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BRIEF COMMUNICATION

An Alternative Lamp for Fluorescence Microscopy

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Summary. There has been marked development in reagents, filters and microscope equipment for fluorescence microscopy and particularly for immunofluorescence studies. The use of a different and more efficient lamp for excitation of fluorochromes is now reported.

Continuing development has made the techniques of immunofluorescence of use in an increasing number of fields of scientific study. Reagents have been studied extensively (Brighton, 1966; Brighton, Taylor, Tomlinson and Wilkinson, 1967; Holborow, 1970; MRC Working Party, 1971), and now at least two European manufacturers of commercial reagents, Nordic Pharmaceuticals, Tilburg, The Netherlands, and Wellcome Reagents Ltd, Beckenham, England, have excellent materials available. The increased specificity of these products has removed limitations on the techniques imposed by less pure reagents.

Concurrently with this development of reagents there has also been marked improvement in the types of excitation filters available. For immunofluorescence studies the most commonly used fluorochrome remains fluorescein, coupled to an immunoglobulin by means of a thiocarbamide bond. It has been shown by Nairn (1964) that the most efficient wavelength of light for stimulating the fluorescence of this compound is around 495 nm, with the light emitted as fluorescence at 523 nm. Formerly the excitation filters available were not designed to separate completely these two wavelengths, but now various manufacturers have evolved a class of interference filters, formed by deposition of thin metal films on glass, and these permit a much greater proportion of light of 495 nm to pass through, with adequate separation from the wavelength of the emitted fluorescence.

Development of the principle of incident illumination by Ploem (1967) has also extended the scope of the microscope. Equipment for this technique is now commercially available. When using either a light-ground or dark-ground condenser for transmitted illumination the flux of light available at the microscope stage is governed partly by the optical system of the microscope, partly by the numerical aperture of the condenser and partly by the intensity of the light source. As all of these factors normally have fixed values the flux of light at the stage is a constant value for each apparatus. This means that although an adequate amount of light is available with lower power objectives, $\times 16$ and $\times 40$, the amount of light is frequently insufficient to give a bright image with the higher power lenses required for most bacteriological work, e.g. $\times 100$ oil immersion. A different situation exists with incident illumination. The light illuminating the specimen is directed

thereto through the objective lens and the amount supplied depends on the numerical aperture of the objective lens, and the light source. In practice, lenses of higher power, × 100, have greater numerical apertures, around 1·25, than those of lower power lenses, and thus increase the flux of illumination of the specimen.

In spite of these improvements to reagents, filters and microscopes, only two types of lamp have been commonly used to provide illumination, the high-pressure mercury arc, which produces considerable ultra-violet (uv) radiation and filament lamps of various types, which produce no uv and an emission at 495 nm which is adequate for some, but by no means all applications, even when combined with the new filters and reagents. It has not been possible to study the internal structure of infected tissue culture cells with high power lenses, because of autofluorescence and fading.

Whilst adequate power for most applications is available from the mercury vapour lamp this type of illumination has other serious disadvantages. Short wave uv radiation is dangerous to the eyes of the microscopist, and necessitates special precautions to avoid retinal damage. The lamps also have only a relatively short useful life of about 50 hours before the uv output shows decline, but the most serious limitation on the use of uv is the autofluorescence introduced in the protein components of the preparation. This shows normally as a pale bluish-grey fluorescence which has the serious disadvantage of masking the green fluorescence of fluorescein in weak-positive preparations.

We have therefore examined other types of lamp, commercially available at the present time, that will permit the considerable improvements in reagents and filters to be exploited further. At first we used a 200 watt tungsten–halogen projection lamp EL 39 TI (G.E.C., London, England). This lamp produces adequate illumination at 495 nm but also so much infra-red (ir) radiation that considerable damage to heat filters and subsequently to optical filters occurred.

The performance of the 250 watt C.S.I. lamp (Phillips, Eindhoven, The Netherlands) was then studied. This lamp is supplied as a standard illuminant by Zeiss for applications such as photomicrography and microprojection, though not for fluorescence. It is basically a mercury vapour discharge lamp which has metallo-halide additives incorporated within the envelope of the arc. The quartz envelope is surrounded with an outer glass sheath, and as a result of the combination of halide incorporation and glass outer sheath the lamp has no output of short wave uv.

The spectrum (Fig. 1) shows a mercury line at 365 nm of reduced height with a much higher continuum between the major peaks in the visible region, from 400 to 750 nm. At 495 nm the lamp delivers 62 $\mu W/cm^2$ per 10 nm, compared with 10 μW for the mercury lamp. When used with a Zeiss Kp500 or Balzer FITC 3 or 4 primary excitation filter excellent stimulation of the fluorescein is observed, against a blue field, which must be removed by a secondary, eyepiece filter. When autofluorescence produced by uv is present the use of a secondary eyepiece filter is an unsatisfactory procedure as the filter permits only the yellow-green fraction of the bluish-grey autofluorescence to pass, and false positive results may be produced. As the C.S.I. lamp does not produce autofluorescence of proteins visible at 400–600 nm, it is quite safe to use an eyepiece filter.

Although fading of fluorescence specimens is still observed with the C.S.I. lamp, it is much less than when uv is used for illumination, presumably due to the much smaller amount of energy absorbed by the proteins of the preparation.

The lamp has a useful life of about 500 hours and operates from the L2 setting of standard mercury arc chokes.

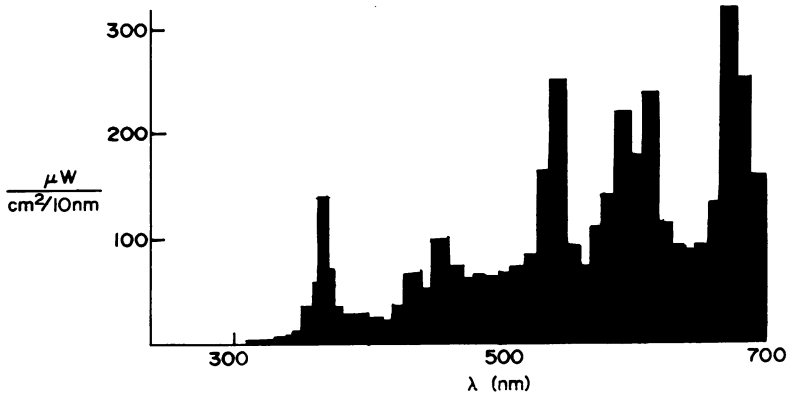

FIG. 1. Absolute spectral energy distribution of C.S.I. lamp, measured at 1 m distance.

On the Zeiss Photomicroscope II we now use the following incident light system for both research and routine purposes. Illumination from the C.S.I. lamp, housed in the mercury arc lamphouse travels first through a heat filter then through either a Zeiss Kp500 or Balzer FITC 4 primary filter. The beam is then split by an interference half-plate, Zeiss 500, inclined at an angle of 45° to the incident beam. This permits light of wavelength greater than 500 nm to pass through, and reflects light of wavelength less than 500 nm downwards, through either a Zeiss ×40 1·00 n.a. planapochromat, a Zeiss ×63 1·25 n.a. Neofluar or a Zeiss ×100 1·25 n.a. planachromat oil immersion objective on to the specimen. After stimulation of the fluorescein the emitted light of 523 nm returns via the objective lens through the half-plate to the eyepieces. A pale yellow 'edge' filter of 510 nm is a suitable secondary filter and produces a brilliant image of the fluorescent specimen against a black background. For those who prefer a red-tinted background the primary excitation filter may be a Balzer FITC 3 which does not remove all of the red light from the source, and sufficient of the harmonic wavelength is reflected by the half-plate to give a dull red background.

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