and its surrounding medium are equal, at least over the visible and long ultra-violet regions of the spectrum (fig. 4, B). A fluorescent object of this nature could then be activated by light from a selected source, no primary or secondary filters of any kind being needed. This was achieved by Barnard and Welch (1936) when they used monochromatic light at 275 mμ and a quartz dark-field condenser to record the autofluorescence of bacterial cell components.

A near approach to this ideal situation has been demonstrated in this laboratory, with fluorochromed specimens of rat spermatozoa (fig. 4, A, B). Smears were made on thin glass slides and fixed with formaldehyde-saline.

FIG. 3 (plate). A, dried black ink-spot in air without cover-glass. Excitation with ultra-violet at 365 mμ. Wratten 18A primary filter. Wratten 2B secondary filter. Condenser, cardioid. Exposure 1 min.

B, same preparation and conditions as A, but with bright-field illumination. Achromatic condenser NA 1·3. Exposure 1 min. Beck 4 mm. NA 0·95 apochromatic objective.

C, D, E, and F, section of cat-stomach fixed and stained with acridine orange No. 788, 1 in 2,000, and mounted in buffer (pH 2·7). Polychromatic fluorescence recorded in black and white with cardioid and bright-field condensers. Objective, Beck 4 mm. NA 0·95 apochromatic.

C, condenser, cardioid. Filters, primary, copper sulphate; and ammonia liquid; secondary, Wratten No. 4. Exposure 30 sec. Remarks: colour contrasts good between nuclei and cytoplasm. Contrasts good between stained and unstained tissues. Black background.

D, condenser, achromatic NA 1·30 oiled to slide. Filter and exposure as for C. Remarks: bright blue background with a high percentage of blue light masking image. General loss of colour and contrasts. Requires much stronger primary and secondary filtration.

E, condenser, cardioid. Filters, primary, Chance OX7; secondary, Wratten No. 2B. Exposure 1½ min. Remarks: Good contrasts maintained as in C. Fluorescence lower especially in nuclei owing to lower absorption of energy at these wavelengths.

F, condenser, achromatic NA 1·30 oiled to slide. Filters, primary, Wratten 18A; secondary, Wratten No. 4. Exposure 6 min. Remarks: lower non-visible wavelengths exciting fluorescence help to improve contrasts. Secondary filter absorbs more efficiently direct light reaching objective. Fluorescence intensity of nuclei very similar to E at these wavelengths. Contrast not as good as in E with some loss of colour purity and resolvable detail. Exposure 4 times as long as E.

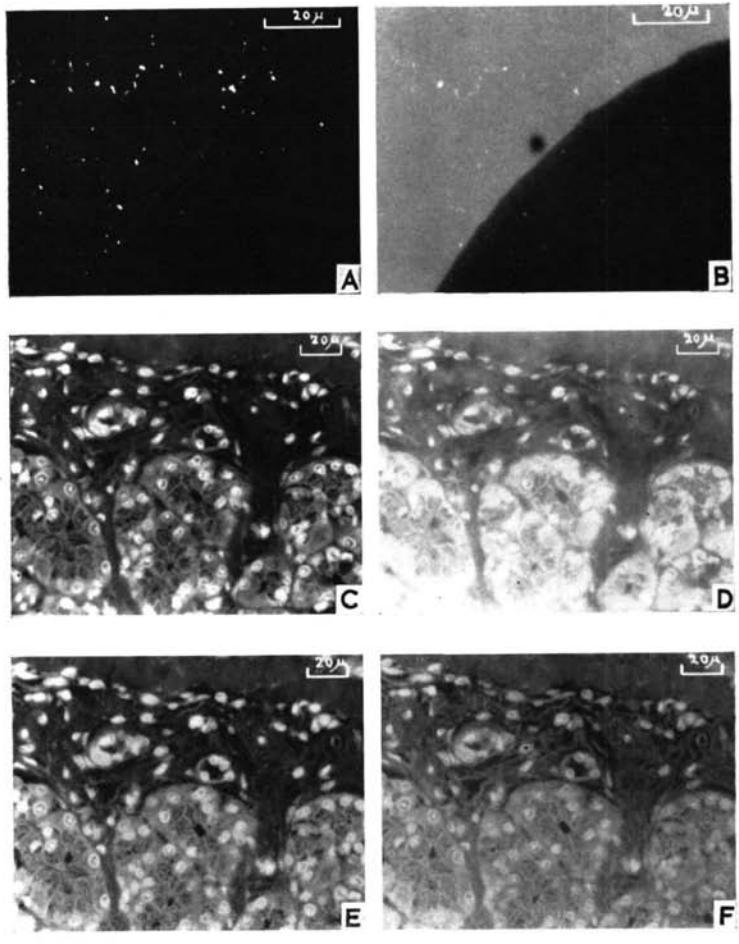

FIG. 3

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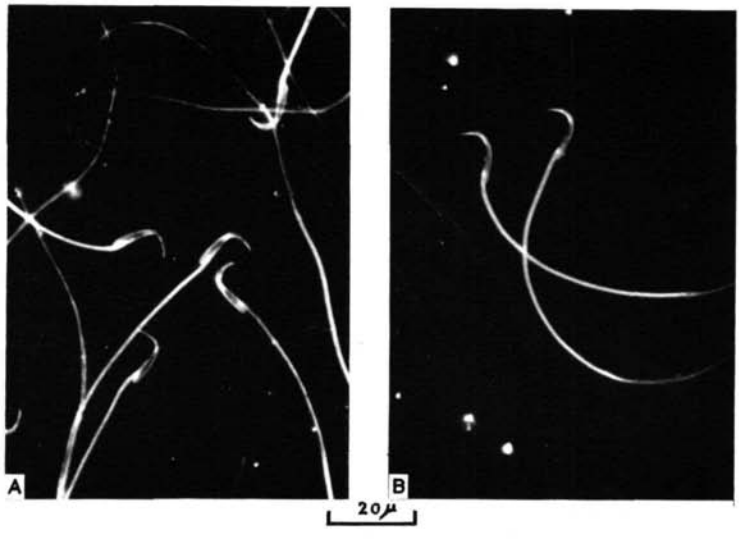

FIG. 4

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After staining for 15 min in 1% rhodamine B (colour index 749), they were washed well in water, dried, and mounted in DPX ($N_D = 1{\cdot}524$) under a glass coverslip. The fluorescence microscope was fitted with a cardioid condenser and a dry objective (Beck 4-mm apochromatic, NA 0·95). The light source was a 250-watt mercury pressure lamp, and no primary or secondary filters were employed. Rhodamine B absorbs light energy strongly in the visible region, with a maximum at 550 mμ. Excellent fluorescent images were seen on a dark background. Where the cells had been stained with the dye the image had good contrast; but the unstained portions were perceptible as a dark-field image due to reflection into the objective of a small part of the incident light. It is inferred that the refractive indices of the medium and of the unstained parts of the cells were close but not identical under the conditions of the experiment. In general, as the difference between the object and medium increases more light is scattered, and the more obtrusive is the dark-field image which is superimposed on that due to fluorescence. This is the reverse of the mounting procedure for dark-field microscopy when resolution is dependent on refractive index differences between specimen and media.

Occasions when all filters can be dispensed with will rarely occur in work on biological specimens; for in fresh and in fixed materials the various cellular components usually differ sufficiently in refractive index to be depicted as a dark-field image. In practice, when ultra-violet wavelengths are required for excitation of fluorescence much of the light impinging on refractile non-fluorescent parts of the specimen will be scattered, and some of this will enter the objective lens. However, most of it is absorbed by the objective and eyepiece lenses and it does not harm the eyes nor interfere with the fluorescent image except in photographic records. Consequently no secondary filter is needed for direct observational work, while for photography it is enough to insert in the eyepiece an almost colourless ultra-violet absorbing filter such as the Wratten 2B. When excitation is with blue-violet or other visible wavelengths it becomes necessary of course to employ slightly stronger secondary filtration to absorb the visible dark-field image. Such filters range in density from the pale yellow Zeiss 'euphos' cover-glass type to the deeper coloured minus-blue filters familiar to photographers. Some selected primary and secondary filters, suitable for use with the mercury pressure lamp + dark-field

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FIG. 4 (plate). A, dark-field photomicrograph of a smear preparation of rat-sperm fixed and stained with 1% rhodamine B, photographed dry in air without a cover-glass.

B, fluorescence photomicrograph of fixed rat-sperm smear stained with 1% rhodamine B solution and mounted in DPX, N_D 1·524. Light source of A and B, 250-watt mercury pressure lamp. No primary or secondary filters used for either record. Condenser, Zeiss cardioid. Objective, Beck 4-mm NA 0·95 apochromatic with correction collar.

C, sample of living and dead spermatozoa stained with equal parts primulin at pH 7·2–8·2 (dye concentration approx. 1 : 30,000) and rhodamine 6G B.D.H. (dye concentration 1 : 30,000). The living spermatozoa fluoresce bright yellow and the dead ones light blue. Living spermatozoa will not absorb the primulin (after Bishop and Smiles, 1957). Primary filter, Chance OX7. Secondary filter, Wratten 2B. Excitation with ultra-violet light at 365 mμ. Condenser, Zeiss cardioid. Objective, Beck 4 mm NA 0·95.

condenser system, are given in table 2. Comparison of these filters with those given in table 1, in connexion with the bright-field condenser system, serves to illustrate the very different role of the secondary filters in the two systems of fluorescence microscopy. In the dark-field system it is less critical, and required only to absorb such light as may be scattered and reflected from the specimen into the observing system. It seems reasonable to suppose that if more tests were made of non-fluorescing mounting media with refractive indices similar to that of the specimen, the need for secondary filtration might be reduced even further in dark-field fluorescence microscopy.

Some additional factors of practical importance concerning primary filtration have been noted during routine use of the dark-field system in this laboratory. It is frequently necessary, when employing the fluorescein-labelled antibody technique (Coons and Kaplan, 1950), to differentiate conclusively between the specific apple-green fluorescence of cell structures binding the conjugate, and the non-specific autofluorescence and blue dark-ground image of non-fluorescing elements which are usually also present. For this technique excitation with the carbon arc or with mercury vapour lamp has proved satisfactory but the former emits a particularly intense spectrum over the entire blue-violet region. Some control on the intensity of the dark-field image can be a valuable aid for discerning the finer details of cell structure and the general anatomical relationships; on the other hand, as a check for the detection of very small amounts of the specific fluorescent dye it may be desirable to suppress the dark-field image below the limit of perception altogether. By increasing the transmission of the primary filter the more refractile elements can be revealed in greater detail, but with some loss of fluorescence visibility where take-up of the dye has been minimal. Conversely, curtailing transmission will diminish the brightness of non-fluorescent refractile elements and allow the weaker fluorescent details to be seen more clearly. For this purpose, in addition to a wide range of interchangeable glass filters, a variable thickness cuvette has been utilized to hold the coloured chemical solutions which can be used as an alternative form of primary filter with a useful range of transmission values.

Attempts have been made to improve the quality and intensity of the activating light, and trials carried out in this laboratory, with 'interference' primary filters, show that they have distinct possibilities, especially when it is desirable to select particular bands of the spectrum to obtain the specific maximum fluorescence of cell components. There are instances, however, with fluorochrome dyes, when high activating intensities are definitely harmful to the staining process and quality of the image. A 'saturation level' is reached and a severe lack of contrasts might result from this phenomenon. In these circumstances activating light of a lower intensity will excite the same level of fluorescence intensity in the specimen. Provided that the primary transmission is adjusted to this level, the degree of scattered light will be appreciably lower and a secondary filter with lower absorption can then be used.

Far more common effects due to intense excitation are 'photo-chemical'

TABLE 2

Dark-field condenser

Primary filters			*Secondary filters*	
Stain technique	*Type*	*Maximum transmission*	*Type*	*Maximum absorption*
Primulin dye for distinguishing between living and dead cells. Also suitable for autofluorescence	*Chance OX7 (glass)	240 to 400 mμ (87% transmission—ultra-violet excitation)	Wratten gelatin No. 2B	at wavelengths shorter than 420 mμ
Fluorescein-labelled antibody (Coons technique)	*Wratten No. 50 (glass-mounted gelatin)	436 mμ (10% transmission—blue light excitation)	Wratten gelatin † No. 8 or Ilford † No. 108	at wavelengths shorter than 460 mμ
and	or			
Acridine orange nucleic acid staining method (Armstrong)	Copper sulphate ammonia solution— $CuSO_4.5H_2O$ 25 g NH_4OH(d = 0·88) 300 ml dist. water 675 ml	400 to 500 mμ (60% transmission at 4-cm cell width—blue light excitation)	Wratten gelatin † No. 8 or Ilford † No. 110	at wavelengths shorter than 480 mμ

* A cell, 2 cm thick, containing a 10% copper sulphate, is always used in conjunction with these filters.

Note. † Autofluorescence of these filters is extremely low and does not interfere with quality of image. A 2B filter may be placed in front of the secondary filter to absorb stray ultra-violet light as a precaution.

changes and 'quenching'. These changes take place in both living and fixed tissues but are more rapid in the former, and may have to be controlled by reducing the intensity of the primary excitation. Overall intensity may be reduced by placing a neutral density wedge in the beam and moving it across the light path to a point where the dye will tolerate excitation without changing colour; alternatively the wavelengths of the exciting radiations may be adjusted to correspond with those absorbed by the lower slopes of the absorption curve of the dye. Primary and secondary filters would have to be selected for this purpose and observations made on lower intensity images. Radiation damage to living specimens is only noticeable after prolonged exposure to long-wave ultra-violet and blue light.

Measurements of the relative efficiency of bright- and dark-field condensers

Condensers used in the early days of fluorescence microscopy were of quartz, so that maximum transmission of the ultra-violet radiations was obtained. The field collector lens, liquid filter cells, and specimen slides were also of quartz for this reason. With improvements in the design of light sources the use of quartz lenses, slides, and cover-glasses now proves to be unnecessary with wavelengths normally employed to excite fluorescence. Absorption of ultra-violet at 360 mμ and at longer wavelengths by glass components is very small compared with the total light losses in the whole system.

In support of bright-field condensers Richards (1955) points out quite rightly that less light is concentrated on the specimen with the dark-field condenser. This is true when the condensers are used in the normal way for bright- and dark-field illumination. The difference, however, in the concentrating power is not as great as would appear. Ellinger (1940) considered the dark-field condenser less efficient because of the much smaller entrance pupil to the system. He failed to note that since the focal length of the bright-field condenser is greater than that of the dark-field, the light is focused over a greater area at a lower intensity.

Light intensity measurements were therefore made of the amount of energy concentrated on the object by each system. Köhler illumination was used and the field-iris reduced so that the area covered by a bright-field achromatic condenser, NA 1·30, was the same as that covered by a cardioid condenser. With a stabilized current for the light source and all other conditions being the same, it was found that the intensity for the bright-field condenser was 3 times as great under these conditions as for the dark-field system. A photo-electric cell (Mullard 90 A.V. vacuum cell) was used to obtain these results. The absolute fluorescent intensity of the image, with ideal filter systems for both types of condenser, cannot be determined accurately owing to the number of complex factors involved. By using a high sensitivity photo-multiplier it was possible to obtain comparative measurements for the two systems employing filter combinations recommended in tables 1 and 2.

A monolayer of tissue culture cells, stained with acridine orange, was chosen as a suitable specimen for the purpose of obtaining these readings. Since the

dye complexes formed in the cells absorb strongly in the ultra-violet and blue-violet regions (see fig. 1), measurements could be made with the same specimen under conditions suitable to each of the condenser systems for these wavelengths. The specimen exhibited typically polychromatic fluorescence. The results obtained are shown in table 3. From these readings it will be seen that the dark-field condenser shows an approximate increase in efficiency of 40% for the two bands of light used. Heavy filtration used for the bright-field condenser to maintain a black background has greatly reduced its efficiency.

TABLE 3

	Bright-field condenser		*Dark-field condenser*	
Excitation wavelength	*Filters*	*Meter reading*	*Filters*	*Meter reading*
Ultra-violet 360 mμ	No. 18A plus No. 4	8	OX7 plus No. 3	11
Blue violet 400–500 mμ	No. 50 plus Nos. 8 and 9	12	Copper sulphate and ammonia plus No. 8	17

Further, it was found to be even less efficient when accurate colour records are required, and to approach results obtained when using the dark-field condenser with blue light excitation of an acridine orange stained specimen, ultra-violet light had to be used (fig. 3). These results are confirmed by exposure times for several kinds of biological specimens stained with fluorochromes having different absorption characteristics. Light intensity measurements and the relative proportional values of energy collected from a self-luminous body are plotted against objective apertures and shown in fig. 2, B. The importance of separating the exciting radiations from the fluorescent image-forming wavelengths at higher numerical apertures is demonstrated by this curve.

Photomicrographic records were also made to illustrate the background intensity differences with ultra-violet and visible light radiations (fig. 3). Photomicrograph A shows the outline of an opaque ink-spot in air extending across half the field. This was illuminated with intense ultra-violet radiations from a dark-field condenser. Photomicrograph B is of the same opaque spot illuminated with a bright-field condenser. Other conditions were the same for both condensers. The objective used was a 4-mm NA 0·95 apochromatic with correction collar. Secondary filtration was not used for either system and the test spot was photographed dry without a cover-glass. As there are no refractile elements present in the test object the dark-field record should only show a black picture without any outline of the spot. There is some evidence of scattered light reaching the objective from the edge of the ink-spot and from dust particles present in the field. Record B clearly shows the high intensity of the unabsorbed light reaching the objective. The remaining records, C, D, E, and F, illustrate image quality and contrast obtained with the two

systems of illumination at different wavelengths. Identical secondary filtration was used for C, D, and F. A colourless secondary filter, Wratten 2B, was used for recording E.

There is no general agreement about which type of condenser is most efficient but it is evident from these results that several real advantages are gained with dark-field illumination in fluorescence microscopy. The most important of these is the maintenance of a black background to the specimen when an intense band of exciting radiations is employed. The role of the secondary filter is simplified and quite different from that used with bright-field illumination, serving only to absorb residual scattered activating light which is reflected into the objective by the specimen. Usually this is of a low intensity and requires only weak secondary filtration. Owing to the inefficiency or lack of suitable light sources, the bright-field system requires exacting filter combinations with consequent losses of activating energy and deterioration of image contrasts.

In studies of vitally fluorochromed cells, photomicrographic records in colour of the fluorescent image are of the utmost importance (Bishop and Austin, 1957; Bishop and Smiles, 1957) (fig. 4, c); to avoid any changes taking place in the cell owing to ultra-violet or blue light radiations the exposures to light must be kept to a minimum. For this work the dark-field condenser has proved superior to the bright-field system and recently it has been found possible to detect and record in colour at 'high magnifications' various species of acid-fast bacilli in tissue sections, stained with auramine and rhodamine (Kuper and May, 1960). The highest possible intensity of activating light is necessary for these critical observations and it is therefore necessary to use a very wide band of activating blue-violet light to excite maximum fluorescence of these dyes with absorption peaks between 400 mμ and 556 mμ. At the highest magnifications with binocular vision ($\times$ 100 objective $\times$ 10 eyepiece) the bacilli can be clearly observed fluorescing a bright golden yellow against a blue dark-ground image of unstained tissue.

In assessing the relative merits of the two systems of illumination, no account has been taken of the relation of visual acuity to the brightness levels compared. Observations should be made under as near normal conditions of lighting as can be comfortably tolerated. The brightness levels obtained with the dark-field system enable one to use the microscope for long periods without undue eye-strain. Differences of visual acuity from one person to another appear to be mostly associated with colour interpretation rather than brightness level.

Use of polarized light. The practical application of polarized light to the fluorescence microscope to replace the usual filters has been investigated. The specimen is illuminated with plane-polarized light of the required wavelengths, and an analyser, with its plane of vibration at right angles to the polarized beam, is placed between the objective and eyepiece. By this means polarized activating light will be prevented from reaching the eye and only fluorescent light from the specimen will be observed. Because of strong

depolarization of light by the dark-field condenser this method of illumination has not been found suitable, but with the bright-field condenser polarized light has certain advantages over ordinary light, especially in combination with suitable filters permitting wide bands of the spectrum to be used to excite fluorescence. There is no restriction to the numerical aperture that can be used and glare is appreciably reduced. The most useful application of this method is to the study of polarized fluorescent light in relation to structure orientation. Nicol prisms must be used with the long ultra-violet, and objectives of up to ×20 (8 mm) with a ×6 eyepiece give good results. Image intensity at higher magnifications than this is poor owing to light losses in the system. Prisms with blue light are particularly useful at high magnifications but generally for medium and low powers polaroid screens give better results.

Light Sources

The essential requirement for an appropriate light source is strong emission in the specific wavelengths required to activate fluorescence of the stained elements of the specimen which are to be studied. It is therefore necessary to consider the emission spectrum of the source in relation to the absorption spectrum of the dye to be used. It is useful to have records available of the absorption spectra, from the short wave ultra-violet into the visual spectrum, of the dyes being used for fluorescence microscopy (see fig. 2). Since the absorbing wavelengths of the dye may be spread over a region of the spectrum, as is the case with fluorescein which absorbs from 460 mμ to 510 mμ, it will then be necessary to consider the energy output of the source in this 'region' in relation to the remaining wavelengths emitted and the primary filtration to be used. It must be remembered that the absorption maxima may be shifted by formation of strong organic dye-complexes within the tissues.

The emission spectra and intrinsic brilliance of suitable lamps can be ascertained from the manufacturers. At present there is no one source which will satisfy all possible requirements of the microscopist.

Four types of light source are available for use with the fluorescence microscope, namely:

(1) High-tension spark-discharge between metal electrodes.
(2) Low- and high-intensity carbon arcs.
(3) Mercury discharge lamps.
(4) Tungsten filament lamps.

High-tension spark. In certain instances the high-tension spark will be necessary if an intense source of energy is required in the ultra-violet region between 200 and 300 mμ (Barnard and Welch, 1936). When it is important that monochromatic radiation should be used to activate fluorescence it will be necessary to employ a monochromator consisting of two quartz prisms. This system will probably be of value in future investigations into microspectroscopic measurements of fluorescent compounds and complexes formed with tissues (Acheson and Orzel, 1956).

Carbon arcs. The carbon arc is well known as a useful source of ultra-violet and blue light radiations. In the low-intensity arc energy is emitted over a wide range of the spectrum from 300 mμ into the infra-red; it has been widely favoured by many workers using the fluorescein-labelled antibody techniques and since it has a high energy output over the whole of the absorbing region of the fluorescein dye, especially in the cyanogen bands at 415, 385, and 375·5 mμ, this source has proved very useful. Unfortunately, considerable energy is emitted at all other wavelengths, particularly in the red, which have to be removed by primary filtration, which greatly reduces the intensity of the exciting radiations. The efficiency of this type of source can be much improved by shielding the electrodes from the collecting lenses. The tips of the electrodes are responsible for the longer wavelength radiations which are not required, and by selectively focusing on to the specimen only the rays emitted by the 'arc', primary filtration is simplified. In high-intensity arcs the positive electrode consists of a carbon rod with a core of rare earth compounds and burns with a current density at the positive pole (D.C. current) about $3\frac{1}{2}$ times that of low-density arcs. All arc lamps correctly adjusted will provide a steady source of energy and should be operated by clockwork with suitable automatic compensation for variations in the rate of burning. A real advance in the application of the carbon arc to fluorescence work could be achieved by designing a suitably compact form of 'enclosed' arc, sometimes called a flame arc.

Mercury discharge lamps. Mercury vapour discharge lamps have proved suitable for most purposes in fluorescence microscopy. In view of the high energy output which may be several times that of the carbon arc, and also their compactness and adaptability, these lamps are preferred in many laboratories. In the ultra-violet the emission spectra have well-separated maxima at 312 mμ, 334 mμ, 365–6 mμ; a series of strong lines from 377 mμ to 408 mμ in the deep violet regions, and an isolated band with very strong emission at 435–6 mμ in the blue-violet. If a well-corrected collector lens is used to focus light from the arc, excluding any image of the electrodes, the red light emission can be minimized. Residual red light and heat are completely absorbed by an acidified 10% solution of $CuSO_4$ incorporated in the primary filtration. This kind of lamp also emits strong lines in the yellow and green which are sufficiently absorbed by primary filters which transmit up to 500 mμ. Mercury discharge lamps can be operated under varying conditions and degrees of pressure. The spectral energy distribution is determined by the operating pressure which can be as low as 0·01 mm or as high as 20 to 30 atmospheres. The main value of the vapour lamp lies in the intensity of irradiation in the blue-violet and ultra-violet regions at suitably separated wavelengths. Lamps that operate at higher pressure emit the maximum energy at longer wavelengths with a stronger continuous background spectrum than those working at lower pressures; there is a relative fall in the intensity of the ultra-violet and heavier primary filters become necessary. Compact source lamps of 1000 watts or more fall into this class and are mainly useful as blue-violet light sources. In certain instances when fluorescence is weak and the excitation

wavelengths are between 400 and 500 mμ, these lamps may have advantages over the lower pressure types. The most useful mercury lamp is the quartz mercury discharge lamp operating at about 20 atmospheres and rated at 150 to 250 watts. Its output and intrinsic brilliance (25,000 candles/cm²) from an arc of 2 or 3 mm length, remains steady for about 400 h and then drops by 40–50%. These lamps have to be cooled after use before they will operate again and maximum brilliance is reached in 10 to 20 minutes. The mercury vapour lamp run at atmospheric pressure has the most favourable spectral energy distribution with practically no background and low emission at the longer wavelengths. Unfortunately, it has not the intrinsic brilliance required for fluorescence microscopy.

Tungsten filament lamps. These are the least efficient type of source to use for any purpose in fluorescence work. The spectrum of the radiation emitted is of the continuous type and lies mostly in the visual and infra-red regions. Although these lamps have been used as a source for exciting radiations in the blue region, a fair proportion of the energy responsible for fluorescence lies between 450 mμ and 520 mμ. Workers claiming success with this lamp have used fluorochromes with strong absorption in this region.

Observation System

Resolution. Provided that glare is reduced to a minimum and a completely black background maintained, it is reasonable to expect, since all observations are carried out with the objective aperture fully utilized, a higher standard of resolution than is obtained with the ordinary light microscope (fig. 6, A). Because the specimen is self-luminous each point on the object emits waves to fill the aperture of the lens and will be imaged by it to form similar points, the closeness of which will depend on the quality of the lens and its NA. This is in accordance with the classical interpretation of resolution as formulated by Abbe,

resolution $= 0{\cdot}61\lambda/\mathrm{NA}$, where λ is the wavelength of the light used to illuminate the specimen.

In fluorescence, λ represents the light emitted by the specimen, independent of illumination, and is usually in the visible region of the spectrum. All the light originates from the specimen without any external rays taking part in the formation of the image and each point of light emitted will be independent of light coming from any other point. The image-forming rays are not therefore capable of interference as they are when an object is externally illuminated. There is always a possibility, however, of light rays from the source reaching the objective and interfering with fluorescent image-forming rays when bright-field illumination is used. For these reasons when visible light is used it is important to have an accurate filter combination if the highest possible resolution is required. By closing the aperture of the bright-field condenser to improve the background and contrasts when high NA objectives are used,

further interference of the image-forming rays is caused and resolution is reduced.

Visibility. The dark-field system is of particular value when detecting light emitted from particles below the limit of resolution. It has been claimed by Levaditi and Panthier (1945) that there is no theoretical limit to the size of particles which can be detected. A notable advance employing new techniques has been made by Venetta (1959).

If it is desirable to obtain quantitative micro-intensity measurements when the fluorescence is below photo-emulsion sensitivity, a photo-image intensifying tube can be used to raise low energy levels to frequency contrast responses that can be recorded. An American instrument, named the 'astracon', has recently been developed and is capable of detecting single photons of light.

Objectives. For most purposes the usual achromatic objectives supplied with microscopes are suitable for fluorescence work. Some loss in image intensity is to be expected when oil-immersion apochromatic objectives are used, owing to the greater number of lens components present in these systems.There may also be a loss of image contrast with the older forms of these objectives because they contain fluorite lenses; this mineral has autofluorescent properties which will cause considerable glare in the image plane. The 4-mm × 40 apochromatic objective of NA 0·95 fitted with a correction collar permitting observations to be carried out on covered and uncovered specimens is, however, to be recommended, provided that it is reasonably new. It has the advantage over other objectives with a similar magnification of having a higher numerical aperture, yielding a much brighter image; it will be found most useful for general purposes as well as for photomicrography. Spherical and chromatic aberrations due to varying thicknesses of cover-glass and mountant above the specimen can be reduced to a minimum by proper adjustment of the correction collar. The presence of these aberrations can be easily detected, since isolated structures seen against a black background are surrounded by a diffuse halo. By rotating the objective collar a little at a time to reduce this effect and then refocusing the microscope, a position will be reached when the image is completely free from halo. Very high quality images are also obtained with the modern fluorite objectives. These give a greater depth of field at the equivalent numerical aperture to an apochromatic lens and with less glare because of the fewer lens components necessary for this objective. Images of good contrast with resolution almost equal to that of the apochromats are possible and they are quite suitable for colour photomicrography.

The water-immersion achromatic series of objectives are very useful, in particular the × 50, NA 1·0 with a working distance of 0·5 mm, when using dark-field illumination. Complete absence of any fluorescence of the distilled water used for immersing the lens is an additional advantage. Tests have been carried out with the flat-field objectives now available in the achromatic and apochromatic series. The dry objectives up to 4 mm, NA 0·65 give excellent images without any loss of intensity. Unfortunately the 'immersion' flat-field objectives are not suitable. There is an appreciable drop in image intensity

owing to the greater number of lens components necessary for this type of objective and no advantage will be gained by their use. Lenses coated against reflection give an image with slightly better contrast than ordinary lenses and this is particularly noticeable with immersion objectives.

Special objectives for fluorescence microscopy are available from certain manufacturers. These objectives have an ultra-violet-absorbing filter permanently mounted immediately in front of the lens system. This is the ideal position for such a filter but limits the range of secondary filtration. It is also important to make certain that these objectives can be used with covered specimens as well as uncovered temporary mounts. When it is necessary to use annular oblique illumination directed from above the specimen, e.g. opaque objects, to excite surface fluorescence, special objectives are necessary. Of these the ultropak and epi-illuminator series incorporating an incident dark-field system of illumination are to be recommended. Other reflecting systems have been found less efficient owing to the high proportion of exciting radiations absorbed by the optical components, causing a serious drop in image intensity. For intravital microscopy it is an advantage to use objectives fitted with special immersion caps and cones which enable one to maintain a focus below the surface of organs. Water or physiological saline are suitable immersion fluids to use for these observations. At medium and low powers with incident illumination there is an appreciable amount of light reflected back into the objective. Oblique lighting is therefore recommended but at higher magnifications light losses may be serious. Normal incident illumination will be necessary to maintain image brightness and good backgrounds are obtained in the ultra-violet. Blue light activation, because of strong reflections, requires an annular oblique system for all magnifications.

Image brightness obtainable from an objective of given NA at a certain fluorescence intensity level varies approximately as the reciprocal of the square of the magnification. It has been found in practice, therefore, better to use medium-power objectives with the highest permissible NA and an eyepiece magnification of $\times 8$ to $\times 12$, thus utilizing a maximum cone of fluorescent light to produce a high contrast image. This is possible with dark-field illumination up to apertures of approximately NA 1·0 with most biological specimens when mounted on slides 1 to 1·2 mm thick in a watery medium with a refractive index of 1·33 to 1·45. As the refractive index of the mounting medium is increased the illuminating cone will become larger and more energy is concentrated on the specimen. Objectives of not more than NA 1·0 are therefore able to gain the advantage of the larger illuminating cone without altering the secondary filtration, since none of these rays enter the objective. Provided that the difference between the refractive indices of the specimen and the mounting medium is not too great, scattered light from the specimen, when excited with blue light, will not interfere with the image. Immersion objectives with numerical apertures up to 1·4 can be used to examine preparations excited with ultra-violet radiations at 365 mμ and only require a colourless Wratten 2B filter for secondary filtration. Beyond 400 mμ stronger filters are

necessary at full apertures. A point is reached with excitation by visible light when the intensity of the direct unobstructed beam entering the objective interferes with the contrast of the image, and it is necessary to reduce the objective aperture to maintain a black background. With correct secondary filtration apertures up to 1·30 NA have been used without any reduction in the image contrast.

Experiments were carried out with suitably stained sections mounted in paraffin oil on cover-glasses (of thickness 0·3 to 0·4 mm) in place of slides, and a Beck dark-field focusing condenser was used to illuminate the sections with blue light. Reducing the slide thickness permitted a more oblique cone of illuminating rays, enabling an objective of NA 1·25 to be used. No direct light entered the objective under these conditions. Image intensity was high against a black background with a marked improvement in the purity of the fluorescent colours. Condensers permitting an NA of 1·3 to 1·4 are not generally suitable for biological work because specimens have to be mounted in a medium of a refractive index higher than the maximum NA given by the illuminating beam to achieve a dark field. For general purposes, however, the dark-field system limits immersion objectives to an NA of 1·1 when visual light is used to excite fluorescence. The question arises whether the dark-field method can be applied to numerical apertures up to 1·4 NA. The only solution would be one in which full cone illumination is used with an objective having a central opaque stop mounted in it to obstruct the direct exciting beam of light. Such a stop would have to be of a diameter to allow the maximum amount of fluorescent light to reach the image plane. Calculation shows that the diameter must be of such a value that $\sin^2 U^1/2 = \sin^2 U/2$ or $\sin U^1/2 = \frac{1}{\sqrt{2}} \sin U/2$, where U is the angular aperture of the objective and U^1 the aperture of the illuminating cone of rays.

Immersion objectives are best fitted with an iris diaphragm rather than a funnel stop, to allow for the rapid adjustment of the lens aperture to the existing conditions. Care must be taken with the use of immersion fluids. With blue light illumination the objective maker's oil must be used to immerse the lens to the cover-slip. Ultra-violet excitation will require the use of a non-fluorescing oil and medicinal paraffin oil is quite satisfactory in this respect. Immersion of high aperture dark-field condensers to the underside of the specimen slide is always necessary whatever aperture objective is used.

Eyepieces. Most modern eyepieces of Huygenian and negative compensating design are quite satisfactory. When using fluorite or apochromatic objectives it is advisable to employ the eyepieces corrected for colour magnification as supplied by the manufacturer. It is a great advantage if the eyepiece lenses are 'bloomed'. Compensating and Kellner eyepieces of older design are not suitable owing to autofluorescence of cemented lens components present which causes a loss of intensity and considerable 'glare'.

Veiling glare. The presence of glare and its effect on the performance of the fluorescence microscope demands particular attention. In an optical system when visible light is used it is confined to the following causes: (*a*) 'mechanical

glare' arising from light reflected from mechanical and non-optical surfaces; (*b*) 'optical glare' produced by light scattered and reflected at and between air/glass surfaces of lens components and slide/cover-slip surfaces; and (*c*) 'specimen glare' due to particulate nature and variations in refractive index in the object plane, causing light to be scattered, diffracted, and reflected. These all contribute to stray light being distributed over the image plane, causing losses in contrast and resolution. In addition, the fluorescence microscope has a more serious form of glare due to the autofluorescence of the lens cements, lenses, and immersion oil in the system. When a bright-field condenser is used, this is transmitted into the microscope from the illuminating system where fluorescing cements, glass surfaces, dust, and grease all contribute to a much higher proportion of total glare in the microscope. Since dark-field illumination limits the primary cause of glare to the preparation, only scattered light in the object plane will produce autofluorescence of the lenses in the microscope, and this was subsequently found to be negligible. Tests were carried out to ascertain the degree of glare present employing (*a*) ultra-violet light, and (*b*) blue light radiations with the dark-field condenser. With radiations at 365 mμ, glare in the microscope was extremely low. Its existence mainly originated from the lens components of the compensating eyepiece of old design used in the test. When a quartz eyepiece was used in its place the glare was eliminated. With blue light a greater degree of glare was detected; it was largely due to unabsorbed activating light. Fluorescence photomicrographs illustrating the effect of glare on finely resolvable detail are shown in fig. 5.

Alignment of the Microscope

Accurate centration of the illuminating apparatus is, of course, necessary for good results in bright- and dark-field microscopy; it is even more essential in fluorescence work owing to the low light intensity levels which often have to be used, and maximum excitation is required. The system is sensitive to the slightest decentration which will drastically alter the fluorescence intensities, and colour photographs may reveal a serious degradation of the true colours. A simple method of setting up the light source and optical components can be adopted and is reliable for any method of microscopy with either the 'critical' or Köhler system of illumination.

The microscope. An optical bench 1 metre long, adapted for fluorescence microscopy, is illustrated in fig. 6, B. This apparatus has proved to be satisfactory for most requirements in fluorescence work and is easily adapted to other methods of observation. The bench should stand on a strong, heavy table to minimize vibrations for photomicrography.

The light source A is the 250-watt high-pressure mercury vapour lamp normally used for this work.

The collector lens is a Nelson type 2 lens aplanatic of $2\frac{1}{2}$-in. focal length.

The heat-absorbing chamber of water necessary when carbon and similar types of open arc are employed. This cell, of glass or perspex, requires to be 3 in. thick to be efficient.

Two stands are required for the primary filters. The first holds glass cells of different thicknesses to contain liquid filters; the second supports a sliding metal frame taking two glass filters, 50 mm × 50 mm × 6 mm, permitting a quick change from one filter to another during examination. The microscope is mounted on a special base-plate screwed to a broad saddle.

Metal shields are provided to screen off any stray light from the lamp, and with a correctly balanced filtration system it is perfectly satisfactory to work in a darkened room with a table standard and 75-watt lamp at 4 ft behind the observer. By dispensing with the microscope mirror and observing with the body-tube in the horizontal position, an appreciable gain in intensity from the light source is achieved, especially with wavelengths from 300 to 400 mμ.

When bench space is limited and it is necessary to use the microscope for general purposes, an optical bench half a metre long will be more convenient. The microscope is then placed in the upright position at a suitable height on the laboratory bench, so that the axis from the light source to the centre of the mirror can be easily established. By substituting the primary fluorescence filters with neutral density screens or visible light filters the microscope can be converted to other methods of observation, e.g. bright-field, phase contrast, dark-ground, &c. To be able to do this quickly without any further adjustment to the illuminating system is of the greatest value in fluorescence studies as it enables the observer to identify localized fluorescent areas or inclusions in terms of the general morphology of the tissues. Directly comparable fluorescence and bright-field observations can then be made. A neutral density screen is necessary in addition to a green filter (Wratten No. 61) to observe the specimen by dark-ground. Fluorescence studies of motile organisms and observation of any changes that may take place owing to irradiation and reaction to staining are easily made with this system. Small changes in refractility are also easily seen (Bishop and Smiles, 1957).

When arc lamps are used, it is necessary to adopt the Köhler system of illumination because of the shape and instability of the light source. With

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FIG. 5 (plate). Examples of veiling glare.

A, the specimen was a monolayer of normal pig kidney-cells stained with acridine orange and photographed on Ilford Pan F 35-mm film. Condenser system, bright-field C. T. & S. achromatic condenser NA 1·30; maximum excitation wavelength, 360 mμ; Beck 4-mm apochromatic objective, dry.

B, same field as A, photographed under identical conditions with the exception of the condenser which was replaced with a quartz system. There is a perceptible loss of contrasts and background density in A caused by the autofluorescence of the glass components at this wavelength.

C, a specimen similar to A. Cells photographed on Ilford Pan F 35-mm film. Condenser, Zeiss dark-field cardioid. Objective, Leitz 2 mm. Immersion oil used, maker's oil supplied for use with this objective at ordinary wavelengths (N_D 1·520). Maximum excitation wavelength at 350 mμ.

D, taken under the same conditions as C with the exception of the immersion oil. Non-fluorescing medicinal paraffin oil was used. The obvious loss of resolution and contrasts in C when compared with D are due to the strong autofluorescent properties of the immersion oil used under these conditions.

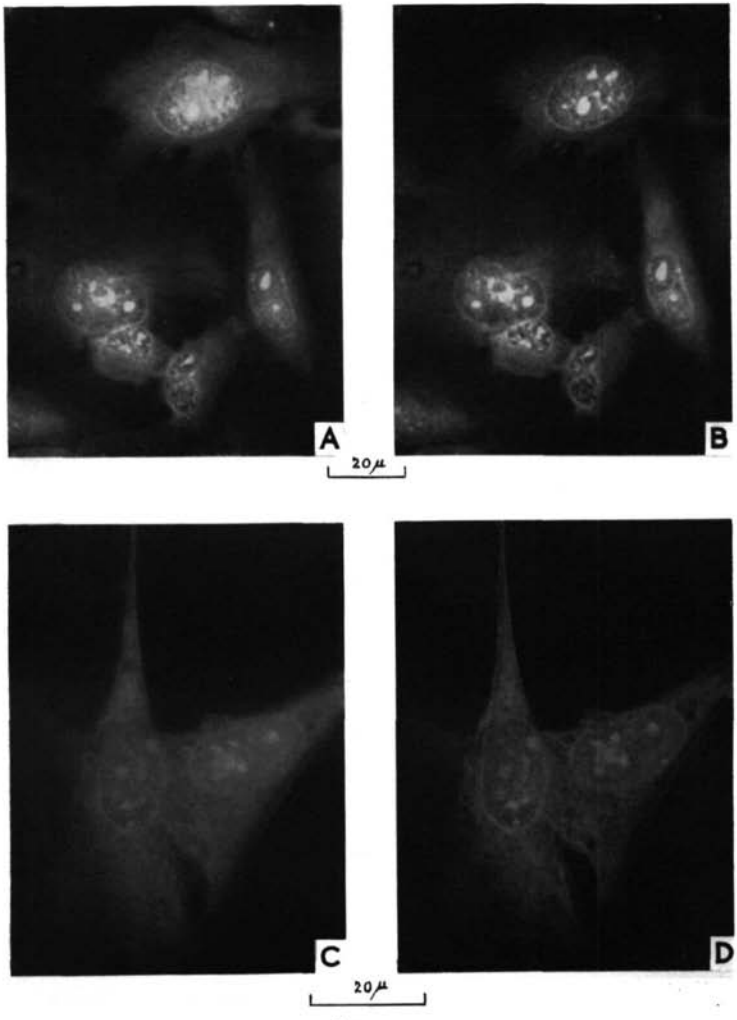

FIG. 5

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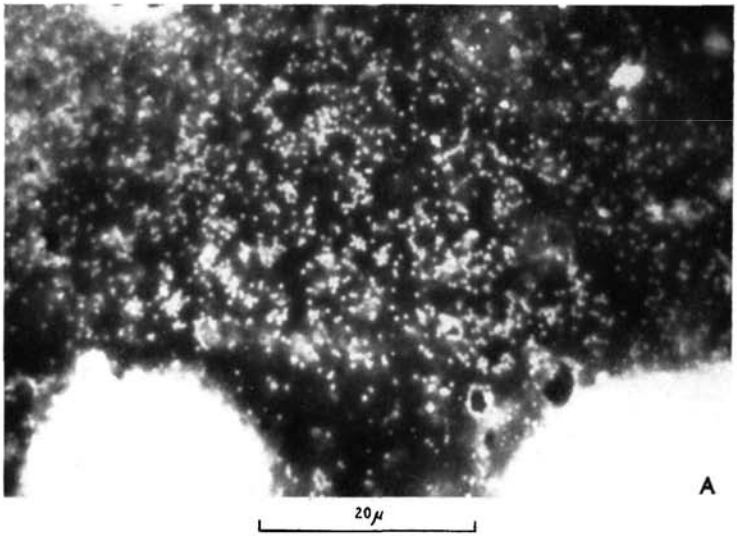

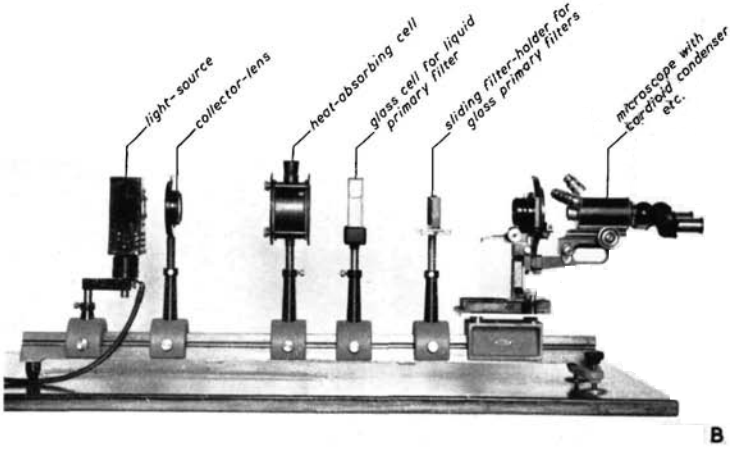

FIG. 6

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oil-immersion objectives of the highest power and eyepieces of medium power (×10 to ×12), it is possible to use 'critical' illumination with this type of source; but it is most important that the field of observation should be evenly illuminated. This is not always possible owing to the fact that the arc is focused in the plane of the object and vignetting at the edges of the field is more noticeable in photographs than by observation. Köhler illumination overcomes these difficulties and ensures an even field of illumination. This is achieved by focusing the condenser on a point slightly in front of the field collector lens and imaging this plane on the object when viewed through the microscope. An iris diaphragm is usually placed in front of the collector lens for this purpose and also for control of the area of the field of illumination.

Alignment with microscope in vertical position. For microscopes of modern design, with inclined binocular head, it is more convenient for the instrument to be set up in the vertical position and if possible mounted on a base-plate attached to the optical bench. Allowance must be made for the height of the instrument, when correctly aligned with the illumination, for comfortable observations to be made. When ultra-violet light is used and the fluorescence intensity is low it will be advantageous to have the surface of the microscope mirror silvered to give the maximum transmission at these wavelengths. It is essential that the light source, collector lens, and microscope lenses should be brought into coaxial relationship with the axis of the body-tube of the microscope. To do this it is necessary to ascertain the axis for these components in the following manner.

1. Remove condenser, objective, and eyepieces from the microscope. Adjust the position of the optical bench in front of the microscope with the single levelling screw, at one end, nearest to the mirror. The microscope should stand with the mirror approximately level with the light source when clamped in position ready for use.

2. Place two short stem saddles, one at each end of the bench (see fig. 7), and clamp firmly with retaining screws. Set the first alignment rod in the stem of the saddle as illustrated, with the point approximately the same height from the bench as the centre of the light source will be when mounted in position. Set the second alignment rod in the second saddle nearest the mirror at exactly the same height as the first rod. It may be necessary to incline the optical bench towards the mirror, roughly aligning the points of the two rods with the centre of the mirror.

3. Place a pinhole eyepiece in the body-tube and look down the microscope; tilt the plane surface of the mirror to direct an image, by reflection of the

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FIG. 6 (plate). A, smear preparation made on a glass slide from the freshly cut surface of a tumour-like lesion in the skin of a Rhesus monkey. Stained with acridine orange at pH 2·7. The lesion was produced by inoculation with a filterable agent allied to the pox viruses. Two cells in the lower part of the field are breaking down to release clusters of elementary bodies, which emit the greenish yellow fluorescence of DNA-containing structures. Diameter of the elementary bodies = 250 mμ approx. (measured with the electron microscope).

B, the fluorescence microscope. (The field iris is not shown.)

alignment rods, up the body-tube. Adjust by raising or lowering the first rod with the levelling screws of the bench until the tip is exactly behind and level with the tip of the second and viewed centrally in the nosepiece aperture. The axis $A\ A^1$ (fig. 7) is now established and will be coaxial with the axis of the body-tube. The position of the mirror is now fixed and must not be moved.

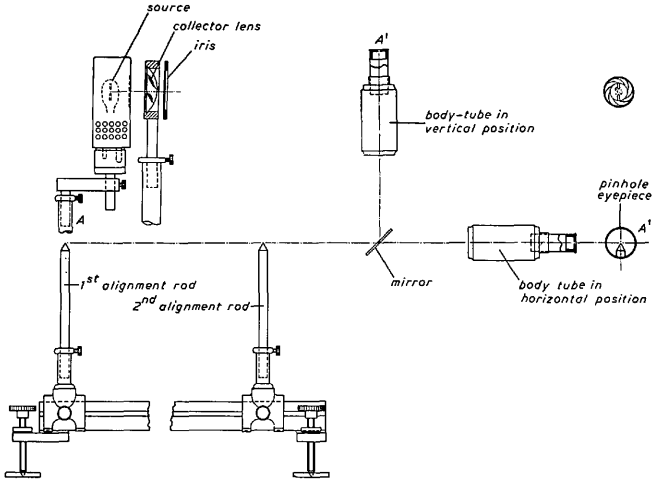

FIG. 7. Diagram of optical bench with alignment rods in position for setting up the microscope in the horizontal and vertical positions. A, A^1, optical axis.

4. Remove the first alignment rod. Place the light source and filter stands on the bench as shown in fig. 6, B. Switch on the source and with a suitable neutral screen in the second filter stand, observe the point of the second alignment rod through the pinhole eyepiece. Adjust the height of the source with the two levelling screws of the bench so that it is centrally aligned with the point of the second rod. Remove this rod and place the collector lens in position in front of the lamp; focus a parallel beam on to the mirror. Observe the image of the source through the pinhole and bring it central in the nosepiece aperture by centring the collector lens with centring screws.

5. Mount the condenser, a low-power objective (16 mm), and eyepiece on the microscope. With a suitable specimen (a section of tissue will do) on the stage, immerse the top lens of the condenser to the lower side of the slide with non-fluorescent immersion oil. Adjust the collector lens so that the beam just fills the entrance pupil of the condenser and then close the field iris diaphragm. Focus a sharp image of the diaphragm in the plane of the object by carefully

raising the condenser and finally centre the diaphragm in the field with the condenser centring screws. Open the field diaphragm fully and with suitable primary and secondary filters in position the microscope is ready for fluorescence observations.

It will be necessary to readjust the focus of the condenser for any variations in slide thickness when changing specimens. Centration of the condenser may be required when changing from one objective to another and should always be checked before making any photomicrographs. To obtain a maximum area of illumination the source and collector lens should be moved closer to the mirror until no further increase in size of the field diaphragm is observed when closed. It should not be necessary to recentre the collector lens if the alignment is carried out accurately. The specimen should always be shielded from the light source when observations are not in progress and searching time kept to a minimum because of the rapid fading and quenching of many fluorochromes when excited with ultra-violet and blue light.

Alignment with microscope in horizontal position. In this arrangement, which is to be recommended for research purposes, the instrumental adjustments are simplified by omission of the mirror, and the microscope become an integral part of the optical bench. The height of the microscope body-tube from the bench will be determined by the type of light source to be used and it is advisable to have an adjustable base-plate made to fit into a strong saddle for the microscope to stand on (fig. 6, B). Since the body-tube axis in the horizontal position may not be exactly parallel with the optical bench, it is important to establish a common axis from the centre of the source to the eyepiece of the microscope. It will only be necessary to re-establish this axis when the light source is changed. The procedure is as follows.

1. Ensure that the microscope is clamped firmly in position on the bench with the body-tube aligned with the optical bench when fully in the horizontal position. Remove the condenser, objective, and eyepiece.

2. Clamp a saddle and the first alignment rod to the opposite end of the bench. Place a pinhole eyepiece in the microscope and adjust the height of the tip of the rod, when viewed through the eyepiece, so that it is in the centre of the body-tube nosepiece aperture. Clamp a second saddle and second rod midway between the first rod and the microscope. By viewing the tip of the first rod, place the tip of the second rod so that it is exactly central in the nosepiece aperture and aligned with the tip of the first. The axis $A\,A^1$ is now established. A sheet of white paper held behind the rods will aid in sighting the tips.

3. Remove the first rod and mount the light source at approximately the same height from the bench in its place. Observe the second rod and the source through the pinhole eyepiece; adjust the height and lateral position of the centre of the source so as to be directly behind the tip of the second rod.

4. Remove the second rod and place the collector lens and filter stands in position on the bench. Focus the collector lens so that the nosepiece is filled

with light. Observe the image of the source (with a dense neutral screen in the filter stand) through the pinhole and bring it central in the nosepiece aperture with the centring adjustment of the collector lens.

Finally carry out the adjustments described in section 5, p. 444. Make a quick check for centration while the microscope is in use, by replacing the specimen with a slide coated with fluorescein to which has been added a proportion of gelatin to form an emulsion. This is allowed to dry on the slide, covered, and sealed. The coating forms a satisfactory screen for observing the image of the field diaphragm with a low-power objective. A thin coating of uranium nitrate crystals is also a useful test object.

Alignment with incident light. To centre a system with normal incident light, i.e. full aperture lighting from above the specimen, or with annular incident oblique light such as is used in the ultropak and epi-illuminators, the same procedure is carried out as outlined for substage illumination. This may not be possible with 'built-in' systems of modern microscopes.

To observe an image of the source with the incident illuminator screwed into position on the body-tube but without objectives, &c., in position (see stage 1 above), place a 3 in. × 1 in. slide (the upper surface of which is silvered) on the stage directly beneath the illuminator. With pinhole eyepiece in position an image of the source can now be clearly viewed. The axis of the illuminating beams from the source should be at 90° to the body-tube axis in the vertical plane and centred on the entrance aperture of the illuminator before assembling the objective. The silvered slide is then removed and a specimen put in its place. With an objective and eyepiece in position the stage is carefully raised to the focus of the objective by means of the stage rack adjustment. The field-diaphragm is adjusted to the same plane of focus as the specimen. Any lateral movement of the field-diaphragm image on the specimen on focusing indicates decentration in the system.

Preparation of Specimens

The specimen and mountant. Observations can be made upon either fresh material or semi-permanent preparations. Unless the specimen can be stored in the dry unmounted state, e.g. crystals, fibres, or fixed smears and films, &c., it is not usually feasible to preserve mounted specimens indefinitely. Satisfactory semi-permanent preparations of stained sections or monolayer tissue cultures can be made by mounting in suitably buffered aqueous solutions, glycerol, or physiological saline. For the study of certain types of biological material blood-serum is a most useful mountant. Observations on blood-parasites are often advantageously made by mounting the specimen in serum taken from the host, which must, of course, be fresh. After a few days serum exhibits a strong bluish-white autofluorescence.

The mounted specimens are sealed with wax (2 parts beeswax, 1 part dental wax) and will often retain their fluorescent qualities for several months without deterioration, especially if stored in the dark. Dry specimens, and in certain

instances fluorochromed sections, may be mounted to advantage in castor or paraffin oils (medicinal). These have only a small amount of autofluorescence at 365 mμ. The most suitable medium for permanent mounts is DPX (N_D 1·524). It has a low autofluorescence and will preserve the fluorochrome dyes over long periods (when a neutral pH is maintained). This medium can only be used with dyes that are not removed by alcohol and similar solvents, which in practice is a serious limitation. If blue light is to be used to excite fluorescence in the specimen, Gurr's fluoremount, Apáthy's medium, or a solution of hyrax (Flatters and Garnett) have also proved successful.

Loss of image contrast can be due to the presence of excess dye which has diffused out from the specimen to form a uniform fluorescent background. This is avoided by adequate washing of the specimen in water or buffered solution before mounting, but specimens can often be washed and remounted if necessary. Saline has been used very effectively to clean away excess dye in old specimens stained with fluorescein-antibody conjugate. These will retain their fluorescence longer if stored at 4° C. Perfection of the image will depend on the thickness of the specimen in relation to the depth of field of the objective in the object space. To obtain images of good colour-contrast the thickness of the specimen should not be more than twice the focal depth of the objective in the object space and should depend on the morphological distribution of the fluorescing structures of the object. For the best results paraffin and freshly cut sections of tissue should be thin, certainly not more than 3 μ thick for higher aperture work. Similar advantages are gained by limiting the optical path between the undersurface of the cover-glass and the specimen. Monolayer cell cultures on cover-glasses are ideal specimens for fluorescence microscopy. Smears and films are best made on the cover-glass and mounted on to the slide so as to eliminate the layer of mountant between the specimen and cover-glass. Only the smallest quantity of mounting medium necessary should be used. Freshly cut frozen sections of tissue are best mounted in glycerol, but if allowed to dry on the slide, paraffin oil has proved a good mountant. Slides coated with agar (2% or 3%, diluted with an equal volume of buffer or serum) have proved extremely useful for mounting living tissues, &c., and enable a clean background to be obtained with the cells spread evenly with just enough pressure from the cover-glass to reveal details of structure. Provided that the coating is sufficiently thin (0·5 to 1·0 mm), light scatter and autofluorescence of the agar are not noticeable. Certain kinds of specimen are best examined dry and this may be done with the specimen uncovered. Bone and tooth sections must be ground and polished. Sections of natural bone can be prepared down to 50 mμ thick and must be examined with the bright-field system owing to scattering of light at wide angles of illumination with dark field. Sections of teeth 25 mμ thick give excellent images by dark field. Before examination with higher-aperture dry objectives, corrections must be made with the correction collar to compensate for the absence of a cover-glass. With refractile specimens, scattered visible light transmitted by the primary filter (red light with the 0×7 filter, unless a solution of copper sulphate is used)

will be reflected into the objective. In this case the specimen must be examined, covered and mounted in a medium of suitable refractive index. Freshly cut frozen sections have very little autofluorescence, but after storage the autofluorescence increases in intensity and a stage will be reached when this may interfere with the colour and strength of any dyes applied to the tissues. For these reasons specimens are best examined, and photographed if necessary, soon after mounting. Studies of autofluorescence must be made with fresh material and precautions taken to preserve the neutral reaction of tissue without extraction of the fluorescent substances during the process of mounting.

Slides and cover-glasses. When the cardioid dark-field condenser is used the thickness of the slides must not exceed 1·2 mm and when the microscope is used in the horizontal position should not be less than 1·0 mm thick. It is necessary to know the cover-glass thickness when high-aperture dry objectives, not fitted with a correction collar, are used. Only those that are 0·16 to 0·18 mm thick should be used. Slides must be chemically clean and free from grease to maintain the true fluorescence colours and image contrast. Certain batches of slides and cover-slips, even after cleaning by the usual methods, take up the basic fluorochrome dyes when used in the normal way for fluorescence microscopy. These may fluoresce quite strongly and produce a coloured background, which reduces contrast and detail in the image. The take-up of the dye is due to an almost insoluble film on the surface of the glass, which can only be effectively removed by polishing. By applying a wet polishing powder of alumina or a commercial glass cleaner, and polishing with the flat end of a wooden rod to which is attached a piece of 'selvyt' cloth, the contaminating film can be removed quite easily. The slides and covers should then be rinsed in several baths of hot water and dried. It is advisable to check each batch of slides and covers for dye 'take-up' before resorting to special cleaning methods.

Only when fluorescence is low and a quartz condenser is used with excitation wavelengths at 365 mμ or below is it necessary to resort to quartz slide and cover-slip preparations. The majority of slides supplied transmit freely down to 350 mμ and any autofluorescence of the glass has been found to be negligible for most purposes. Effective secondary filtration of ultra-violet light for temporary mounts in water, buffered solutions, glycerol, &c., is obtained with ultra-violet-absorbing cover-glass (Zeiss 'euplus' covers). These are used in place of ordinary cover-glasses and absorb strongly wavelengths up to 400 mμ and transmit 80% of the remaining spectrum.

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